The Development of *In vivo* Comprehensive Multiphase NMR and Its Potential for the Investigation of Aquatic Toxicity

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

NMR based metabolomics has been extensively used to provide a molecular-level understanding of underlying biological processes due to environmental stimuli. Previously reported *in vivo* NMR studies were limited to detecting dynamic and soluble entities, resulting in the need for an approach to detect all bonds (solids, gels and liquids *in vivo*) and thus provide a comprehensive overview as to all components inside a living organism. CMP-NMR integrates all the electronics from HR-MAS (gel-phase NMR), with high power RF handling to study rigid solids, into a single probe. Therefore, *in vivo* CMP-NMR holds potential to correlate rapid stress responses (metabolite fluxes) to slower structural changes (thinning of shells). In this regard, the focus of this dissertation was the development of CMP-NMR for the study of living systems. In Chapter 2, CMP-NMR
combined with spectral editing isolates the signals arising from the rigid solids (chitin), to gel (lipid membranes) and liquids (amino acids) in *Hyalella azteca* into discrete spectra for each sub-component. Additionally, $^{13}$C isotopic enrichment and 2D NMR enabled the assignment of nearly 40 metabolites. However, the stress exerted on the organisms due to the fast spinning (2.5 KHz) remained a major challenge of the approach. To address this, Chapter 3 introduces a slow spinning technique by concatenating PURGE and TOSS.243, which leads to high resolution *in vivo* 1D $^1$H NMR profiles down to 300 Hz. In Chapter 4 hetero-nuclear ($^{31}$P, $^{19}$F $^2$H, $^{15}$N, NMR) experiments are introduced to follow the fate of the contaminants inside the living organism and 2D correlations ($^1$H-$^{13}$C, $^2$H-$^{13}$C) monitor the biomolecular responses of the organism to the nutritional substrates. In Chapter 5 $^2$H/$^{13}$C isotopic enrichment of the organisms, is combined with 2D $^2$H-$^{13}$C NMR leading to the first high resolution sideband free spectra at 50 Hz and extending the experimental time up to 48 h. Combined *in vivo* CMP NMR techniques show great potential for future application in environmental toxicity risk assessments.
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Preface

The contents presented in this dissertation are a collection of research performed by the author with important contribution with the co-authors. The dissertation is based upon four original manuscripts, Chapters 2, 3 and 5 have been published in peer-reviewed journals and Chapter 4 is under review for publication. This would result in an overlap between chapters and particularly chapters’ introductions contain some repeated material throughout the thesis. The studies were designed in collaboration between Yalda Liaghati Mobarhan and André J. Simpson. All data have been acquired and interpreted by Yalda Liaghati Mobarhan under the guidance of André J. Simpson. All manuscripts were written by Yalda Liaghati Mobarhan, with critical comments and assistance provided by André J. Simpson. The contributions of additional authors are described below.

Chapter 2: Comprehensive Multiphase NMR Applied to a Living Organism

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Author Contributions

Yalda Liaghati Mobarhan conducted sample preparation, experimental work, data analysis, interpretation, and preparation of the manuscript under the direct guidance of André J. Simpson. Myrna Simpson assisted in project design and provided access to NMR spectrometer. Blythe Fortier-McGill provided technical support. Ronald Soong, Werner E. Maas, Michael Fey, Martine Monette, Henry J. Stronks, Warren Norwood provided technical assistance. Sebastian Schmidt, Hermann Heumann provided assistance with the organisms’ supplements.
Chapter 3: Effective Combined Water and Sideband Suppression for Low-Speed Tissue and In vivo MAS NMR

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Yalda Liaghati Mobarhan conducted sample preparation, experimental work, data analysis, and interpretation, and prepared the manuscript under the direct guidance of André J. Simpson. Jochem Struppe and Blythe E. Fortier-McGill provided technical support.

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Chapter 5: In vivo Ultra Slow MAS $^2$H-$^{13}$C NMR Emphasizes Metabolites in Dynamic Flux

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Author Contributions

Yalda Liaghati Mobarhan conducted sample preparation, experimental work, data analysis, interpretation, and preparation the manuscript under the guidance of Andre J. Simpson. Ronald Soong assisted with experimental design. Myrna Simpson assisted in project design and provided access to NMR spectrometer. All other co-authors provided technical support.
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List of Abbreviations

1D: One Dimensional

2D: Two Dimensional

AMIX: Analysis of Mixtures

ASTM: American Society for Testing and Materials

ATP: Adenosine Triphosphate

COSY: Correlation Spectroscopy

CMP-NMR: Comprehensive Multiphase Nuclear Magnetic Resonance

CPMG: Carr-Purcell-Meiboom-Gill

CP: Cross Polarization

CSA: Chemical Shift Anisotropy

EC: Environment Canada

GABA: Gamma-Aminobutyric Acid

GC: Gas Chromatography

HETCOR: Hetero-nuclear Chemical shift Correlation

HR-MAS: High Resolution Magic Angle Spinning

HPLC: High Performance Liquid Chromatography

HSQC: Hetero-nuclear Single Quantum Coherence

HMQC: Hetero-nuclear Multiple Quantum Coherence

J-RES: J-resolved Spectroscopy
LC: Liquid Chromatography

LD50: Half Maximal Lethality Dose

MAS: Magic Angle Spinning

MOA: Mode of Action

MS: Mass Spectrometry

NMR: Nuclear Magnetic Resonance

PASS: Phase Adjusted Spinning Sidebands

PCA: Principal Component Analysis

PFOA: Perfluorooctanoic Acid

PFOS: Perfluorosulfonic Acid

PRESAT: Presaturation

RF: Radio Frequency

S/N: Signal-To-Noise

SS: Slow Spinning

TOCSY: Total Correlation Spectroscopy

TOSS: Total Sideband Suppression

USEPA: United States Environmental Protection Agency
Chapter 1

Introduction
1.1 Environmental Toxicity

The ever-growing number of anthropogenic compounds utilized and discharged by industry, agriculture and human activities, poses a great potential threat to both human and ecosystem health. Hazardous compounds from various sources are introduced into the air, soil and water and can become widely distributed into the environment within different physical phases. For example, emissions to air can result in atmospheric deposition to soil and water, and runoff from soils can eventually contaminate water bodies. Water contamination directly influences both human health via drinking water supplies and environmental health by impacting the biodiversity and function of aquatic ecosystems. In aquatic environments many of the waste materials disposed into the surface water, eventually accumulate in the sediments and can be taken up by aquatic organisms, both from the surrounding medium or via trophic transfer [1].

Toxins often have poorly understood environmental fate and often their impact on organisms (i.e. toxic-mode-of-action, binding receptors, biotransformation) are not well understood. Persistent Organic Pollutants (POPs), are a class of synthetic chemicals resistant towards degradation and with long half-lives in water and sediment that can span decades. Inside organisms POPs can potentially bioaccumulate in the organism and biomagnify throughout the food chain posing serious health impacts once consumed by higher order predators or humans. A group of POPs, perfluorinated compounds (PFCs), have been commercially used since 1950s in a variety of consumer products, including clothing, Teflon and food packaging. These chemicals are known to cause cancer and numerous other detrimental health effects and do not readily biodegrade or biotransform [2]. The linear chain species, perfluorooctanoic acid (PFOA) and perfluorosulfonic acid (PFOS), have a hydrophobic tail and a hydrophilic head and are highly soluble in water and travel to remote locations as far as the Arctic [2]. Once inside the body they mimick lipids and bind to the fatty acid binding sites on proteins, bioaccumulate and can be transferred to future generations [3]. Although PFOA and PFOS have been phased out in some parts of the world,
emerging derivatized replacements which share similar chemical characteristics are subject to new
global concerns [4].

Pharmaceuticals also have potential impact on both human and environmental health. It is reported
that due to insufficient removal from wastewater up to 90% of pharmaceuticals and their by-
products end up in water bodies [5]. Although evidence regarding their health impacts is still not
clear, water quality experts and environmental advocates are increasingly concerned about their
hazards on aquatic life [6].

Pesticides and herbicides are another major group of organic contaminates that are introduced into
the water threatening aquatic life. While extensive literature has already documented the
deleterious effects of many of these contaminants, their long-term impacts are not well understood.
A current example is the case of Canada’s most popular herbicide, glyphosate. While many
commercializing companies support its re-approval by Health Canada’s Pest Management
Regulatory Agency in 2017 and claim it is safe within the permissible limits. In contrast,
independent scientists correspond chronic diseases (such as cancers, neuropathies, infertility and
birth defects) to long-term exposure to glyphosate at below the regulatory limits with potential
unknown adverse effects on aquatic environments. This group critically accuse the shortcomings
in the current regulatory risks evaluation techniques [7].

Furthermore, industrial facilities continuously release heavy metals which have complex impacts
on aquatic life [8]. While high concentrations of metals can be toxic to biota, bioavailability of
these metals in sediments is greatly variable, and direct concentration measurements are not always
a valid indicator of their potential toxicity. For example, in crustacean’s cuticle defects were
initially correlated to genetic problems, but later considered to be correlated to exposure to trace
heavy metals [9]. These suggest an immediate need for an analytical approach that can act as a
“molecular interpreter” to relate such physical symptoms to the chemistry and biochemical
processes that cause them. In addition, due to unknown reasons, the concurrent presence of
contaminants such as heavy metals in an effluent, has been shown to exert synergistic adverse
effects on organisms [10].

Emerging scientific evidence continues to link the increasing occurrences of many chronic
diseases to exposures to contaminants [11,12], through consuming water contaminated aquatic
food sources. Often these diseases have few physical signs prior to outbreak, suggesting sensitive molecular based tools need to be developed as potential “early warning indicators” of exposure [13].

National environmental agencies such as Environment Canada (EC) and Environmental Protection Agency (EPA) are responsible for setting policies to protect the environment and ensure a safe environment for all the species. These policies consist of assessment of the potential risk of emerging substances to the environment and/or human health prior to their widespread release in industry [14], re-evaluation of the previously tested chemicals in order to ensure compliance with current scientific standards based on scientific evidence and enforce laws and regulations governing the use of toxic substances, and their disposal into the environment. Derivation of water quality guidelines for ecosystems rely on several assessment tools including biological assessments and toxicity tests in aquatic systems as part of an overall risk assessment framework.

Conventional toxicity assessments in aquatic ecosystems are mainly based on the data generated from standardized laboratory test protocols performed on model organisms. Many of these protocols have been developed in the twentieth century [15] and are still applied as they are easy to conduct and reflect the organism’s health [16]. For targeted studies designed to evaluate the potential for specific effects typically, different doses of the substance of interest are administered to groups of selected organisms to determine the dose lethal to 50% for the test population (LD50) [17]. In these tests the relative toxicity of a substance is established, while chronic tests reflect the long-term exposure using endpoints such as growth and reproduction. The acute and chronic test durations vary among different species life-cycle and usually inter-lab inconsistencies are observed within the results [18]. The acute tests define the threshold toxicity for a test organism, potentially overlook sub-lethal affects. The majority of studies are conducted over a short time course (48 hours-21 days) and are typically bias towards acute toxicity that does not address the long-term effects of the exposure and potentially, contaminants responsible for chronic diseases are largely neglected in the current methods. Additionally, these tests lack the necessary information to explain the biochemical basis of the toxicity. In order to establish a safe level of exposure and provide an effective initiative for prevention of the adverse effects surrounding exposure to a toxin, a more in-depth molecular-level understanding of the potential risks, mechanisms of action, and organism responses is desperately needed. This should be able to answer questions such as, which
chemicals can bioaccumulate and cause long term disease/deficiencies? How do toxins biotransform and are their products potentially more toxic? What is the toxic mode-of-action and what biochemical pathways are perturbed within the organism? which underpin many of the possible adverse impacts associated with sub-lethal toxicity. This has been manifested in separate reports by EPA “Toxicity Testing in the 21st Century” which states “The new paradigm should facilitate evaluating the susceptibility of different life stages, understanding the mechanisms by which toxicity occurs, and considering the risks of concurrent, cumulative exposure to multiple and diverse chemicals”. And another paper which denoted that due to limitations in the current approaches we are experiencing “Dark ages of toxicology”, since "...aquatic toxicity data derived with old testing methods are no longer adequate” [19]. According to these reports substantially revised modern approaches of toxicity evaluations would need to be capable of directly evaluating the mode-of-action, bioaccumulation, biotransformation, molecular reactivity, excretion and binding in response to the surroundings.

1.2 Metabolomics

Biological systems are highly dynamic, and their cellular functions are influenced by a variety of internal and environmental factors. An alternative way to characterize environmental toxicity is to understand the molecular level impacts and biological mechanisms responses caused by a stressor. Metabolites are the direct response of complex cellular and biological processes often fluxes in this metabolite pool occur prior to detectable changes in the genome, or proteome [20]. Therefore, the metabolome consisting of many small (<1500 Da) endogenous (amino acids, carbohydrates, organics acids) and exogenous (newly formed metabolites), represents a sensitive and rapid indicator of the functional status of an organism. Thus, metabolomics, the study of these metabolites has emerged as valuable tool to characterize inter/intra cellular and dynamic molecular changes in biological systems [21,22]. During recent years metabolomics has found extensive applications in fields spanning from toxicology, drug discovery [23], nutrition, natural product [24] and in medical studies both for disease diagnosis and biomarkers discovery [22,25,26].
Environmental metabolomics is a sub-discipline of metabolomics with a focus on the impact of environmental stimuli such as natural stressors and anthropogenic pollutants. This approach evaluates potential adverse effects resulting from the stressor by discerning the metabolic changes between the healthy and exposed organisms with time, concentration or other variables. By correlating the metabolomic fluxes to the biological pathways affected it is possible to delineate the mode of action of the contaminant. The greater biological insight brought about by these approaches holds great promise for science-based assessment, that can lead to improved regulation. Environmental metabolomics has been applied on both aquatic and terrestrial organisms such as earthworms [27-29].

Given the vast amount of data produced in metabolomics, these studies are usually combined with statistical analysis methods to allow researchers to gain biological insight from complex data in a timely manner. Principal Component Analysis (PCA) is one of the most common unsupervised data analysis tools used to identify data patterns and reveal the hidden relationships among the data sets [26]. The fundamental idea behind PCA is to reduce a set of possibly correlated features of a given data set to (linearly) uncorrelated features in separate PC plots based on their importance. This results in the reduction of the dimensionality of a large data set. PCA captures the overall variability or separation in the metabolome of various groups at different conditions from the control.

Conventionally, metabolomics uses Mass Spectrometry (MS) or Nuclear Magnetic Resonance (NMR) to detect the variations in metabolite profiles in tissue extracts or biofluids [30]. Advances in these analytical techniques have given rise to the rapid development of metabolomics during the last decade. Both platforms are capable of high sample throughput and automated analysis. MS-based approaches are inherently more sensitive than NMR based techniques, providing access to lower levels of metabolites. MS techniques have detection limits in the range of picograms while typical $^1$H NMR detection is at the $\mu$g/mL to ng/mL level [31]. Moreover, MS approaches are coupled to gas chromatography (GC) or liquid chromatography (LC) preceding the analysis to facilitate separation. Often these hyphenated techniques require extensive sample preparation for compatibility with a specific sample introduction technique. For example, GC often requires chemical derivatization of the analyte to increase the volatility in non-volatile sample and LC requires dissolution and ionization. Therefore, MS techniques are more commonly applied in
targeted metabolomics studies for the selective identification and quantification of defined groups of known metabolites of interest within a large sample.

Another fact is that these sample preparation steps are destructive and hence MS detection is not applicable for the analysis of the living organisms which is the scope of the current thesis.

Application of NMR based metabolomics in toxicology was pioneered by Nicholson [32]. NMR-based metabolomics has been established as key tool in toxicity studies and exploring the effect of various natural and anthropogenic stressors on a range of different species including mussels, craps, fish and earthworms [15,27,33-35] exposed to contaminated air, water and soil. The non-invasive nature of NMR permits in vivo studies, which is unparalleled by any other analytical tool.

1.3 Nuclear Magnetic Spectroscopy

1.3.1 Platform of Choice

The present dissertation makes extensive use of NMR to investigate the metabolomic and structural frameworks in intact living systems. NMR is arguably the most powerful instrumental platform in modern research, providing unprecedented levels of molecular information on the structures, dynamics, and inter/intra molecular interactions. NMR is non-selective for a given nucleus within the applied excitation bandwidth and is a powerful tool in structural determination of unknown components. In addition, NMR is high throughput, requires little sample preparation and is non-destructive [25]. NMR is highly versatile and unlike other analytical methods which typically require that sample meets certain criteria (physical properties) it can accommodate all samples types. NMR can provide a broad overview on a wide range of metabolites in biological samples in situ. Therefore, it is particularly useful for untargeted metabolomics, a comprehensive analysis of all the observable analytes including unknown chemicals within a complex sample. Untargeted analysis offers the opportunity for potential novel target discovery. NMR based metabolomics can generate reliable and reproducible data which is crucial in environmental risk assessment studies [36]. Given the fact that biomolecules mainly consist of magnetically active
nuclei that are commonly studied by NMR $^1$H, $^{13}$C, $^{15}$N and $^{31}$P, $^{19}$F[37] makes NMR a well-suited platform for studies on biological and natural samples. Further, as NMR uses low energy radio frequency pulses, to excite these nuclei [25] existing in the sample it is the only non-destructive and non-invasive modern analytical tool that can provide a detailed metabolic profiling on living organisms.

In practice NMR includes various methodologies that each unravel part of the structure, and by integrating their output can elucidate a wealth of molecular and structural information on complex analytes. Significant progress in the NMR field in the hardware and pulse sequences have been made over the last 3 decades. Specifically, much of the progress is related to the development of various 2D experiments. These experiments extend the spectral dispersion into an additional dimension and provide information on correlations for robust identification of metabolites. The additional information contained in nD NMR (for example through space correlations, through bond correlations, diffusivity, etc.) can be combined to assign structures de novo, if databases are not available. Therefore, NMR is one of the only tools that can solve a complete molecular structure if novel or unidentified metabolites arise. Furthermore, the use of stable isotopes ($^{13}$C or $^2$H, $^{15}$N) incorporated selectively or non-selectively into the organisms or into the external stimuli in conjunction with multidimensional techniques makes NMR spectroscopy the method of choice for exploring selective metabolic pathways and site specific studies [38,39].

1.3.2 Basics and Theory

This dissertation involves the application of NMR as an analytical tool on living organisms, and thus an advanced understanding of the quantum physics and NMR theory fall outside the scope of the thesis. However, in the following section a brief background of NMR theory will be presented to obtain a basic understanding of the underlying principles and for a more detailed discussion readers are referred to related textbooks such as “Understanding NMR” by Keeler or “Spin Dynamics” by Levitt [40,41]. Subsequently, NMR spectroscopy as an analytical tool will be discussed along with the recent progress in NMR approaches. Finally, the most relevant experiments performed in vivo will be reviewed.
To introduce the underlying theory of NMR, first we need to explain a few concepts from quantum physics.

The observable energy of a quantum system is characterized by the Hamiltonian operator (or simply Hamiltonian). In fact, Hamiltonian of a quantum system has a quantized set of eigenvalues and every measurement of energy of the system will end up to one of these values according to (time-independent) Schrodinger equation:

$$\hat{H}\psi = E\psi$$

(1)

The Hamiltonian operator of a nuclear spin in a magnetic field of strength $B_0$ along the $z$-axis is given by

$$\hat{H} = -\gamma B_0 \hat{I}_z$$

(2)

where $\gamma$ is called gyromagnetic ratio and is an intrinsic property of the nucleus. The operator $\hat{I}_z$ is the $z$-component of the spin angular momentum. One needs to note that angular momentum in quantum mechanics has two parts, one is spin angular momentum and the other, orbital angular momentum. It turns out that spin angular momentum is an intrinsic property of certain type of nuclei and it is fundamental as mass and charge. The eigenvalues of spin angular momentum can be specified by the spin quantum number $I$, that can be either integer or half-integer, (i.e. $I = 0, \frac{1}{2}, 1, \frac{3}{2}, \ldots$). The spin angular momentum operator has $2I + 1$ eigenvalues ranging from $-I$ to $I$ in integer steps.

NMR is based on this spin angular momentum which is an intrinsic property of the nuclear subatomic particles, nuclear spin. Individual unpaired subatomic particles electrons, protons, and neutrons each possesses a spin value of $\frac{1}{2}$. The net spin number of the nuclei depends on the number of their neutrons and protons (mass). The addition of these two particles can give rise to three general conditions:

Even number: $I = 0$ (i.e. $^{12}\text{C}$, not detected by NMR),
An unpaired $I = ½$ (i.e. $^1\text{H}$, $^{13}\text{C}$, $^{15}\text{N}$, $^{19}\text{F}$ and $^{31}\text{P}$, NMR active)

Odd number $I > ½$ (i.e. $^2\text{H}$, $^{3/2}\text{B}$, $^{5/2}\text{O}$, NMR active, quadrupolar nuclei)

Nuclei with $I = 0$ are not NMR active and will not be further discussed. All nuclei $I \neq 0$ have a magnetic dipole moment ($\mu$) and are considered NMR active. $I = ½$ nuclei have a spherical charge distribution, that results in a simpler NMR behavior. Fortunately, most relevant nuclei which are also more frequently studied nuclei in NMR spectroscopy have $I = ½$. Nuclei with $I > ½$ have non-spherical charge distributions, and hence have an additional electric quadrupole moment. Going back to quantum physics;

As we are mostly focus on spin $^1\!\!\!_2$ nuclei, eigenvalues and eigenfunctions of $\hat{I}_z$ is given by

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

where $m = \pm^1\!\!\!_2$ and $\hbar$ is the reduced Planck’s constant $\hbar = \frac{h}{2\pi}$. This will show that eigenvalues of Hamiltonian are given by $m\hbar\gamma\text{B}_0$, that is,

$$\hat{H}\psi_m = -m\hbar\gamma\text{B}_0\psi_m$$

In the absence of a strong field and at a thermal equilibrium, the nuclei are degenerate. In the presence of an external magnetic field ($\text{B}_0$), there are two possible energy levels for spin-half nuclei as

$$E_m = -m\hbar\gamma\text{B}_0, m = \pm^1\!\!\!_2$$

Conventionally, the energy level of these two spins have been denoted by $E_\alpha$ for $m = ^1\!\!\!_2$ and $E_\beta$ for $m = -^1\!\!\!_2$. The energy gap between these two levels is given by

$$\Delta E = E_\beta - E_\alpha = \hbar\gamma\text{B}_0$$
As energy is related to frequency by \( E = \hbar \nu = \hbar \omega \), the above energy gap can be written in terms of frequencies.

\[
\Delta \omega = \gamma \mathcal{B}_0
\]  

(7)

Here \( \omega \) is the angular frequency and \( \gamma \) is the gyromagnetic ratio which is an isotopic specific constant. The negative of this frequency is the so-called Larmor frequency and is denoted by \( \omega_0 \):

\[
\omega_0 = -\gamma \mathcal{B}_0 \left( \frac{\text{rad}}{\text{sec}} \right)
\]  

(8)

In this view the energy gap between up (\( \alpha \)) and down (\( \beta \)) spin states is exactly equals the negative of Larmor frequency. Moreover, system’s Hamiltonian can be written in terms of \( \omega_0 \) as energy and frequency are proportional, we can write energy in terms of Hz as

\[
\mathbf{H}_{\text{one-spin}} = \omega_0 \hat{I}_z
\]  

(9)

1.3.3 Bulk Magnetization and Vector Model

As have been discussed above, some nuclei possess spin angular momentum property, which by itself causes a phenomenon called nuclear spin magnetic moment. This means that each nucleus generates a slight magnetic field in its surroundings similar to a small bar magnet. This magnetic field will interact with the applied magnetic field and NMR studies the interaction between the magnetic moment of each of nucleus spins and an applied large magnetic field.

It is only quantum mechanics which can give us a full understanding of NMR, however, in this section we chose a simpler and more vivid approach called vector model that would be enough for our current needs. Vector model, uses a 3D model view of nucleus’ properties, applied magnetic fields and RF pulses which will give us a more concrete idea of a few fundamental NMR experiments.
For a given sample of nuclei spins placed in a magnetic field, each spin acts like a small bar magnet, however, the observed magnetization is the average of all magnetic fields. The overall average of magnetic moments produced by all spins is called bulk magnetization. For simplicity, it is often assumed that the external magnetic field $B_0$ is along the $z$–axis. The energy of interaction between each spin nuclei and the magnetic field characterized by $\cos \theta$, where $\theta$ is the angle between the spin axis and the field $B_0$. Therefore, each magnetic moment is in the highest energy level if it is aligned with the magnetic field $B_0$ and it is in the lowest energy level if aligned against $B_0$.

Placing a sample of nuclei spins in a magnetic field, a slight preference in $\alpha$ state gives rise to a macroscopic magnetic moment (net magnetization). This number is usually very small and thus, the sensitivity of NMR is generally low [42]. This proportional tendency is given by Boltzmann distribution as

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

Here, $N_\alpha$, $N_\beta$ represent the number of spins up and spins down magnets, $\Delta E$ is the energy level difference, $k$ is the Boltzmann constant and $T$ is temperature in Kelvin.

Note that there is no preference of spins magnetization in $x$– and $y$– directions, so the bulk magnetization will be observed only along $z$-axis. Then the bulk magnetization will stay at that steady state which is the so-called magnetization equilibrium. This process of reaching magnetization equilibrium is called relaxation and will be discussed later in this section. At steady state there is no magnetization changes over time to be studied. Only if the bulk magnetization could be tipped away from $z$–axis, then the bulk magnetization goes in a precession motion(rotation) about the $z$–axis. This tipping from $z$–axis is generated by a rather weak magnetic field oscillating along $x$–direction at Larmor frequency. In fact, this resonant frequency equals to energy gap between two energy levels. Therefore, the precessional frequency of the bulk magnetization is exactly Larmor frequency

$$\omega_0 = -\gamma B_0.$$
This presessional magnetization would become detectable as it changes over time. In fact, if a coil exists along the x-axis, then the rotating magnetization intersecting the coil will induce a current within the coil which can be measured. This is called free induction decay signal (FID) which can be detected in NMR experiments. Signal detection will be covered in the following section.

Table 1.1 compares the magnetic properties of several relevant NMR active nuclei commonly employed in metabolomics.

### Table 1.1 Magnetic properties of biologically relevant NMR nuclei

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Spin Quantum Number</th>
<th>Natural Abundance</th>
<th>NMR Frequency ((^{1}\text{H}=500 \text{ MHz}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{1}\text{H})</td>
<td>1/2</td>
<td>99.99</td>
<td>26.752</td>
</tr>
<tr>
<td>(^{2}\text{H})</td>
<td>1</td>
<td>0.01</td>
<td>4.106</td>
</tr>
<tr>
<td>(^{13}\text{C})</td>
<td>1/2</td>
<td>1.07</td>
<td>6.728</td>
</tr>
<tr>
<td>(^{15}\text{N})</td>
<td>1/2</td>
<td>0.365</td>
<td>-2.712</td>
</tr>
<tr>
<td>(^{19}\text{F})</td>
<td>1/2</td>
<td>100.00</td>
<td>25.162</td>
</tr>
<tr>
<td>(^{31}\text{P})</td>
<td>1/2</td>
<td>100.00</td>
<td>10.839</td>
</tr>
</tbody>
</table>
1.3.3.1 NMR Signal Detection

In order to obtain better understanding of the signal produced and detected in a NMR experiment a macroscopic view is presented using a 3D coordinate system. NMR works by applying a strong magnetic field to perturb the equilibrium state of nuclear spins. By convention the external magnetic field is applied along the z axis of a coordinate system and it is the z component \( I_z \) of the spins that align with the \( B_z \) as depicted in the Figure 1.1.

A radio frequency pulse with equal energy to the gap between the two states at the correct angle, is used to excite the nuclei of interest and manipulate a net bulk magnetization in the xy plane. At any instant in time, this magnetization can be represented by a magnetization vector following the right-hand rule. For example, when a 90º pulse is applied to protons within a system in the +y the net magnetization is rotated into the x-y plane and along x. Only the frequencies corresponding to \( \Delta E \) at which the nuclear spins transition among the energy states are absorbed. This energy transfer is depicted with a band and reported as chemical shift [25].

1.3.3.2 Chemical shift

The main output of any NMR experiment is the resonance frequency of specific nuclei existing within the sample at \( B \).

\[
\nu = \frac{\gamma B}{2\pi}
\]

(12)

The precise values of this frequency absorbed by same nuclei within a sample varies depending on its chemical environment (electron distribution and bonding of neighboring atoms). The applied magnetic field \( B_0 \), induces an opposed electrical field in electrons surrounding the nucleus. This causes nuclei in a region of high electron density to become more shielded from the applied field. On the contrary, if the neighboring molecule reduces the electron density on the nuclei shift to higher frequencies(deshielding) is expected. The combined effect of the strong magnetic field \( B \) and these local fields give rise to slightly shifted Larmor frequencies observed.
The resulting position of an absorption frequency of nucleus in accordance with other nuclei is the separation between its resonance frequency from a reference standard relative to the frequency of the reference is called its chemical shift and reported in ppm rather than in frequency.

\[
\delta = \frac{(\nu - \nu_{REF}) \times 10^6}{\nu_{REF}}
\]  

(13)

This ratio is thus, independent from the field strength and can be universally used regardless of the strength of the magnet.

In addition to chemical shift, other information intensity, widths and multiplicity of the signals can be derived from the spectra to obtain unique quantitative information, regarding the molecular structure. The resulting spectra is unique to each molecule and in the simplest form these signatures can be matched against databases to characterize the compounds [40]. Specifically, in metabolomics the emergence of new resonances and the degradation products are identified using the chemical shifts.

1.3.3.3 Relaxation

By far we have briefly explained that subsequently to the application of a RF pulse the spins are aligned together (perturbed from z axis to the x-y plane) and precess with same phase (transverse magnetization), to produce detectable signals in the receiver coils. Overtime, the excited spins will eventually relax back to the ground state and reestablish the thermal equilibrium. There are two types of relaxation by which a spin system loses energy; namely T_1 and T_2 relaxation. Since the spontaneous emission is negligible the relaxation comes about with the non-radiative interaction of the nuclei with their neighbor spins (the “lattice”). The longitudinal relaxation reestablishes the z component of the magnetization back to the equilibrium state, therefore it directly affects the sensitivity of the signal. This decay occurs with a rate proportional to 1/T_1. T_1 in liquids usually ranges between 10^{-2} to 100 s.
Figure 1. 1 A) Bulk magnetization in the presence of external field, B) Establishment of the coherence in the x-y plane C) The longitudinal relaxation reestablishes the z component of the magnetization back along the z axis (T₁ relaxation) D) After RF pulse is stopped the spins begin to rotate with their own Larmor frequency and become decoherent in the x-y plane (T₂ relaxation)

When the RF pulse is discontinued due to a slightly different magnetic field experienced by each spin (due to inhomogeneity of B₀) they begin to rotate at their own Larmor frequency therefore their coherence starts to decay in the x-y plane. This phenomenon known as spin-spin relaxation occurs with a rate proportional to 1/T₂. The time constant by which the spins return to equilibrium of the transverse magnetization, is T₂. T₂ is always smaller than or equal to T₁ and hence the limiting factor in the acquisition time. The shorter T₂ (the faster the signal decays) the shorter the
acquisition time and the broader the lines become. In systems with slower rotational diffusion such as bulky and large molecules with $T_2$ values in the order of ms, there is no point for acquisition times in the order of seconds. A shorter acquisition time limits the certainty of the Larmor frequency and therefore, reduces spectral resolution.

In general, NMR spectroscopy relaxation is comparably a slow process (in the order of millisecond to seconds), which provides ample time to allow manipulations and differentiation of the spins based on their relaxation differences. NMR signals are acquired by repeating the experiments many times. Between the scans there is need for a delay to allow the spins to relax back to their equilibrium prior to the next manipulation.

1.3.3.4 NMR Instrumentation

The most important part of the NMR spectrometer is the superconducting magnet that produces the $B_0$ field necessary for the NMR experiments. The probe head and the shim coils for homogenizing the $B_0$ field are placed within the bore of the magnet. The probe consists of RF coils (in the x-y plane), that excite the nuclei by producing an RF pulse. The sample vessel is positioned within the RF coil of the probe. In the HR-MAS systems this forms a magic angle with the system (explained in section 1.3.4.3).

The signal emitted from the sample enters the receiver coils. A readout system consisting of a computer records and analyses the data. The signal is then converted from intensity/time pairs to intensity/frequency pairs (NMR spectrum) by Fourier transformation.

1.3.4 NMR Methodologies

Traditionally NMR was performed on separate phases, in either solution state or solid state [43]. In 1996 HR-MAS was developed [44] that allowed the study of both solutions and gel-like components. Finally, in 2012 CMP-NMR [45] was developed that permits all phases in a multiphase sample to be studied simultaneously.
1.3.4.1 Solid state NMR

Solid state NMR is performed on solids or dried samples packed into small rotors with sample quantities of ~100 mg. The same way that two actual magnets in close proximity behave towards each other, two nuclei with spins tightly packed within the structure of a rigid system exert magnetic moments on each other. The resulting magnetic field felt by nucleus 1 is defined as

\[ h_{\text{loc}} = \frac{\mu}{r_{12}^2} (3 \cos^2 \theta_{12} - 1) \]  

(14)

Where \( r_{12} \) is the distance between the nuclei, \( \theta \) is the angle between the internuclear vector, the external B, and \( \mu \). These magnetic dipole moments are strong, permanent and their existence hinders the resolution and sensitivity of the solid NMR spectra. Solid state NMR probes have capabilities to handle high powers in the range of hundreds of Watts.

The other broadening effect in the solids is called chemical shift anisotropy, caused by the interaction of nuclei with the surrounding electrons. Within a solid, molecular groups may exist in different orientations, which in turn gives rise to a spread of the frequencies.

Currently, solid state NMR techniques takes advantage of Magic Angle Spinning (MAS), to overcome these broadening effects, giving rise to solution like sharp peaks. However, homonuclear \(^1\text{H}-^1\text{H} \) dipolar interactions in true solids are very strong and difficult to remove even by MAS, broadening the spectra up to few KHz, therefore, \(^{13}\text{C} \) NMR detection is more commonly performed using solid state NMR. MAS will be discussed in detail in the following sections. Although solid state NMR has been extensively used in studies such as soil most metabolites do not exist in a purely solid form.

1.3.4.2 Solution State NMR

In solution state NMR rapid tumbling averages out the broadening effects observed within solids, resulting in high resolution spectra for liquids. Often samples need to be extracted into a homogenous solution prior to analysis by solution state NMR. The isolation of the phases improves
the resolution and the sensitivity of the spectra. Solution state NMR probes have the capability to use a lock signal to account for the magnetic drift overtime and further enhances the line shape. In addition, they are equipped with pulsed field gradient coils along the sample that allow for diffusion-based measurements, improved 2D NMR and more effective water suppression. Examples of their use will be further explained in section 1.5. Solution state NMR has found great popularity in biofluids, blood, urine cells and tissue extracts to detect a wide range of metabolites within the dissolved phases in different fields [46]. In fact, 1D $^1$H NMR solution state metabolomics is one of the most widely used NMR technique [28,47-49]. Solution in vivo NMR has also been developed[50,51] with the ability to look at the dissolved metabolites in whole organisms, this technique will be discussed in more detail in the section “in vivo NMR”.

1.3.4.3 HR-MAS NMR

Intact natural samples (soil, plants, and tissues) are the most challenging samples to study by NMR as they consist of multiple phases. The varying magnetic susceptibilities across these phases (e.g. boundaries in the inter/intracellular phases) along with anisotropy (uneven electron distribution around the nucleus) and dipolar interactions (i.e. $^1$H-$^1$H through space interactions) are often a source of spectral broadening and loss of resolution in NMR spectra. In the solution state these broadening effects are often averaged to zero due to high molecular motion, however, in solids or molecules with restricted motion this averaging does not occur.

Developed for solid state, MAS is used to effectively remove the spectral line broadening in intact samples. If the sample is rotated about an angle (known as the magic angle, $\theta = 54.73^\circ$) relative to the static external magnetic field ($B_0$), the $(3 \cos^2\theta - 1)/2$ part of the equation is averaged to zero resulting in enhanced line shapes.

High Resolution Magic Angle Spinning (HR-MAS) introduced in 1996, is a hybrid of the solid and solution state NMR techniques [44]. It combines the magic angle spinning from solids NMR probes [52] with the low power radio frequency (RF), lock, susceptibility matched stators and pulse field gradients found in liquid-state NMR probes.
The advantage of HR-MAS lies in its ability to identify the structures that exhibit minimal local mobility (dissolved and swollen components) in heterogeneous samples allowing for the non-invasive investigation of intact biological systems such as tissues or whole cells. Further, the ability to obtain high resolution spectra on a small quantity (~ 5 to 20 mg), prompted HR-MAS NMR application for biopsy analysis in the medical field. In 1996 HR-MAS provided high resolution spectra of lymph node tissues with high fat content, in a reduced experimental time that prevented sample degradation [53]. Since then, HR-MAS has been applied in several fields and extensively biological in the medical field to study the metabolic profiles of different tumor tissues for biomarker identification [54,55]. Using HR-MAS alone is not ideal, while it provides excellent information on soluble and gel components, no information is obtained for the true solids.

1.3.4.4 Comprehensive Multiphase (CMP) NMR

To truly gain a comprehensive view on biological systems (environmental issues or biological purposes), one should be able to follow the organisms and the processes that happens in all their components in the intact and native state. CMP-NMR offers such a holistic view of systems and provides the ability to study all phases simultaneously, with an insight beyond just the various phases, but the arrangements, conformations, and organization between them.

Comprehensive Multiphase NMR (CMP) probes developed in 2012 [45] incorporates the components of a HR-MAS probe with added ability of high power RF handling to study the true solids. The technique allows all components within the different phases (liquids, gels and solids) of a multiphase sample to be studied and differentiated in a universal approach. CMP-NMR can be thought of as changing the NMR technology to match the sample rather than the more traditional vice–versa. The approach does not require sample preparation such as extractions or drying and samples are studied in their native phase, as is.

CMP was initially applied to a soil sample where separate 1D $^1$H and $^{13}$C NMR spectra were obtained for each phase [56]. This work also demonstrated that it can serve to study the binding interactions between soil/contaminant domains. CMP has since been successfully applied to study molecular structures in natural samples such as soil [56] and intact plants [57], the transport of
molecules across phases [58], swelling within phases [59,60] of rubber by biofuels [57,61,62] and to follow biological mechanisms in growing seeds [61].

The novel level of information provided by CMP encouraged its application further to *in vivo* studies on a whole living system [63]. Before proceeding to NMR studies of living organisms, a brief discussion on *in vivo* NMR and its distinct advantages will be provided.

### 1.4 *In vivo* NMR

#### 1.4.1 Background

Although *in vivo* NMR was initially introduced in 1981, at the present NMR is not routinely employed in environmental assessment (i.e., not widely used by environmental agencies). *In vivo* studies using a high-resolution NMR system combined with 2D NMR and isotopically enriched organisms are a new methodology with examples only reported recently [51,64] and great promise for future applications.

The focus of this research was to highlight the potential of analyzing small invertebrates organisms (commonly used for environmental toxicity testing) using high resolution vertical high-field NMR spectrometers rather than MRI systems. Magnetic Resonance Imaging (MRI), a routinely used technique in the medical field, is based on the application of a low field magnet (2T). Although MRI can provide *in vivo* information on the contrast and distribution of $^1$H signals arising from the lipid and water contents of different organs, it provides limited biochemical information, due to magnetic susceptibility distortions in static samples and is mainly used to identify physical anomalies for diagnostic purposes. However, with surface coils close to the object to reduce field inhomogeneity, additional information on individual metabolites can be obtained [65].

In contrast, the high-field NMR spectrometers, 11.7 T, used in this thesis have numerous advantages over the aforementioned techniques. In addition to higher external field which provides high resolution data and increased sensitivity, extensive shim systems that contribute to considerably higher magnetic homogeneity (i.e. improved line shape and more chemical
information), multiple channels (i.e. permit H-C studies to improve spectral dispersion) and lock systems (to provide stability over long experimental acquisitions) provide considerable advantages. In this regard, use of smaller organisms such as *D. magna* and *H. azteca* and the additional molecular information afforded by *in vivo* NMR give insight into “how” and “why” specific chemicals are toxic, information necessary for developing robust environmental regulations.

The first *in vivo* NMR on whole organisms was reported in early 80’s [66] suggesting high potential for aquatic toxicity evaluations. However, the next *in vivo* NMR was reported almost a decade later where ^31^P NMR was used to detect the energy status through the phosphorylated metabolite fluxes in *Oryzias latipes* during embryogenesis and hypoxia. The fish was kept within a flow set up that enabled delivery of oxygen and controlled the hypoxia conditions [67]. However, one major drawback of these NMR techniques was that magnetic susceptibility mismatches across the organism lead to broad lines and little metabolic information in the ^1^H NMR spectra.

A particularly useful tool for bridging the analytical gap between solution state and *in vivo* NMR is HR-MAS. The technique proved to be highly versatile in metabolomic analysis of biopsy samples where *in situ* monitoring of lipid and aqueous metabolites is crucial in the diagnosis. The increasing interest towards metabolic profiling of the intact tissues [68] and the strong correlations observed between metabolites and disease pathologies encouraged the application of HR-MAS to living systems. In 2003 in a breakthrough study an ultra-slow spinning HR-MAS was applied to a mouse using low field (1.5 T), horizontal magnets at spinning rate as low as few Hz [69], albeit the approach was poorly resolved to identify discrete metabolites. HR-MAS was then further applied to endogeic earthworms (*Aporrectodea caliginosa*) where *in vivo* ^31^P and ^1^H NMR metabolic profiles of body parts were obtained using conventional NMR spectra and were compared to HR-MAS spectra [70]. The results showed a significant improvement in terms of the line shape and resolution. This study applied 2D NMR for *in situ* assessment of gel-like domains and lipids, along with ^31^P NMR to monitor energy metabolism at 2 KHz. Later the *in vivo* 1D ^1^H NMR lipid profiles of sedated *D. magna* were studied and correlated to physiological state using HR-MAS. The lipid profiles were compared among *D. magna* at different life stages, as well as pregnant versus non-pregnant populations [64]. The NMR spectra mainly comprised overlapping lipid resonances with limited information as to other metabolites. In 2014 1D ^1^H NMR HR-MAS
was applied to identify the changes in the lipid layers of mutants compared to healthy flies (*Drosophila melanogaster*). In addition, $^1$H HR-MAS NMR combined with localized imaging was applied to identify the metabolite composition of different anatomic parts of fly by placing the organism inside an insert positioned alongside the spinning axis of the rotor, allowed spinning up to 2.7 KHz. *In 2014, a micro HR-MAS using low volume of whole Saccharomyces cervisiae* cells and coil spinning at 300 Hz, studied the metabolites correlated with growth rate under osmotic stress and aging [71]. However since then only a limited number of studies have been dedicated to the metabolomic profiling of a living organism under HR-MAS conditions [70].

1.4.2 Why *in vivo* NMR?

*In vitro* and *in vivo* NMR approaches both allow molecular-level investigation of the chemical mechanisms in the cells, tissues and organism [65,72], however each with its own advantages. Although, *in vivo* NMR provides unprecedented levels of information in the most realistic state, established metabolic *in vitro* methods using extracts or biofluids are more commonly applied [73]. *In vitro* studies are popular as they can be conducted relatively fast and a lot of data can be collected at once. *In conventional in vitro* metabolomic studies, samples are extracted or the whole organisms are homogenized for study. Therefore, compared to whole living organisms, they have reduced susceptibility distortions which leads to sharper lines and ultimately easier spectral interpretation. However, their limitation originates from the fact that real-time biochemical fluxes cannot be monitored. Furthermore, hydrophobic and hydrophilic metabolites often have to be studied separately (due to solubility in different solvents) which raises the question as to "how representative these extractions are to the original composition of the tissue?". As an example the synaptic vesicles in neurons are poorly soluble in water and by extraction lose their structural integrity [74]. Additionally, there is always the uncertainty as to whether or not some biomolecules have been modified as a result of the extraction procedures.

*In vitro* studies capture a snapshot of the metabolome profile at one point in time [27,73,75]. In order to follow the fate of the contaminant, a series of different organisms are sampled at different time intervals. *In vivo* studies minimizes systematic errors due to the natural variability, as it allows for an organism before exposure to serve as its own "control" [76].

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As a result, smaller fluctuations in the metabolite structural composition can be more easily discerned [67]. Additionally, in vivo studies provide unrivalled information, as it permits the potential for time course investigations from pre-exposure to post-exposure and into a recovery phase with high temporal data acquisition, which helps to build the connectivity between the biological processes involved. The cellular level of information concerning both the mode of toxic action (a common set of physiological and behavioral signs that characterize a type of adverse biological response) and the mechanism of action (the biochemical processes underlying a given mode of action) obtained through in vivo studies are key to understanding the nature of the contaminants in question [77]. This type of information is crucial for policy makers, as it will allow differentiation between a temporary shock response (deviation from, and subsequent return to homeostasis) from a permanent biochemical change (no return to homeostasis, i.e. likely a precursor to disease).

Another advantage to in vivo HR-MAS NMR is compliance with the recent initiatives by the environmental toxicity regulations that recommend establishing new approaches to reduce the number of animals used in toxicity testing [78]. However, there are inherent challenges associated with in vivo NMR including (challenging water suppression, low signal intensity, requirement for oxygen and food delivery during the experiment, and continual movement of the organisms in and out of the detection coil, heterogeneity of whole organisms etc. (discussed later)). While requiring technically challenging solutions these drawbacks are generally minor compared to the potential wealth of novel scientific insight gained. In vivo NMR also has great potential as a complementary approach to conventional in vitro studies. For example, an in vitro approach to initially identify potential biomarkers or screen a wide range of stressors, followed by more targeted in vivo studies to provide more information on the most problematic contaminants and mixtures.

Currently in vivo NMR can be performed using two different approaches: in the solution state (i.e. organisms freely swim in NMR tube) [50,51] or under MAS conditions (organisms inside a rotor) [64]. The key technical difference between the two approaches is that, solution state in vivo NMR represents a static system, whereas the MAS approach requires the organism to be spun inside a rotor at the magic angle. Each system provides a unique environment to study organisms in vivo.
1.4.3 Static \textit{in vivo} NMR

The static system provides a low stress environment where the organism can be kept alive indefinitely. In this method a flow system (as shown in Figure 1.2) supplies food and/or oxygen and doses of contaminant to the organism and permits the study of environmental variables such as different food sources or toxins on-line. The limitation is that only dissolved metabolites can be detected and other components such as bound metabolites, tissues, and structural components remain undetectable. Furthermore, susceptibility mismatches across the organism lead to broad proton line shape, which means currently the best way to recover metabolic information is through 2D $^{1}$H-$^{13}$C correlations.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1_2.png}
\caption{Comparison of the two \textit{in vivo} NMR methodologies, A. Schematically shows the flow system used in the static system to provide oxygen/food as well as the organisms inside the NMR tube B. Represent the rotor in spinning position inside the coil and the organisms in MAS based studies.}
\end{figure}
1.4.4 *In Vivo* CMP-NMR

Biological samples are highly heterogeneous multiphase samples; specifically, living systems are a synergism of liquids, gels and solid components [79,80]. While, it is the interactions among these phases that regulates the complicated biological activity, a comprehensive non-invasive multiphase analysis tool is required to truly understand living processes. Traditionally, due to different NMR hardware requirements heterogeneous samples had to be either dried for solid state or extracted homogenously for solution-based NMR. While previous HR-MAS studies provide excellent information on soluble and gel components, no information is provided for the true solids. Each group of components provides complementary information; dissolved metabolites provide an information-rich molecular fingerprint that can help explain biochemical fluxes induced by environmental stressors. Similarly, gels (i.e. membranes, proteins) and solids (i.e. cell walls, shells) hold the potential to comprehend changes to the structural framework of a living system.

Environmental stressors can adversely impact organisms, and changes can manifest as a metabolomic response or cause epigenetic changes that eventually result in phenotypic variation [81]. Studies on crustaceans subject to excessive environmental pollutants in addition to gametogenesis and metabolism changes, have revealed alterations in the exoskeleton integrity [82,83]. These structural changes in turn, might increase permeability to other toxins in the polluted waters and endanger the species ([81] [84]). The same way, bone degradation (osteoporosis) can lead to fragility in human [85]. However, many of these structural changes (within the aquatic organisms) cannot be investigated through conventional NMR methodologies or static flow studies. As such there is a considerable need for an analytical approach to bridge the gap between the rapid stress responses (metabolomic fluxes) to slower “structural” impacts (for example changes in the solid exoskeleton/shell) due to long term exposure [82,83]. Ideally the solution is a "molecular interpreter" to connect the physical and structural indications of the disease with the underlying biological processes in a non-invasive way.

*In vivo* CMP-NMR permits both, real-time measurement of metabolic fluxes in response to environmental changes, as well as, the structural changes (i.e. shell thinning) and cellular framework (proteome, bioaccumulation in membranes). The recent advances in NMR's capabilities *in vivo* makes it an indispensable analytical tool for environmental scientists.
technique is ideal for studying *in vivo* transformation, bioaccumulation and excretion of contaminants. In addition, synchronized responses in the metabolome explain the impact of the chemicals on living systems and the biochemical pathways affected (precursors to disease) and thus holds great promise to correlate the environmental impacts and medical implications of exposure.

While flow based studies are more feasible, complementary CMP studies are capable to study contaminant species that bind to proteins, partition into cells walls etc. and provides an ideal platform to study contaminant binding, distinct type of information and when used in combination in the future, the techniques will provide a more complete picture and deeper insight to the processes such as chemical exposure or dietary changes.

1.4.5 Subject Organisms

Very limited number of clinical toxicology studies are permitted on humans. However, the central pathways of metabolism such as glycolysis and citric acid cycle and many cellular functions have been highly conserved during eukaryotic evolution in invertebrates and vertebrates, Therefore, often smaller animals can be utilized as surrogates in evaluation studies [86]. The use of surrogates allows for controlled environmental variables with controlled dosage/route of exposures to be tested. The organisms are selected based on experimental goals, accessibility and ease of culture in the lab environment, as well as, the amenability to the desired manipulations. For the purpose of this research, commonly used model organisms in aquatic and sediment toxicity testing that were also small enough to fit a 4 mm rotor were employed.

*Daphnia magna* (water flea) is a planktonic crustacean with an average of 4 mm in length and 2 mg of dry mass. It is a keystone species in aquatic systems and as a herbivore highly valuable in the trophic transfer of both nutrients and toxins to larger predators in the food chain. It is highly sensitive to contamination and therefore, has been extensively deployed in aquatic toxicity [87-89]. Their bodies are enclosed by a shell largely consisting of chitin which provides additional structural support (for MAS studies).
*Hyalella azteca* (fresh water shrimp), is an amphipod crustacean that swims freely, as well as burrow into sediments, and is widely distributed throughout North American fresh water. *H. azteca* are few millimeters long and well-fitted for high resolution NMR spectrometers. They feed on algae or bacteria that adhere to sediment particles [90] and are an important link in the aquatic food web consumed by fish and larger invertebrates. Benthic organisms contain multiple exposure routes (i.e. diffusion from both water and sediment, ingestion of sediment) and can open new windows into toxicity. The widespread distribution of *H. azteca* in fresh water sediments together with their high sensitivity to biotic and abiotic variables and measurable response made this species one of the most frequently used organisms for both aquatic and sediment toxicity assessments by Environment Canada and USEPA. *H. azteca* was initially used as model organism by Canadian researchers Burgman and Munawar in 1989 [91]. The first protocol regarding their use in toxicity measurements was established by Environment Canada in 1992 followed by USEPA in 1994. Following the concluding remarks, that sediment contamination poses a potential hazard for the animals and humans feeding on fish, American Society for Testing and Materials (ASTM) [92], developed sediment-associated toxicity test procedures in 2002.

Over the past few decades numerous studies have been conducted on *H. azteca* to investigate sediment and aquatic toxicities [1,93-97], while studies on *D. magna* are limited to aquatic studies [87,88,98]. The mechanisms of metal pollutants bioaccumulation and uptake in various combinations of metals have been studied using *H. azteca*. Acute and delayed effects of pulse exposure of pesticides, on *H. azteca* has been also examined [99][94,97]. *H. azteca* has been used as a field biomonitor to study the effects of metal contaminations in the rivers near the mines in northwestern Quebec [100]. *H. azteca* has been used to investigate the correlation between the toxicity of metals in overlaying water and their bioaccumulation in the sediments. In addition, it has been used to define toxicity thresholds for different metals [101].
1.5 NMR Experiments

This section outlines basic experimental techniques most relevant to the study of whole living organisms.

1.5.1 Spectral Editing Techniques

This series of experiments use molecular diffusion and relaxation filters to spectroscopically isolate the different phases within the intact heterogeneous samples without chemical or physical separation in the sample. The experiments can be applied on both $^1$H and $^{13}$C NMR spectra resulting in separate spectra for the various phases.

1.5.1.1 $^1$H NMR Detection

With close to a 100% abundance and high gyromagnetic ratio, $^1$H NMR spectroscopy has the highest sensitivity in NMR studies. However, given that all the biological molecules contain $^1$H, the $^1$H 1D NMR of complex samples can be poorly resolved overlapping signals within the relatively narrow chemical shift range of only 0-10 ppm. In conventional $^1$H NMR spectra of biological samples, consisting of components with varying abundance, size and relative mobility, small metabolites are usually masked by the more abundant molecules such as lipids. Therefore different methods have been developed in order to discriminate the different components. However, under MAS $^1$H NMR detects bonds that exhibit some mobility (i.e. liquids, gels and semi-solids), while true solids are broadened out by strong $^1$H - $^1$H couplings. As such for true crystalline solids $^{13}$C detection is more commonly used (see later). The signals from solution, gels and semi-solids can be discriminated based on relaxation or diffusion. More detailed discussion is given in Chapter 5 as such here only a brief summary is provided.
Diffusion Filtering: By applying diffusion-based experiments molecules are separated based on their size, shape and movement over a controlled time in magnetic field gradient. First a reference spectrum identical to a conventional single pulse $^1$H NMR with no gradient field and no diffusion time. The reference experiments detect all the molecules from solution through to semi-solids.

In diffusion editing, the gradient encodes the spatial position of signals at the start of the experiment and then decodes them at the end [102]. The signal from molecules that diffuse and move positions are not refocused and are attenuated. The result is the DE spectrum represents the gel-like components with enough dynamic to survive the diffusion delays, and limited diffusion. Subtraction of the DE from the control spectra yields in the most dynamic and fast diffusing components referred to as Inverse Diffusion Editing (IDE) which contains the dissolved fraction which freely moves within the sample.

In DE experiments, the long delays required to allow the diffusion to take place may also result in the loss of semi-rigid species through fast transverse relaxation. If a full monitoring of the components is desired a subsequent experiment Relaxation Arising from Diffusion Editing (RADE) is performed. The spectrum is produced by comparing the reference spectrum (gradient and diffusion time off) to a second spectrum where the (gradient is off, but the delays are set as required for diffusion editing). The experiment is discussed more in Chapter 5 but is designed to recover components with very fast relaxation, i.e., the semi-solids.

1.5.1.2 $^{13}$C NMR Detection (Semi and rigid solids)

$^{13}$C NMR provides an overview of the carbon containing metabolites and structural components. Despite the improved resolution in $^{13}$C NMR due to higher chemical shift range (0-200 ppm), the low natural abundance and low gyromagnetic ratio of $^{13}$C lead to unacceptably long data acquisition times, limiting its application in vivo. Therefore, isotopic enrichment is necessary to enhance the sensitivity in $^{13}$C NMR spectra. Another important consideration in application of $^{13}$C NMR is the large $^1$H-$^{13}$C couplings (typically range from 100 to 250 Hz), which reduces the sensitivity and complicates the spectra especially in solids. CMP probes have the ability to handle the intense RF fields required by high decoupling (narrows $^{13}$C lines) and cross polarization
(enhances the $^{13}$C signal via magnetization from protons in proximity) powers required for the optimal $^{13}$C detection of the solid phases within the sample.

1.5.1.2.1 $^{13}$C Cross Polarization (CP-MAS)

CP-MAS is a solid state NMR experiment used to enhance an insensitive nucleus increasing magnetization by polarization transfer from neighboring spins of higher gyromagnetic ratio [103]. This approach uses the strong dipolar couplings within the solid structures to transfer the magnetization of an abundant spin system to a dilute spin system ($^1$H to $^{13}$C) and enhances the sensitivity of the lower spins by maximal signal enhancement factor of $\gamma/\gamma$ (~ 4 for $^1$H-$^{13}$C). CP-MAS can be applied to identify all solid components such as true solids (bones, shells) and rigid-gels (structural lipids) in a living system. As CP-MAS transfers signal through rigid dipoles it is only efficient for rigid bonds and cannot detect mobile gels or solutions.

1.5.1.2.2 Relaxation Filtering of solids

Relaxation filtering can be applied to differentiate true crystalline solids from the semi-solids. The $T_2$ relaxation time varies among the nuclei in different molecular environments. For example, true solids exhibit very fast $T_2$ relaxation, while the bond motion in semi-solids extends the relaxation time significantly. By applying a short $T_2$ filtered the signals from true solids can be suppressed leaving just the semi-solids. The true solids can be recovered by difference, via subtraction of the $T_2$-filter CP experiments (semi-solids only) from the CP experiment (both semi and rigid solids). The experiments are applied to a living organism in Chapter 2 and discussed in great details in chapter 5.

1.5.2 2D NMR

The additional resolution afforded by second dimension in 2D NMR spectra reduces spectral overlap and provides correlation information for the identification of a range of metabolites. The peak capacity of NMR is significantly increased from 5000 and 30,000 respectively in 1D $^1$H and
in $^{13}$C NMR, to a reported resolving power of 2,000,000 per unit in 2D $^1$H–$^{13}$C HSQC [104]. A 2D molecular profile of a sample is unique under the same conditions and provides a fingerprint as to chemical composition and metabolites. There is an increasing number of pulse sequences developed that each highlight different correlations. Some of the most commonly used 2D approaches include Correlation spectroscopy (COSY), Hetero-nuclear Single Quantum Coherence (HSQC) and Hetero-nuclear Multiple Quantum Coherence (HMQC) and are introduced here.

1.5.2.1 Correlation Spectroscopy (COSY)

Is the simplest two dimensional homonuclear experiment in which the cross peak represents $^1$H-$^1$H correlations in both dimensions. It provides information on the spin scalar coupling (in simple terms identifies connections between protons on neighboring carbons) and can be used further as a complementary technique to confirm assignments made using other 2D experiments.

1.5.2.2 Hetero-nuclear Single Quantum Coherence

Two dimensional $^1$H observe experiments are widely applied for identification of $^1$H–$^{13}$C and $^{15}$N–$^1$H correlations in biological samples. The horizontal axis describes the $^1$H chemical shift and the vertical axis represents the hetero-nuclei. Effectively each cross peak can be defined by two coordinate chemical shifts and is useful for structural assignments. Hetero-nuclear correlations can arise via a single quantum coherence pathway (HSQC) or a multiple quantum pathway (HMQC). Theoretically HMQC and HSQC should result in similar spectra, however, in practice the exact performance is often sample specific. HSQC theoretically gives narrower proton lines as the $^1$H-$^1$H coupling is refocused during the experiment [105], while HMQC generally provides better water suppression [106,107].
1.5.3 Water suppression

Solvent suppression is essentially required for \(^1\)H NMR detection techniques. Living organisms swim in 100% water. In addition, within a living system the water exists in bound, gel-like and free environments that further broaden the water baseline even under MAS. If water suppression is not performed, then the large signal distorts the NMR receiver signal. Once suppressed the gain of the receiver can be increase providing improved dynamic range and better detection of low concentration components.

Many solvent suppression techniques have evolved over the years to accommodate a variety of samples in different conditions [157]. Presaturation is a simple two-pulse experiment that utilizes a relatively long, low power RF pulse to selectively saturate the water, and a non-selective 45- 90° pulse to excite the desired resonances. This pulse sequence is particularly useful for aqueous samples but fails in the presence of a very intense water signal [158].

Pulsed field gradients have introduced new possibilities of water suppression techniques such as W5-WATERGATE [108] [165] and PURGE [109]. At MAS speeds of 300-1000Hz, PURGE [109], which combines gradients, echoes and relaxation to cancel the water is highly efficient as discussed in Chapter 3. The most effective water suppression sequence at spinning speeds >1.5 KHz applied \textit{in vivo}, is Shaped Presaturation W5-WATERGATE. This technique is simple to implement and builds upon the W5-WATERGATE sequence by adding a train of shaped water presaturation pulses before the W5-WATERGATE [145] to enhance the line shape. Subsequently, the DANTE blocks within the W5-WATERGATE [110] invert the sample signals and the residual water is dephased using pulse field gradients. With proper optimization, the resulting spectrum can be mostly free of the residual water signal and lead to improved Signal-to-Noise (S/N) for solute resonances.
1.6 Research objectives

The scope of the current thesis is to develop a CMP-NMR based in vivo NMR spectroscopy method. This thesis included the first applications of CMP-NMR to a living organism and allows for the first time all bonds (solutions, gels and solids) to be detected in vivo.

The results of this dissertation should directly appeal to researchers in environmental fields looking to understand what compounds in the environment adversely affect the species health. The implications of the results will also considerably impact many fields involving the living systems or the processes relating to living systems. In vivo CMP-NMR, introduced here, has great potential to monitor the transformation, excretion and bioaccumulation of the chemicals, the organism’s metabolic response to the chemical, any subsequent structural changes (proteins synthesized, changes in bone/shell etc.) and follow a possible recovery from exposure. In turn such information is likely to become key to setting the most effective future policies on chemical use and permissible levels in the environment. Moreover, the medical field will greatly benefit from CMP-NMR’s unique ability to study all components; the approach provides the framework to better diagnose, treat, and develop novel treatments for many diseases.

In spite of numerous advantages of MAS based methods, organisms can be adversely impacted by the high rotation speeds required to enhance the line shapes. Therefore, the main drawback of the MAS based approaches is the limitation imposed by the speed and duration of the spinning. Also, the limited oxygen level inside a rotor causes hypoxia reducing the experimental time. These shortcomings have been addressed in the chapters 3 and 5. In Chapter 4 novel approaches for monitoring biological pathways were explored by deploying hetero-nuclei and isotopic enrichment of the organism.

In summary, given NMR’s capability to examine within a living organism with unprecedented information on its metabolome, in vivo NMR has great potential to evolve as a key tool for understanding biological processes and how living systems are impacted by toxic chemicals.
1.6.1 Research Topic 1 *In vivo* CMP-NMR Applied to a Whole Organism

**Research Hypotheses:** Can CMP-NMR be applied to a living Organism and observe all its components?

As explained earlier NMR has developed as separate methodologies to study solids, liquids and gels. The CMP-NMR combines all these methodologies and allows all phases to be detected at once. The objective of this chapter was to apply the CMP-NMR to a living organism for the first time. *H. azteca* (freshwater shrimp) was used due to its importance in aquatic and sediment toxicity testing. The goal was to develop a technique to differentiate the full range of components, from rigid polysaccharides (chitin in the shell) at one extreme, to soluble metabolites at the other in a whole intact organism. In this method spectral editing approaches combined with 2D NMR and $^{13}$C isotopic enrichment of the shrimp allowed over 40 metabolites and a range of structural components in the various phases to be identified. Customized rotor caps were used to allow
oxygen delivery into the rotor and elevate the organism survival rates. This approach can serve as a complementary method to conventional metabolomic studies on extracts rather than intact organisms to investigate the molecular-level sub-lethal toxicity [47]. The developed technique has great potential to study molecular binding, identify receptors, measure diffusion and trace dynamics across the interfaces from liquid to gel to solid phases. The result is an unprecedented window into the fate of molecules inside living organisms and natural intact samples [56].

1.6.2 Effective combined water and sideband suppression for low-speed tissue and

\textit{in vivo} MAS NMR

Research hypotheses: How will the reduced spinning impact the stress exerted on the organism in \textit{in vivo} CMP studies? Can \textit{in vivo} CMP-NMR be applied at lower spin rates with preserved resolution?

In CMP-NMR the high spinning speeds, normally applied on non-living samples (5-20 KHz), are not viable for living organisms or tissues since the centrifugal forces destroy the integrity of the living cells. As such, Chapter 3 introduces a high resolution 1D $^1$H NMR methodology at low spinning speeds (< 500 Hz), that permit longer experimental times and a wider range of biological samples; cells, tissues or organisms to be studied.

Slow spinning gives rise to spinning sidebands (artifacts) that mask the real signals. In addition, in the presence of the sidebands, the isotropic signal is split amongst sideband components leading to loss of signal sensitivity. The sidebands arise from the modulation of the Larmor frequency during the spinning caused by chemical shift anisotropy and relocate according to the spinning rate. At high spinning rates (2.5 KHz and above) water sidebands are shifted outside the $^1$H spectral region (0 and 10 ppm) at 11.7 T (500 MHz). In order to perform studies at lower spin rates these sidebands need to be selectively attenuated with minimum signal loss.

In this chapter, water peaks and the sidebands were successfully suppressed by concatenating two sideband suppression sequences, Total Suppression of Side Bands (TOSS) (TOSS.243) [111], and Phase-Adjusted Spinning Sidebands (PASS) [112] with Presaturation Utilizing Relaxation
Gradients and Echoes (PURGE) [109]. Initially, the pulse sequences were optimized on an earthworm, with high biomass to precisely locate the intense sidebands. Subsequently, the suppression techniques are further optimized on delicate samples using a rotor of whole D. magna, ex vivo. Finally, when applied in vivo to H. azteca, highly resolved 1D spectra in spinning rates as low as 500 Hz are resulted [113,114]. When compared the two sideband suppression techniques at 500 Hz, PURGE-TOSS.243 shows 2.5 times higher signal to noise ratio in vivo and is a more robust experiment that can be readily applied to 2D NMR. Furthermore, the resulting spectra are comparable to 1H spectrum of H. azteca at 2500 Hz with amino acids and proteins and many other resonances observed. Interestingly, viability tests at 500 Hz spinning, revealed a 100% survival rate for H. azteca for over 24 h which will significantly extend the experimental time from 12 h (in Chapter 2) to over 24 h.

1.6.3 Research Topic 3 Investigating Multinuclear Approaches in in vivo Comprehensive Multiphase NMR

Research hypothesis: Can CMP-NMR and hetero-nuclear NMR be combined to provide a more comprehensive understanding of in vivo metabolism and contaminant fate in a living organism?

In Chapter 2 we concluded that CMP-NMR can be utilized to examine the metabolic profile of an organism. While in the previous chapters the focus was mainly on 1H NMR, in this chapter, the bond specific incorporation of hetero-nuclei, including 2H, 13C, 15N and 31P into H. azteca were investigated in vivo.

This chapter follows two main objectives: to study biomolecular changes within the organism due to exposure to isotopically enriched nutritional/contaminant impacts substrates or to trace the fate of contaminants, themselves. CMP probes have the ability to follow the contaminant fate inside the body of an organism in close to real-time. NMR can potentially explain the mechanisms by which contaminants bind in vivo, identify binding sites, and study bioaccumulation and transformation. This holds specifically true if the stressor contains a NMR active nuclei such as 2H, 19F or 31P. Present in many pharmaceuticals (19F) and environmental contaminants (19F and 31P), hetero-nuclei a range of 1D (directly detected 31P NMR and 19F NMR) provide a unique probe
for more selective studies by NMR to elucidate their dynamics. $^1$H can be replaced by $^2$H as a label in practically any organic molecule (i.e. amino acids studied here) without changing its chemistry.

NMR based metabolomics has immense potential for studying the correlation between the metabolite fluxes due to exposure to a range of nutrients, pathogens or toxins. In this chapter stable isotopes ($^{15}$N, $^{13}$C, $^2$H) from different algae sources are incorporated into the organisms and the resulting labelled metabolites are identified in vivo by taking advantage of 2D correlation experiments (i.e., $^1$H-$^1$C, $^1$H-$^{15}$N, $^2$H-$^{13}$C) to reveal the potential nutritional/contaminant impacts on the organism. In addition, the full solids capabilities of CMP provide the option to investigate rigid nitrogen components within the organism. Combined this information provides a unique window into the metabolic pathways, carbon transfer and growth processes in vivo, as well as, elucidate the biotransformation, bioaccumulation and excretion of the stressor molecules [115].

1.6.4 Research Topic 4: In vivo Ultra Slow MAS $^2$H-$^{13}$C NMR Emphasizes Metabolites in Dynamic Flux.

Research hypothesis: Can CMP-NMR and 2D $^2$H NMR follow metabolomic processes at ultra-slow spinning and extend the experimental time?

In this chapter concerted efforts were made to overcome the main drawback of utilizing CMP-NMR for in vivo applications by reducing the stress exerted from sample spinning in a novel 2D NMR. With $^1$H detection, if the sample is spun too slow, spinning sidebands obscure the isotropic information. In this chapter, a new approach using $^2$H detection is introduced using organisms that are both $^2$H and $^{13}$C enriched. While $^2$H possess a significant quadrupole coupling, for metabolites under isotopic condition, their quadrupole couplings are often averaged out to zero. Larger structural components exhibit wide $^2$H profiles and are not detected via a 2D $^2$H-$^{13}$C experiment. As such it is discovered that 2D $^2$H-$^{13}$C NMR provides a selective window emphasizing the dynamic fully dissolved metabolites (i.e. those most likely in flux) while suppressing the structural framework of the organism. As the dissolved fraction that is selected by $^2$H-$^{13}$C 2D is relatively homogeneous, it produces negligible sideband intensity. In addition, the absence of a dominant water peak makes this technique applicable at 50 Hz. Concludingly, high resolution 2D $^2$H-$^{13}$C
fingerprints can be collected *in vivo* while spinning at only 50 Hz. This greatly reduces the spinning stress and allows organisms to be kept alive for over 48 h inside the NMR. Further, we briefly compare 2D sequences in terms of their ability to collect $^2$H-$^{13}$C NMR data *in vivo*. This study monitors the biomolecular responses in both *D. magna* and *H. azteca* under starvation and feeding conditions for almost a day. To our knowledge this method represents the only ultra-slow MAS (<100 Hz) approach to date with detailed metabolic information.
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Chapter 2

Comprehensive Multiphase NMR Applied to a Living Organism

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2.1 Abstract

Comprehensive Multiphase (CMP) NMR is a novel technology that integrates all the hardware from solution-, gel- and solid state into a single NMR probe, permitting all phases to be studied in intact samples. Here, Comprehensive Multiphase (CMP) NMR is used to study all components in a living organism for the first time. This chapter describes 4 new scientific accomplishments summarized as: 1) CMP-NMR is applied to a living animal, 2) an effective method to deliver oxygen to the organisms is described which permits longer studies essential for in-depth NMR analysis in general, 3) a range of spectral editing approaches are applied to fully differentiate the various phases (solutions (metabolites) through to solids (shell)) 4) $^{13}$C isotopic labelling and multidimensional NMR are combined to provide detailed assignment of metabolites and structural components in vivo. While not explicitly studied here the multiphase capabilities of the technique offer future possibilities to study kinetic transfer between phases (e.g. nutrient assimilation, contaminant sequestration), molecular binding at interfaces (e.g. drug or contaminant binding) and bonding across and between phases (e.g. muscle to bone) in vivo. Future work will need to focus on decreasing the spinning speed to reduce organism stress during analysis.

2.2 Introduction

Living organisms are defined as systems where metabolism is identifiable. A wide range of environmental factors including physical (temperature, light), chemical (nutrients, drugs, contaminants) and biological (pathogens) can impact metabolism and lead to subsequent disease or change in physiology [1]. Living organisms encompass a range of materials from soluble metabolites at one extreme, through gel-like components (e.g. proteins, muscle, membranes) to true solids (bone, shells) at the other. Changes in shell thickness and structure in the organism can result from environmental and contaminant stress, similarly in human, changes in bone function develop conditions such as osteoporosis [2] and Paget’s disease [3]. The overall integrating theme is that living systems are multifarious and it is the delicate synergism between physical
organization, chemical reactivity, and biological processes that give rise to life. As such, to truly understand biological function and response, analytical tools that can comprehensively study all components within living organisms at the molecular-level resolution are desperately required.

Solution-state NMR and MRI have traditionally been used to study living organisms. To date MRI has been arguably the most powerful tool for the study of living systems. Routine MRI studies map water concentrations giving rise to images from which critical diagnoses can be made [4]. Advanced studies even permit localized spectroscopy which can identify dissolved metabolites. However, there are numerous limitations to MRI based methods which include: 1) Only true dissolved molecules can be observed 2) Magnetic susceptibilities lead to broad spectra making identification and quantification challenging 3) The larger sample volume in MRI systems results in less homogeneous magnetic fields 4) A lack of lock circuitry reduces stability overtime (broad lines) 5) Limited spectrometer channels and multinuclear capabilities impede the collection of multinuclear correlation spectroscopy critical for providing spectral dispersion and molecular assignment in complex samples. One simple option is therefore to study living organisms with high resolution solution state NMR spectrometers. Indeed, such studies are highly informative, however, as with MRI, information can only be extracted from the truly dissolved components [5].

When considering molecular processes (for example, incorporation of dissolved nutrients into solid bone, the crystallization of soluble amyloids to form crystalline fibrils in Alzheimer’s, Huntington's and Parkinson's disease) or bioaccumulation of dissolved contaminant or drug within the tissue, the ability to monitor the conversion of one phase into another or the transport across different phase boundaries is essential.

MAS-NMR is required to average chemical shift anisotropy and reduce magnetic susceptibilities in swollen samples permitting both solution state and swellable (dynamic gels) materials to be investigated. Pioneering studies have been performed showing that organisms can be kept alive under MAS conditions for short periods and high resolution spectral information can be extracted [6]. However, all studies to date have utilized High Resolution Magic Angle Spinning probes (HR-MAS) which can only handle low power radio frequency (RF) fields and thus cannot be used to study true solids or rigid gels which require high power decoupling and cross polarization for detection [7].
Figure 2. A) Survival rate in percentage is plotted against several different spin rates (1 h of spinning). B) The influence of oxygen delivery using a customized cap on improving survival rates is depicted. C) The effect of loading different number of organisms on the survival is studied. At 5 °C, with lower oxygen consumption survival rates increase to almost 100%. D) Survival rate up to 16 h is monitored at different temperatures. When the organisms are loaded head first into the rotor there is 100% survival up to 12 h. All experiments were performed in replicates of 3. All studies were performed using the NMR probe external to magnet controlled by a separate variable temperature unit. This permitted use of the NMR spectrometer while these spinning tests were ongoing.
In this study CMP-NMR spectroscopy, introduced in 2012, is applied for the first time to a living system. CMP probes contain a lock, pulse field gradients, high power RF circuitry and are fully susceptibility matched [8]. The result is that all components in all phases can be studied and differentiated in vivo and the full range of solution state, gel-state and solid-state NMR experiments applied without compromise. To demonstrate the proof of principle in this study the organism H. azteca is investigated. One of critically important applications of in vivo NMR is to understand environmental toxicity and stress [9]. Traditionally toxicity has been routinely assessed using growth, reproduction rates or mortality as endpoints. However, over time it has been recognized that this approach alone is insufficient and more information regarding toxic mechanisms and the biochemical pathways perturbed is critically needed to explain, how and why specific chemicals are toxic. This need is summarized in a report “Toxicity Testing in the 21st Century” by the National Academy of Sciences (commissioned by the Environmental Protection Agency) which concludes “The new paradigm should facilitate evaluating the susceptibility of different life stages, understanding the mechanisms by which toxicity occurs, and considering the risks of concurrent, cumulative exposure to multiple and diverse chemicals” [10].

An in vivo approach with the ability to study all phases would be capable of directly evaluating the mode-of-action, bioaccumulation, biotransformation, molecular reactivity, excretion and binding in vivo in response to the organisms’ surroundings. If specific biological responses can be correlated to certain toxins, then it may eventually be possible to interpret what stressors are truly problematic in a contaminated environment by interpreting the biological fingerprints of the native organisms.

H. azteca is considered one of the most sensitive organisms to its environmental conditions and is frequently used and recommended for aquatic and sediment toxicity testing by environmental organizations [10,11]. The organism is used here as a model organism to demonstrate the application of CMP-NMR to study of living organisms in general.
2.3 Results and Discussion

2.3.1 Conditions for Survival

To permit a wide range of NMR experiments required to extract detailed metabolic and structural information, it is imperative that the physical conditions required for \textit{in vivo} magic angle spinning be optimized. The most obvious source of stress is the spinning itself. Figure 2.1A depicts the survival rate at various spinning speeds. Numerous repetitions demonstrate that 2.5 KHz is the highest speed that can be used without affecting survival and to ensure the $^1$H spinning sidebands are outside the spectral region using a 500 MHz NMR spectrometer. In future, while considerable additional research is required to reduce spinning sidebands and suppress water, it should be possible to combine slow magic angle spinning approaches such as PHORMAT [12], which have been shown effective at spinning speeds as low as 1 Hz and for animals as large as rats to be studied. While successful studies so far have identified a handful of metabolites [12] a full multiphase approach could provide information on all components within the organisms and low spinning speed should eliminate spinning stress. In this proof of principle study due to complications of sideband and water suppression at low speeds 2.5 KHz spinning was selected as compromise between NMR spectral information and organism stress.

In addition to spinning, oxygen availability was found to be extremely important to the longevity of the organisms. In a conventional NMR rotor, the sample is sealed air-tight to prevent the water from leaking. While a wide range of approaches were tested to permit oxygen exchange, the simplest solution was to drill a central hole in the rotor cap and proved to be most effective (inset Figure 2.1B). Conventional logic would suggest drilling a hole in the cap would lead to water leakage, however, it was found that while spinning, water pushes against the walls of the seal and a tiny vortex permits air exchange in the center of the rotor. Figure 2.1B shows that the use of a modified cap significantly improves the survival rate. The data for this figure was collected without spinning, such that the influence of spinning (Figure 2.1A) and oxygen availability (Figure 2.1B) can be easily discerned.
The number of organisms per rotor was found to be very important. Tests were carried out with 2-6-week-old organisms, and results revealed that *H. azteca* survive the spinning rate at any age. However, packing more than three organisms result in higher mortality (Figure 2.1C). The tests were performed using the modified cap and at 2.5 KHz spinning for 1 h. In a previous *in vivo* study under MAS, anaesthetization was used to improve the recovery rate of the organisms. However, the use of anaesthetic is not ideal as it alters metabolisms and introduces strong signals into the NMR spectra masking key information. As an alternative, here the experiments were performed at a lower temperature. Figure 2.1C shows that reducing the temperature to 5 °C is beneficial with a 100% survival rate at 2.5 KHz at this temperature observed (Figure 2.1C). To further investigate the effects of temperature organisms were spun at three different temperatures (Figure 2.1D) and their survival was monitored overtime. Organisms at 5 °C had a much higher survival rate, in part due to their reduced heart rate and oxygen consumption at lower temperature [13]. Ultimately, it was found that for the majority of cases spinning at 2.5 KHz for up to 12 h at 5 °C were good experimental conditions yet often one organism out of 4 would die. Further, investigations demonstrated this was caused by evaporation of water through the aeration hole, which is accelerated with spinning. The organism closest to the cap would die while organisms lower in the rotor survived. The simplest solution is to use two medium-large organisms or one very large organism and place them with their head down towards the bottom of the rotor.

Throughout this study, NMR data for a single adult shrimp is reported. Figure A-3 compares the NMR profile for 3 replicates of a single large shrimp (to demonstrate the reproducibility of the approach) with the profile of a single rotor of 3 smaller shrimp for comparison. In general, it was found that smaller shrimp would move in an out of the coil while a single larger shrimp always remained within the coil region throughout. In addition, larger numbers of shrimp resulted in a lactate signal appearing in the NMR as a result of anaerobic stress [5], as well as, alanine, a general stress indicator [14]. The lactate arises likely due to the higher oxygen consumption from a larger number of organisms, also with more organisms packed into the rotor the organisms on top may be forced up towards the water surface. As such we recommend a single large shrimp be used and placed head down such that the gills remain submerged even if some evaporation occurs.

Figure 2.1D shows 100% survival at 2.5 KHz for over 12 h at 5 °C. Using larger specimens prevents them from turning inside the rotor, keeping their gills always submerged. The fact that organisms
with their gills submerged survive longer demonstrates the limiting factor in longer experimental time is not the spinning itself, but due to water evaporation from the hole in the cap. Future work focusing on air permeable, water impermeable membranes may be able to solve this. Appendix Video A-1 shows the *H. azteca* used to collect most of data. (Video A-2) shows the *H. azteca* after 14 h of spinning at 2.5 KHz and 5 °C using the modified cap, 3 weeks following the end of experiments.
Figure 2. A series of 1D direct NMR experiments are performed, A) $^1$H NMR represents an overview of dynamic components (solutions and gels) in *H. azteca* with overlapping resonances dominated by lipids. B) $^{13}$C NMR collected with low power $^1$H and provides an overview as to all the carbon in the organism with the true solids suppressed. C) $^1$H–$^{13}$C CP
2.3.2 Basic NMR Data

\(^1\)H 1D Spectroscopy

Figure 2.2A shows the \(^1\)H NMR spectrum of a single *H. azteca*. Due to the intense water signal from both the external media and water within the organism, W5 WATERGATE (SPR-W5-WATERGATE) developed for the study of natural samples is employed [15]. The \(^1\)H spectrum is dominated by lipid resonances, with protein and metabolite signals also present. As such, the \(^1\)H NMR data in Figure 2.2A represents an overview of the dissolved, gel-like and semi-solid components in the sample, with true solid phases attenuated. \(^1\)H-\(^1\)H dipolar interactions in true solids can lead to proton line widths of many KHz making them too broad for detection using standard \(^1\)H NMR approaches [8].

The solid component, more detailed assignments, and full spectral editing approaches (to separate the solution, gel, semi-solids and solids phase) will be discussed later. The spectrum here was collected using 4096 scans taking 2 h 47 mins. Figure A-1 compares the signal obtained in 256 scans (10.5 mins). It is clear that in the aliphatic region the vast majority of information is retained with only 256 scans, but unfortunately in the aromatic region the signal-to-noise is too low for in-depth analysis. As such if relatively concentrated and resolved resonances in the \(^1\)H spectrum is of interest then collecting real-time (every ~10 min) data should be possible. This however becomes more challenging for metabolites signals buried under the lipid profile which are discussed further in the section “Inverse Diffusion Editing (IDE)”.

1D “High Resolution” \(^{13}\)C NMR with low power decoupling

Figure 2.2B shows the high resolution \(^{13}\)C NMR spectrum collected with low power \(^1\)H decoupling, such that the carbons from the true solids will be discriminated against. The reader can think of the experiment as an overview of all the carbon in the organism albeit with the true solids suppressed (essentially a \(^{13}\)C analogue of the \(^1\)H NMR previously discussed). Using low power decoupling the band width is too narrow to effectively decouple the broad spectral profile of attached protons in the true solid state [8]. Note that in this experiment due to the relatively low signal in the carbon experiments, combined with the relatively mobile nature of the carbon detected with low power decoupling, spinning sidebands were not detected and measures to suppress them were not required. As with the proton spectrum, a strong contribution from lipids is characterized
by the C=C and (CH₂)ₙ resonances. In addition, contributions from carbohydrates and protein (α-carbons and broad aliphatic distribution 10-40 ppm) are apparent.

1H-13C Cross Polarization

1H-13C CP-MAS of a living organism will strongly bias solid-like materials while suppressing mobile components [8]. 13C CP-MAS is a solid state NMR technique that transfers magnetization from proton to carbon via 1H-13C dipoles. The permanent H-C dipoles which exist in solid structures are ideal for cross polarization and CP-MAS is very efficient for true solids. However, upon swelling, water introduces local dynamics which modulate the H-C dipolar interactions in turn reducing CP-MAS efficiency. As such the 1H-13C CP-MAS of a living organism will strongly bias solid-like materials including crystalline solids, amorphous solids and rigid-gels. When applied to the shrimp, strong spinning sidebands were observed consistent with solid material with considerable anisotropy. To suppress the sidebands and permit extraction of the isotropic chemical shift information, total suppression of spinning sidebands (TOSS) was employed [16].

The 1H-13C CP-MAS of the shrimp (Figure 2.2C) shows strong contributions from lipids, and the carbohydrate chitin (the major component of shrimps’ shell). These components are further discussed later. When considered holistically, the 1H, 13C and CP-MAS NMR provide an overview of all the components inside the living H. azteca from soluble metabolites to the most rigid solids. However, due to spectral overlap detailed molecular information is challenging to extract. Hence, a series of spectral editing approaches can be extremely useful, in both reducing overlap and providing complementary information, by highlighting the dissolved, gel, semi-solid, and solid sub-components within the sample.

2.3.3 Spectral Editing

2.3.3.1 Metabolite Spectrum (Inverse Diffusion Editing (IDE))

Inverse Diffusion Editing (IDE) selects molecules that are truly in solution and have unrestricted diffusion. IDE is created by subtracting a diffusion edited spectrum (discussed next) from a reference spectrum without diffusion weighting. As such only molecules that move position in
space (i.e. free to diffuse) are retained and all other molecules are suppressed. In the unedited $^1$H spectrum (Figure 2.2A) lipids (many from cell walls, micelles etc.) dominate the spectral profile masking key resonances from metabolites, whereas in the IDE spectra (Figure 2.3A) they are suppressed. In the IDE spectrum (Figure 2.3A) the lipids (which do not show free diffusion) are suppressed and a range of metabolite signals that were previously suppressed can now be fully extracted [8]. This is critically important as a whole field of research termed metabolomics has evolved, that aims to correlate metabolite response to a range of external variables and stressors [17,18]. Therefore, the ability to extract a high resolution $^1$H profile of just the metabolites in vivo is of utmost importance. Unfortunately, however the IDE spectrum does require collecting a diffusion edited spectrum for subtraction which generally has low signal and requires a large number of scans (4096 required for H. azteca here). As such this limits the temporal resolution at which IDE can be acquired and makes “real-time” flux of these “buried” metabolites signals difficult to obtain. Additional future research into lipid suppression NMR sequences will be required to more effectively extract metabolite profiles in the presence of large overlapping lipid signals.

2.3.3.2 Restricted Diffusion (Diffusion Editing (DE))

Diffusion editing selects components with restricted diffusion. DE involves encoding the spatial position of signals at the start of the experiment and then decodes their position at the end [19]. The signals from molecules that diffuse are attenuated resulting in a selective spectrum of components that do not undergo diffusion, such as; bound species, large macromolecules, biopolymers, lipid vesicles, etc. [8]. Figure 2.3B shows the DE spectrum for the H. azteca which is dominated by lipid signals. These signals also dominate the conventional $^1$H NMR spectrum (Figure 2.2A) and likely arise from cell walls or lipids stored mainly in the muscle tissue or hepatopancreas for energy metabolism [20]. Interestingly, the distinct signal from omega-3 (ω3) fatty acids is present at ~1 ppm, which was previously identified in NMR spectra of Daphnia [21] (see later for more discussion). The aromatic region of the diffusion edited spectrum contains few signals with only a very weak broad profile which is consistent with protein resonances.
2.3.3.3 RADE: Recovering lost $^1$H NMR Semi-solid Signals

RADE is an experiment that recovers the loss of relaxation during the diffusion delays and highlights more rigid/semi-solid components such as semi-solid cell membranes, structural proteins etc. [8]. Diffusion editing based NMR sequences require relatively long delays during which diffusion is allowed to occur. However, during these delays, molecules with fast relaxation can also return to equilibrium, and if not accounted for these species could go undetected by diffusion based spectral editing approaches. To create a RADE spectrum a control spectrum is collected with all delays and gradient lengths set to zero (essentially the same as a conventional $^1$H NMR spectrum). A second spectrum is then collected with the delays and gradients set as they would be for diffusion editing but the power of the diffusion encoding/decoding gradients set to zero. The difference between the 2 spectra is the signal lost through relaxation which is represented in the RADE spectrum. The RADE spectrum (Figure 2.3C) has a strong contribution from lipids similar to the diffusion editing, indicating that along with a range of relatively dynamic lipids (with longer relaxation, appearing in Figure 2.3B) a faster relaxing component also exists consistent with more rigid lipids (for example cell walls and less hydrated fat stores). The aromatic region of the RADE shows clear signals from amide and aromatic amino acids from protein. The appearance of protein in RADE and not in DE demonstrates these components undergo fast relaxation consistent with semi-solids and may arise from the muscle (a major protein locale in crustaceans).

2.3.3.4 $T_2$ filtered CP-MAS ($^{13}$C Detected Semi-solids)

$^1$H RADE presents the most rigid components that can be detected by $^1$H NMR spectroscopy. However, as discussed above, standard “low-power” $^1$H detect experiments will not identify true solids. Cross polarization (CP) can be used as a filter for the detection of solids via $^{13}$C. In previous work we have shown the CP can detect both rigid-gels and true solids, while more dynamic gels and solutions are not observed [8]. The signals from dynamic solids can be separated from true crystalline solids through the use of a very short (~30 μs) $T_2$ filter prior to cross polarization. The result is that only signals from true crystalline solids are suppressed while bonds exhibiting dynamics are retained. This spectrum is depicted in Figure 2.3D and largely contains lipids, similar to the $^1$H RADE (Figure 2.3C). This is expected as previous work has demonstrated that while no
signals are missed using the editing schemes outlined here, the components detected by $^1$H RADE and $^{13}$C $T_2$ filtered CP-MAS tend to overlap with both techniques observing semi-solids and rigid gel-like materials [8].

2.3.3.5 Inverse $T_2$ filtered CP-MAS (True Rigid Solids)

Inverse $T_2$ filtered CP-MAS selects only true solids by subtracting the semi-solid component ($^{13}$C $T_2$ filtered CP-MAS) from the all solid components (conventional $^{13}$C CP-MAS) leaving the true solids by difference. Figure 2.3E shows the rigid solids which are dominated by carbohydrate structures consistent with chitin, the main components of $H. azteca$’s shell [22]. A proportion of the CH$_3$ resonance can also be attributed to chitin. However, the overlap of resonances from ~55-20 ppm (likely including some of the CH$_3$ intensity) also suggests a contribution from rigid proteins that are in the form of chitin-protein fibres or lipid-protein matrices that lie within the cuticles of shrimp and account for the structural strength [23].
Figure 2. A series of spectral editing approaches applied to differentiate the overlapping resonances are presented. A) The dissolved metabolites in $^1$H NMR spectra of *H. azteca* are observed in Inversed Diffusion Editing (IDE), B) Gel like metabolites are emphasized in the Diffusion Edited experiments (DE), C) Resonances from the semi-solid components are observed in the $^1$H RADE NMR spectrum, “proteins” refers to aromatic amino acids in macromolecular structures, D) The semi-solid components are highlighted in $^{13}$C (CPT$_2$) and E) The most rigid components are selected in the inverse CPT$_2$ experiment.
2.3.4 2D NMR Identification of Metabolites

2D NMR provides both additional molecular connectivity information (critical for structural assignment) and affords increased spectral dispersion (reducing spectral overlap). Hetero-nuclear Single-Quantum Coherence (HSQC) correlates directly bonded $^1$H and $^{13}$C units and can, in simple terms, be thought of as a high resolution (theoretical peak capacity as a measure of resolution per unit is reported as~2,000,000 for HSQC [23]) fingerprint of the H-C framework in a complex mixture. $^1$H-$^1$H COrrelation SpectroscopY identifies protons on adjacent carbons. When combined, HSQC identifies the H-C fragments and COSY identifies how these units connect to form a molecule.
Figure 2.4 In vivo $^1$H-$^{13}$C HSQC of $^{13}$C enriched *H. azteca*. A) HSQC with ~40 metabolites assigned using Bruker’s Bio-reference databases. B) Aliphatic region C) Aromatic region D) Color coded assignments corresponding to the dots in B and C. *These signal dominate the semi-solids spectrum (Figure 2.3D) and are consistent with aromatic residues in proteins.
Figure 2.4A shows a HSQC spectrum of the *H. azteca* with major regions labelled; aromatics, nucleic acids, carbohydrates, peptides/proteins, lipids and various other aliphatic units. Exact assignments can be made by matching both HSQC and COSY data against bio-reference NMR databases. Approximately 40 metabolites including a range of amino acids, carbohydrates, nucleotides, and other bioactive compounds were identified (Figures 2.2B-2.2D and supporting section Figure A.5 for assignment confirmation). The potential role of these compounds as stress biomarkers is addressed below. The identification of additional metabolites is challenging, in part due to spectral overlap arising from the complexity of the whole organisms and in part due to limitations of currently available NMR databases. To our knowledge the Bruker Bioreference Databases are one of the most comprehensive metabolites databases commercially available. Still, they only contain 1D and 2D NMR information for ~650 compounds and no metabolites specific to *H. azteca*. However, a few years ago, there were no commercially NMR databases available and with the rapid evolution of NMR based metabolomics the development of novel databases and open source repositories such as; [http://mmcd.nmrfam.wisc.edu/](http://mmcd.nmrfam.wisc.edu/) are starting to evolve. With this in mind, careful inspection of the HSQC shows a truly *in vivo* system, this is actually highly encouraging and exemplifies the wealth of information that eventually will be accessible from MAS *in vivo* spectroscopy as assignment resources improve. Further experiments that should facilitate more detailed assignments are considered in supporting discussion.

2.3.5 Biological Significance

Nearly 40 metabolites were identified in the current study, the most noteworthy of which are discussed briefly here. Close to 20 free amino acids, such as leucine, isoleucine, valine, lysine, and other essential amino acids observed in previous metabolomic studies, appear in the IDE and are confirmed by HSQC [24]. Other identified metabolites include, lipids, choline, nucleosides, carbohydrates, osmolytes as well as, structural components such as chitin. For a complete list of the identified metabolites see Figure 2.4D.

Although amino acids are the building blocks of proteins, most amino acids also play major roles in multiple bio-molecular events. The three branched amino acids (leucine, isoleucine and valine) for example are crucial in muscular protein and neurotransmitter synthesis through regulating the
mRNA translation [25]. They can also potentially act as novel biomarkers that may aid in understanding cardiometabolic health [26]. Corresponding resonances from free tyrosine and phenylalanine are evident in the expanded aromatic region, these two amino acids act as main precursors in many biological processes such as dopamine biosynthesis, which is an intercellular transmitter in multicellular organisms and nervous systems in larger animals.

Aspartic acid, glutamic acid and alanine are found largely as free amino acids mainly in the axoplasm of the nervous systems of marine crustacean and account for 20% of the dry weight of the nerves [27]. Free taurine and glutamic acid may serve as receptors in crustacean antennae and aid in locating the prey in the depth of the ocean or lakes in benthic organisms [28]. Taurine, glycine, proline, glutamic and alanine may likely be important substances for the regulation of osmotic pressure in the crustaceans muscles [29]. Other free amino acids are being targeted as potential biomarkers and differentiators of cancer cells [30] and other disease.

The diffusion edited spectra identify an abundance of lipid reserves which were dominated by triacylglycerides (TAG) as major energy storage in most plants and animals [31,32]. The ability to monitor the lipid composition is very important as they are intimately tied to energy metabolism, a process commonly perturbed by a wide range of contaminants and other external stressors [33]. Lipids also serve as a source of essential biomolecules such as hormones. Many invertebrates such as *D. magna* are of particular interest as they cannot synthesize lipids *de novo* and therefore, rely on food sources for these molecules [34]. However, molecular understanding of the process is not well understood and being able to monitor the consumption of food (algae) *in vivo* or examine the transfer of carbon between trophic levels represents a key tool to better understand the ecology and chemistry of food webs. Particularly interesting, are the resonances corresponding to ω3 fatty acids in the diffusion edited data, which are an important component of cell membranes as well as precursors to many other substances such as the hormones central to reproduction in many organisms. In addition, the ω3 content of the marine organisms is a “quality indicator” in the food industry, since they are the primary source of ω3 for human consumption and cannot be biosynthesized by mammals [35].

The diamines putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane) [36] produced by the deamination of ornithine and lysine [37] are found in high abundance in all major groups
of marine organisms such as invertebrates. They are naturally occurring polyamines, which regulate the concentrations of the cations in macromolecular structure of DNA and RNA or their transport through cellular membranes.

The nucleosides adenosine and uridine represent important biomarkers related to DNA/RNA damage, which have been implicated to be an indication of carcinogenesis. DNA/RNA monitoring can act as a measure of hereditary risk of cancer or many other diseases [38]. Choline is another identified metabolite that appears in the head groups of some phospholipids. It is also a precursor needed to form acetylcholine which is a neurotransmitter that controls memory and muscle movements. Therefore, choline plays major part in many nervous system related disorders [39]. Also, fluctuation in choline level is a well-established biomarker for different cancer types [30].

Chitin is the main structural polysaccharide that forms the protective exoskeleton in all arthropods, and makes up to 60% of their mass and serves as an important structural component in their body. It is periodically shed and causes their growth in several stages of molting ([40] and [41]). Several steps take place to convert trehalose to chitin in which mainly glucose, glutamine and uridine triphosphate (UTP) serve as precursors of its synthesis. The presence of most of the precursors and products through these stages are identified in the 2D spectra. The ability to study soluble precursors, through conversion into a gel and finally a solid shell is only possible using a CMP-NMR probe where all phases can be studied and differentiated in situ. The approach opens exciting possibilities to follow process such as bone formation. Indeed, not only the formation but also, the degradation of the solid materials could be an important complimentary source of information [42] itself key to stress induced thinning in bird egg shells and shells of marine animals, as well as the degradation of bone with age.

2.3.6 Towards \textit{In vivo} Organism Monitoring using CMP-NMR

The major drawback of \textit{in vivo} CMP-NMR is the potential stress from the spinning itself. Figure A-2 compares the $^1$H NMR over a 20-hour period of continuous spinning at 2.5 KHz. In this case a large single shrimp was used, and the organism remained alive after 20 h of spinning. The main $^1$H NMR profile remains consistent. The main difference is the appearance of alanine, which has
been reported as a general indicator of stress [14]. The lack of lactate (an indicator of anaerobic stress) observed when numerous organisms are placed in a rotor (see Figure A-3D), suggests that spinning rate (rather than lack of oxygen) likely plays a role in the alanine production. While it can be argued that moving forward, spinning stress could be reduced (e.g. applying lower spinning rates) eliminating it completely may never be possible. As such it is important to discuss potential ways in which this stress could be accounted and the best way to incorporate CMP-NMR into an in vivo monitoring program. This can be achieved through using CMP-NMR as a stand-alone tool or as part of a larger in vivo NMR framework, both approaches will be discussed briefly.

2.3.7 Using CMP-NMR as a part of a larger in vivo NMR framework

Recently our group has demonstrated that flow based in vivo NMR is an excellent low-stress approach to understand metabolic change [5]. Organisms are placed in a NMR flow tube (which is kept static) and oxygenated water (with or without food) is continually recirculated. Contaminants, stressors or nutrients can be easily introduced into the flow making it ideal for monitoring metabolic response to stress. The drawback, however, is the low resolution from the $^1$H NMR spectra. While this can be overcome via spectral dispersion afforded by $^1$H-$^{13}$C HSQC, information is still only obtained for the truly dissolved metabolites. Conversely, in CMP-NMR the NMR profile is sharpened through MAS and information on the gels and solids can also be obtained. CMP-NMR would be best employed at time point when flow-based NMR identifies responses to provide additional resolution to aid with metabolite assignment as well as, provide information on gels and solids only detectable by CMP-NMR. In this way CMP-NMR plays more of a supporting role in terms of metabolic changes, and a primary tool for observing structural change. This approach leverages the low stress attributes of flow NMR to monitor metabolic change while taking advantage from CMP-NMR for assignment. In such a combined approach as conclusions regarding stress fluxes are drawn from the low stress technique, additional stress from the spinning in CMP-NMR becomes less critical.
2.3.8 CMP-NMR as a stand-alone tool

While the combined approach would be recommended for large studies aimed at truly targeting the metabolic stress, there are situations where the approach may not be feasible or available. In such a scenario spinning stress would have to be differentiated from impacts of exposure (contaminant, drug etc.) via controls. The controls should be identical to the dosed organisms with the exception of the addition of the stressor. In its simplest form differences after chemometrics analysis between the control and dosed organisms, spun for the same amount of time, should arise solely from the stressor as stress arising from spinning should cancel. Such studies should be carefully controlled in terms of spinning time and if multiple NMR experiments are performed, they should be run in the same order and started at the same time after spin initiation on both the dosed and control subjects such that spinning effects cancel. As the stress from spinning likely manifests over time (evidenced by the increase in alanine in Figure A-2) more advanced studies could use time based chemometric trajectory analysis to further differentiate metabolites co-involved in the separate processes [43]. Of particular interest may be to introduce the chemical stressor midway the analysis such that trajectory from spinning remains linear throughout while the influence of the stressor impacts only the later datasets [44]. Currently, our group is working toward protocols for using CMP-NMR effectively to understand stress processes. Given the unique and comprehensive in vivo information that CMP-NMR can provide over more traditional NMR and other non-NMR based methods stress from spinning is an unavoidable side-effect that must be accounted for.

2.4 Experimental

Results (Figure 2.1) suggest that H. azteca spun at a reasonably slow rate 2.5 KHz are limited by the oxygen content in approximately 80 μL of water in the rotor, but are not limited by the spinning itself. To minimize this oxidative stress a hole is drilled into the cap. Rotor caps with o-ring seals were purchased from Wilmad Glass and drilled with a 0.022”-inch drill bit to produce a hole allowing for air exchange thereby extending the survival of H. azteca under MAS conditions.
Viability studies were performed in triplicate and the percentage survival rate along with the standard deviation were reported (see Figure 2.1).

The original *H. azteca* culture was provided by Environment Canada from their main colony. The organisms were then cultured within the University of Toronto laboratory and living conditions for the specimen were controlled according to methodologies by Environment Canada [10] and kept similar to their natural habitat in fresh water. They were kept in 20 L tanks with a 2 cm layer of sand in dechlorinated, aged tap water, continuously aerated as their medium. 20% of the overlaying water was changed 3 times a week before feeding time. The tanks were exposed to 16:8 h light to dark photoperiod using a fluorescence commercial lamp and the temperature was kept at constant 24 °C. Carbonate hardness of 124 mg CaCO$_3$ L$^{-1}$ (consistent with local freshwater conditions). $^{13}$C isotopically labelled were cultivated in a small-scale closed loop system. A custom photobioreactor built by Silantes GmbH was used for production of algae biomass. Each fermentation has been conducted autotrophically and exclusively with $^{13}$CO$_2$ (98% enriched with stable Isotope $^{13}$C) (for more details regarding the $^{13}$C labeling of the algae please refer to appendix section). The cultivation parameters including media, temperature, light intensity, and pH were adjusted to gain a maximum growth rate. The harvested algal biomass was fed at a rate of 2 mg per *H. azteca*, 3 times a week over their life span and represents their sole carbon source.

A 4 mm zirconium rotor was filled with water. For offline viability studies (Figure 2.1) 1-6 *H. azteca* were used. The *H. azteca* were selected based on size with <2 mm representing smaller shrimp, 2-5 mm medium shrimp and >5 mm large shrimp. Size was gauged using a microscope and males were selected for analysis. Different sexes can be recognized by the absence of an enlarged gnathopod or presence of egg case in females. For our experimental purpose adult males were selected due to large body size, and to avoid the inconsistencies due to additional lipid storage within the eggs in the females. For all the NMR data reported here (with the exception of Figure A-3D) a single large shrimp (length ~7 mm) was used and placed in the rotor with its gills towards the bottom. *H. azteca* were loaded from the droplet at the tip of a plastic pipette and let swim into the rotor (refer to (Video A-1) in the appendix). A trace of D$_2$O (~3 μL) was added to act as a spectrometer lock. The rotor was sealed using a top Kel-F cap with an o-ring seal customized with a hole as described above. All NMR spectra were acquired using a Bruker Avance III spectrometer operating at 11.7 T, observing $^1$H 500.13 MHz fitted with a prototype CMP MAS 4 mm $^1$H–$^{13}$C–
$^2$H probe with an actively shielded magic angle gradient (Bruker BioSpin). All experiments were performed at 5 °C, spinning rate of 2.5 KHz and locked on D$_2$O. Sample temperature was not seen to increase more than 1 °C during the experiments. The lock was maintained for all experiments including solids experiments. After each run the organism was monitored for 1 week before being returned to the main colony. Only NMR data from organisms that survived and fully recovered were used in this study.

Additional information including total experimental time, T$_1$ values and delays used are provided in Table A-1. $^1$H NMR SPR-W5 WATERGATE pulse sequence was used for water suppression [15,45] with garp-4 decoupling to remove $^{13}$C splitting. The shaped pre-saturation prior to W5 was achieved using a train of selective pulses: 1000, 2 ms, calibrated rectangular pulses were used, each separated by a 4 µs delay. 256-4096 scans (see Table A-1) were collected with 8192 time domain points, 20 ppm sweep width, a 125 µs binomial delay and a recycle delay 5 x T$_1$. Spectra were processed using an exponential function corresponding to a line broadening of 2 Hz in the transformed spectrum and a filling factor of 2. Carbon spectra were obtained using spectral width of 400 ppm, 16384 time domain points, 5000 scans inverse gated decoupling (Waltz-16) and a recycle delay 5 x T$_1$. Spectra were processed using an exponential function corresponding to a line broadening of 5 Hz in the transformed spectrum and a filling factor of 2.

$^1$H diffusion based editing was performed with a bipolar pulse pair longitudinal encode-decode (BPPLED) sequence [19]. Scans were collected using an encoding/decoding gradient of 1.8 ms at ~50 gauss/cm (65 gauss/cm max for current probe) and a diffusion time of 180 ms. Inverse diffusion editing (IDE), relaxation Recovery Arising from Diffusion Editing (RADE) and inverse T$_2$ filtered $^{13}$C CP/MAS were done by appropriate spectral subtraction as previously described [8]. Briefly, the spectra are scaled until the spectra being subtracted was nulled with the resulting difference spectra containing positive peaks.

CP/MAS was performed using linear ramp defined by 1000 points from 80-100% during a contact time of 2 ms, high power composite pulse decoupling (Spinal 64) and Total Suppression of Sidebands (TOSS) [16]. Decoupling was applied using an RF field of 50 KHz, improvements were not seen beyond this as such the RF field strength was applied at 50 KHz to prevent additional sample heating. 30720 scans were collected using 1024 points and a recycle delay of ~5 x T$_1$ ($^1$H
macromolecules, see Table A-1). Spectra were processed using an exponential function corresponding to a line broadening of 25 Hz in the transformed spectrum and a filling factor of 2. For the selection of semi-solids (CP-T2) a train of 2 x 7.5 µs CPMG echoes were applied to the $^1$H spins prior to contact [8]. The inverse CP-T2 (solids) was created by difference. Singular-value decomposition [46] was applied to CPT2 and ΔCP to reduce spectral noise (an example of which is shown in supporting Figure A.4).

$^1$H–$^{13}$C HSQC spectra were collected in phase sensitive mode using Echo/Antiecho-TPPI gradient selection, with 600 transients for each of the 128 increments in the F1 dimension. 2048 time domain points were collected in F2 and a 1J $^1$H–$^{13}$C of 145 Hz. F2 was processed using an exponential function corresponding to line broadening of 15 Hz and F1 using sine-squared functions with a π/2 phase shift. 2D COSY (Correlation SpectroscopY) spectrum was acquired to confirm HSQC assignments of metabolites. The COSY experiments were collected using an in-phase approach [47], using gradients for coherence selection and low power $^{13}$C garp decoupling throughout. 128 scans and 2048 data points were collected for each of the 196 increments in the F1. Both dimensions were processed using sine-squared functions shifted by 90°, zero filling factor of 2. Compound identification and quantification were performed using AMIX (Analysis of MIXtures software package, version 3.9.15, Bruker BioSpin, in combination with the Bruker Bioreference NMR databases, versions 2-0-0 to 2-0-4) as previously reported [48]. Only assignments with an R$^2$ correlation >0.99 between the observed and databases shifts were retained. Where possible correlations were also confirmed with COSY.

### 2.5 Conclusions

In this study the main objective was to demonstrate the feasibility and applicability of CMP-NMR to a whole living organism, while retaining the biological function. The study demonstrates all structural components can be identified, from metabolites to true solids. The ability to monitor potential real-time molecular fluxes and relate this directly to biological response, activity or behavior provides an essential link to better understand biological processes and ultimately the
biochemistry and chemistry that define these events. The potential of the current work goes beyond the freshwater shrimp studied here and could theoretically be applied to a wide range of small organisms, intact tissue sections (including bone), and to follow almost any biological process, including, growth, reproduction, disease, stress, and others. To our knowledge, this approach arguably provides the greatest amount of molecular information \textit{in vivo} compared to any modern scientific tool, and the versatility to extract information from all components, solutions, gels and solids. However, the approach has limitations, some of which are discussed and addressed in appendix.

In summary, this study demonstrates the possibility to study a whole living organism by CMP-NMR. The unique capabilities afford the possibilities to study and differentiate all phases (solutions, gels, semi-solids, true solids) \textit{in vivo}. Theoretically, this provides the potential to study all organic components within a living organism, providing an unprecedented window into biological processes and stress responses. Considering the huge potential along with the unique and unprecedented molecular information the approach affords, it is clear that \textit{in vivo} CMP-NMR will play an important role in many areas of research. The technique acts as the ideal “molecular interpreter” providing the desperately needed connection between the physical (for example environmental stress and disease) to the chemistry that ultimately defines these processes.
2.6 References


3 Chapter 3

Effective Combined Water and Sideband Suppression for Low-Speed Tissue and \textit{In vivo} MAS NMR

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3.1 Abstract

High Resolution Magic Angle Spinning (HR-MAS) NMR is a powerful technique that can provide metabolic profiles and structural constraints on intact biological and environmental samples such as cells, tissues and living organisms. However, centripetal force from fast spinning can lead to a loss of sample integrity. In analyses focusing on structural organization, metabolite compartmentalization or in vivo studies, it is critical to keep the sample intact. As such there is growing interest in slow spinning studies that preserve sample longevity. In this study, for example, reducing the spinning rate from 2500 Hz to 500 Hz during the analysis of a living freshwater shrimp increased the 100% survivability threshold from ~14 h to 40 h. Unfortunately, reducing spinning rate decreases the intensity of the isotropic signals and increases both the intensity and number of spinning side-bands, which mask spectral information. Interestingly, water suppression approaches such as excitation sculpting and W5 WATERGATE, which are effective at higher spinning rates, fail at lower spinning rates (<2500 Hz). Simpler approaches such as presaturation are not able to effectively suppress water when the ratio of water to biomass is very high, as is the case in vivo. As such there is a considerable gap in NMR approaches which can be used to suppress water signals and sidebands in biological samples at lower spinning rates. This research presents simple but practically important sequences that combine PURGE water suppression with both PASS and an analogue of TOSS termed TOSS.243. The result is simple and effective water and sideband suppression even in extremely dilute samples in pure water down to ~100 Hz spinning rate. The approach is introduced, described and applied to a range of samples including, ex vivo worm tissue, D. magna, and in vivo H. azteca.

3.2 Introduction

Molecular-level characterization of tissues, intact samples and in vivo specimens is essential for understanding structural organization, conformation and binding, which are major processes to biological function. Specifically, the metabolic profile of an organism represents one of the most
sensitive and rapid indicators to a range of environmental, toxicological or pathogenic perturbations [1,2] and is therefore an important tool to detect early responses prior to detectable changes in the genes or at the phenotypic level [3]. In the environmental field, NMR-based metabolomics has emerged as a key tool for toxicological evaluation and exploration of the environmental stressors on organism health [4,5]. In biomedical research, common diagnostic methods such as biopsy or MRI are extremely useful in characterizing the physical changes. Although, they provide little high resolution molecular-level information on the biochemical activity of the tissues. As such, NMR spectroscopy can potentially serve as a complementary high resolution technique by providing insight on the distribution of the metabolites, as well as additional structural and quantitative information [6]. With the additional dispersion afforded by multi-dimensional NMR techniques, individual metabolites can be assigned to describe the metabolic profile in greater detail [7].

Intact heterogeneous samples such as cells, tissues and living organisms are some of the most challenging samples to be investigated by NMR spectroscopy [8]. The varying magnetic susceptibilities across different phases in such “multiphase” samples, (e.g. boundaries in the inter and intracellular phases (cell-water interface etc.)) along with chemical shift anisotropy (uneven electron distribution around the nucleus) and dipolar interactions (i.e. $^1$H-$^1$H through space dipolar couplings) are the main source of spectral broadening and loss of resolution in NMR spectra [9,10]. In true solutions (extracts or biofluids), many of these sources of broadening are often averaged to zero due to high isotropic molecular tumbling. In solids or molecules with restricted motion, this averaging does not occur, and spectra tend to be broader and lacking the spectral resolution required for effective metabolic discrimination. Therefore, the most common utility of $^1$H NMR metabolic profiling in biomedical research is largely restricted to biofluids (e.g. serum and urine) and solution extracts. Although using cell extracts or excised organs eliminates the susceptibility variations, the extraction procedure is destructive and often introduces post-mortem changes in the metabolic profiles. Additionally, such in vitro methods are limited in that they cannot observe the real-time biochemical flux within the organism and the question "how representative these extractions are to the original composition of the tissue?" always remains.

In 1996 a novel form of NMR spectroscopy termed high resolution magic angle spinning (HR-MAS) NMR was introduced to the field [11,12]. If the sample is rotated at magic angle ($\Theta=54.74^\circ$)
relative to the external magnetic field, the broadening effects due to magnetic susceptibility gradients and chemical shift anisotropy can be reduced. This results in improved $^1$H spectral resolution [13] permitting the detection and quantitation of many metabolites that are not clearly resolved in static methodologies, as well as providing additional information on swollen components that may be too broad to observe without MAS. Due to strong $^1$H-$^1$H dipole interactions the $^1$H spectral profile of true solids is often too broad to detect by HR-MAS. Readers interested in accessing all components in a living system should use CMP NMR which combines both $^1$H detection for liquids and gels, with $^{13}$C detection for true solids [14] and was recently applied to living shrimp [15]. In general terms, faster spinning leads to narrower line shape and reduction in sideband intensity resulting in more intense isotropic signals (i.e. the main signals of interest that provide chemical shift information). For example, using a 500 MHz ($^1$H Larmor frequency) spectrometer requires a spinning rate of 5 KHz to shift the sidebands by 10 ppm. As the main spectral window for $^1$H chemical shifts is ~0-10 ppm, to remove the sidebands from the sample of interest a spinning rate of at least 5 KHz is required. This is not a problem for most samples, however, if the samples being analyzed are delicate and maintaining the sample integrity during analysis is essential, then fast spinning is not an option. The centripetal force resulting from the high spinning speeds in MAS based studies can destroy the integrity of the samples. This force is directly related to the mass of the sample, the spinning speed, and the distance from the rotation axis. Previous studies have found that, human prostate tissue could be damaged above 3 KHz and cell lysis has been observed at 4 KHz spinning rates [16,17] using standard 4 mm rotors. A study has shown that larger cells such as, lipid-laden adipocytes are more prone to damages induced by spinning and show much lower viability compared to smaller cells such as preadipocytes after 2 h spinning at 3.5 KHz [18]. Recently, a spinning rate of 2.5 KHz allowed the NMR analysis of living *H. azteca* for 14 h with full recovery [15], while higher spinning rates led to mortality during analysis. Similar results were reported for HR-MAS studies of *D. magna* where 3.6 KHz spinning lead to preferential mortality when compared to 2 KHz [19].

In addition to mortality, spinning stress itself can lead to changes in the metabolome. Indeed, alanine, which has been shown to be a general indicator of stress, was shown in a recent study to increase when organisms experienced spinning stress [15]. Therefore, if the goal of the *in vivo* NMR research is to, for example detect biological stress caused by external stimuli (drugs, contaminants, heat, salts etc.), it is important to minimize the effect of spinning stress such that the
other impacts of stress are easier to differentiate. In order to reduce such stress, slow spinning \textit{in vivo} was proposed [20]. Arguably the most impressive study performed was that of Wind \textit{et al.}, on the midbody of an anesthetized mouse using an 85 MHz NMR system spinning at 4 Hz using localized spectroscopy and imaging detection [21]. While the technique, termed localized magic angle turning (LOCMAT) produced significantly improved resolution over a static sample, the spectra were dominated by a broad lipid profile which prevented assignment of an array of individual metabolites. Due to challenging aspects of \textit{in vivo} studies and limitations of slow spinning MAS technology, there have been very few studies to date. However, studies at higher spinning rates are increasing. In last 10 years \textit{in vivo} HR-MAS studies have been performed on a range of organisms including bacterial cells [22], common fruit fly [23], earthworms [24], D. magna [19], and H. azteca [15]. In each case the maximal survivable spin rate was employed as this provides the maximum amount of spectral information without the need for sideband suppression techniques, required at slower spinning rates.

Conversely, the strong correlation between free metabolites and disease pathology have inspired many studies focusing on delicate intact tissue samples that require the use of low-speed spinning methods in order to preserve the integrity of the specimens [25-28]. Slower spinning gives rise to more intense and numerous sidebands which overlap with the real signals and make interpretations difficult or impossible. In a heterogeneous sample, sidebands arise from the local anisotropic magnetic susceptibility changes and relocate according to the spinning rate and need to be selectively suppressed to extract the isotropic spectral information otherwise masked. Unfortunately, there is a distinct lack of simple and efficient combined water and sideband suppression approaches that provide high resolution information with minimal sensitivity losses.

Early methods such as phase-corrected magic angle turning (PHORMAT) have been applied to biological tissues or even live organisms such as mice [21]. While effective in producing sideband free spectra, PHORMAT evolves the chemical shift information in the indirect dimension of a 2D NMR, making experiments prohibitively long (many hours, even days) while suffering from low signal and poor resolution. As such, most studies have focused on Phase Adjusted Spinning Sidebands (PASS) experiment. PASS is also a 2D approach but the $^1$H spectrum evolves in the direct dimension and sidebands are separated along the indirect dimension. The result is a robust and effective way to separate sidebands from isotropic chemicals shifts. In biological samples,
sideband suppression needs to be combined with water suppression. Most commonly, PASS has been combined with presaturation or Delays Alternating for Nutation for Tailored Excitation (DANTE). Presaturation involves selectively irradiating the water frequency during the relaxation delay, and while it is simple to implement, it is generally not effective for very challenging samples with intense and often broad water signals [29]. Presaturation-PASS has most commonly been used, yet it has been reported as inadequate in various cases [30,31], with limitations such as large residual water peaks or attenuation of resonances close to the solvent region being reported. Although DANTE is quite effective at suppressing both the water and its sidebands, unfortunately, sample signals that resonate at the same frequencies, as the water sidebands are also suppressed leading to numerous windows from which spectral information is lost. Other studies have explored the factors that contribute to intense sidebands in HR-MAS. It has been demonstrated that by reducing the sample size the intensity and distribution of the sidebands is also reduced [32,33]. Sidebands in large part originate from magnetic susceptibility distortions arising from air bubbles trapped within the sample [34]. In another study, it was indicated that restricting the sample to a 12 μL spherical shape reduces sidebands to the point where simple water suppression approaches such as presaturation are effective. Other studies have performed micro HR-MAS using a high resolution microprobe to study live bacteria [35] or sub- millimeter organisms which sufficiently reduces the sidebands at spin rates as low as 300 Hz [36]. While critically important and the ideal solution in many cases, these methods are not practical for in vivo samples of larger organisms, often much greater than 12 μL [37] and require oxygen delivery [15] (i.e. a hole is required in the rotor cap) or a large excess of water inside the rotor (i.e. the sample shape cannot be modified because the organisms require the maximum space possible) for the organism to survive. In our laboratory despite extensive shimming it was not possible to reduce the sidebands to the point where readily available pulse sequences provided usable NMR data for larger living organisms. As such there is a need for effective sideband and water suppression approaches to extend the applicability of modern HR-MAS NMR. Beyond in vivo research, the approaches would allow full standard rotors to be analyzed (important for maximum signal) and allow fast or automated sample preparation. The later could be very important when HR-MAS is applied to differentiate cancerous tissue rapidly during surgery [29,38]. In such applications, the goal is to collect data as fast as possible and using larger samples permits good signal in a short time. As autosamplers are often used for sample preparation and transfer completely removing trapped air may not be possible. In
such cases the ability to suppress both the sidebands and water at low spinning rates which preserve the tissues integrity would be highly advantageous. As such, low speed MAS has the potential to become a significant analytical tool to study *ex vivo* and *in vivo* metabolomics with applications in a wide range of disciplines. At present the lack of robust, effective and simple to use combined sideband and water suppression approaches are limiting the application of low-speed HR-MAS on larger delicate natural samples. The focus in the current study is to establish an effective methodology that will result in sideband free spectra incorporated with efficient water suppression at lower spinning rates. This will allow the use of lower spin rates reducing the negative impacts from spinning on the tissues or living organisms and preserve their physical integrity. In turn, reduced stress from the spinning process can permit longer experimental times (especially for living organisms) permitting more comprehensive characterization often important for the accurate differentiation of stressed vs. healthy, or normal vs. diseased states.

3.3 Materials and Methods

3.3.1 Survival with Spinning Speed

In order to determine the increase in survivability at 500 Hz vs 2500 Hz for freshwater shrimp (Figure 3.1), 1-6 shrimps (depending on the experiment) were loaded into a rotor and spun for different durations using an NMR probe external to the magnet, following a previously published approach [15]. The temperature was controlled and kept at 5 °C using a variable temperature unit. The organisms were recovered by placing the rotors in holding tanks and allowing the organisms to freely swim out. The organisms were monitored for 48 h after spinning and the % of organisms freely swimming in the tank at 48 h was used to determine survivability. All studies were performed in triplicate and the percentage survival, rate along with the standard deviation are reported.
The *Hyalella azteca* culture was initially provided by Environment Canada from their main colony. The organisms were further cultured and maintained according to guidelines suggested by Environment Canada (Burlington, ON, Canada) [39] as previously reported elsewhere [15].

### 3.3.2 Sample Preparation

For the *ex vivo* worm studies, one whole *Eisenia fetida* with a length of ~3 cm small enough to fit (folded) into a rotor, was inserted into a 4-mm rotor in H$_2$O/D$_2$O (95:5, v/v) solution. *E. fetida* were purchased from The Worm Factory (Perth ON, Canada) and raised as previously described elsewhere [40,41].

For the study of water fleas, 20 *D. magna* were freeze dried and crushed prior to the experiments and subsequently transferred to a 4 mm rotor in H$_2$O/D$_2$O (95:5, v/v) solution. The cultures were originally supplied by Ward's Scientific (Rochester, NY, USA) and kept in fresh water under a 16:8 light:dark photoperiod and fed with algae [7].

For the *in vivo* studies 1-5 *H. azteca* were loaded into a rotor with their heads kept down and let swim from a droplet at the tip of plastic pipette. Handling and culturing of *H. azteca* has been previously reported [15,39]. A trace of D$_2$O (~3 μL) added to each sample acted as the spectrometer $^2$H field lock. The rotor was sealed using a top Kel-F cap with an o-ring seal. In order to prevent hypoxia for *in vivo* studies the cap was customized with a hole as described previously [15].

### 3.3.3 NMR Instrumentation

All $^1$H NMR spectra were acquired on a Bruker Avance III spectrometer operating at 11.7 T, observing $^1$H 500.13 MHz fitted with a Comprehensive Multiphase (CMP) MAS 4-mm three channel ($^1$H–$^{13}$C and $^2$H) probe [14] with an actively shielded Z-gradient. For all samples with the exception of the *in vivo* sample, 243 scans were collected (for the *in vivo* sample 8262 scans were used) with 16384 points in the time domain, 20 ppm sweep width and a recycle delay of 2 s. Presaturation was applied to the isotropic water peak using 0.1 mW throughout the recycle delay.
PURGE contains both delays where the magnetization is along XY ($\tau_2$ delays) and delays where the magnetization is along Z ($\tau_1$ delays). The $\tau_2$ delays were set to 20 $\mu$s such that they had a negligible impact on the sequence. The $\tau_{1A}$ and $\tau_{1B}$ delays (see Figure 3.4, refer to PULSE program) were optimized in real-time (gs mode) to obtain a minimal water signal. The optimal setting helps null the water through $T_1$ effects [29] as the magnetization is stored along Z during these delays. Values varied depending on the sample, probe and spectrometer used, but were generally around 3 ms ($\tau_{1A}$) and 12 ms ($\tau_{1B}$) for optimal water cancelation. Identical settings were used for all versions of PURGE-TOSS and PURGE-PASS. In the case of 2D PASS, 16 scans per 16 increments were collected for all experiments except for in vivo. For “long” in vivo experiments 64 scans per 128 increments were used. Total scans in for PURGE-TOSS were adjusted to match those in PURGE-PASS to permit comparison. In the TOSS.243 sequence it is possible to shift the last pulse such that it forms an echo over an additional rotor period. All samples in this chapter were run without this additional echo. At higher spinning speeds (>500 Hz) this optional echo can aid water suppression, however at spinning speeds <500 Hz the rotor period is long enough such that J-coupling evolution and additional relaxation become significant and is generally not recommended.

Spectra were processed by applying an exponential multiplication of FIDs by a factor of 2 Hz in the transformed spectrum and a filling factor of 2 followed by Fourier transform. All experiments were performed at 5 °C for better tissue preservation and consistency with the previous in vivo study [15].

In addition to biological/environmental samples, the RF pulse sequences were optimized using a standard sample of 5 mM sucrose in H$_2$O/D$_2$O (95:5, v/v). Example spectra are provided in the supporting information.
3.4 Results and Discussion

3.4.1 Survival of organisms \textit{in vivo} under MAS

The high centripetal forces imposed on living organisms or the biological tissues at high spin rates may damage the organizational tissues, even if the cells are not disrupted [17]. Many studies have tried to identify the limits to the spinning speed on different tissues, as they may have varying tolerance towards spinning. In a previous \textit{in vivo} MAS study on \textit{H. azteca}, survival rate at different spinning speeds was monitored over time. It was found that in order to move the water sidebands outside the spectral window at 500 MHz (\textsuperscript{1}H) a minimum spinning speed of 2.5 KHz was required. At this speed the organisms showed 100\% survival for \textasciitilde14 h.

Interestingly, if the spinning speed is reduced to 500 Hz, 100\% survival rate is increased to 40 h providing a longer time window for in-depth \textit{in vivo} analysis (Figure 3.1A). This is significant as most acute toxicity tests are performed the first 48 h and this window is considered the most important in understanding toxic responses to environmental stressors [42-44]. Since the centrifugal forces are proportional to the square of the spin rate, a 5-10-fold reduction in spinning rates can substantially decrease the induced effect of spinning speed by 25-100 times. Figure 3.1B demonstrates that it is even possible to analyze a larger number of organisms at a lower spinning speed, likely in part due to the lower forces the organisms induce on each other with slower spinning. Furthermore, as previously reported, these studies use customized cap drilled with a 500 \textmu m hole to allow air exchange, causing some evaporation identified as a considerable drawback [15]. At lower spinning speed evaporation is reduced due to less drive air blowing across cap helping maintain the water level inside the rotor.
Figure 3.1 A) The impact of decreasing the spinning rate on survival rate is depicted. B) Demonstrates that at slower spinning rate more organisms can be analyzed in a single rotor.

Unfortunately, while reducing the spinning rate has considerable benefits for the organisms, the opposite is true with respect to the quality of NMR data that can be obtained. Figure 3.2A shows that at a relatively high spinning rate 2.5 KHz (or above), at 500 MHz, the water sidebands are shifted outside the spectral region of interest (10 ppm spectral width) and do not interfere with spectral analysis. With a reduced spinning rate (Figures 3.B-E) the number, as well as, the intensity of these sidebands increases. As the isotropic centerband is split amongst sideband components, slower spinning speeds lead to intensity loss in of the isotropic peaks. In addition, it is important to note that while Figure 3.2 highlights only the water sidebands, sidebands also arise from the sample itself leading to a situation where little to no isotropic information can be extracted from the simple $^1$H NMR spectra. Consequently, there is great need in the environmental and medical fields for a combined water suppression and sideband approach that can remove the spectral artefacts while preserving the isotropic information.
3.4.2 Water Suppression

In the case of living organisms or intact tissues water signal can be many orders of magnitude more intense compared to trace concentration of the metabolites. The simplest solution is to replace the H$_2$O with D$_2$O [15,19] to reduce the water signal. However, such exchanges can lead to leaching of components from the sample and as D$_2$O is toxic at high concentrations [44], it will negatively affect long term in vivo analysis for many species. For the optimal detection of low concentration species, the NMR receiver needs to be set to its maximum value and with samples with high water contents this requires effective water suppression.

\[ \text{Figure 3.2} \] The intensity of the isotropic signal, as well as the number, intensity and the locations of the sidebands change with respect to the different spinning rates. Sidebands are identified with blue triangles, and red asterisks indicate the isotropic peak for water.
In this chapter, the effectiveness of various common water suppression approaches at low spinning speed (<1000 Hz) were compared. Early research employed a rotor-synchronized sequence, namely DANTE for water suppression at low speed [15,18-23]. However, such approaches lead to nulling not only the water at the sideband locations but also the corresponding analyte signals [17]. While DANTE is easy to perform, the loss of spectral information from the sample was considered too great a compromise and DANTE was not pursued further here. Early methods such as DANTE have largely been replaced by modern water suppression methods utilizing pulse field gradients to dephase the water. In our lab, shaped pre-saturation (SPR-W5 WATERGATE) [45] and excitation sculpting (ES) [46], have both proved excellent and robust at higher spinning rates [15,47].

Unfortunately, the aforementioned sequences proved inadequate at low spinning speeds. Figure 3.3 compares various water suppression approaches using a whole worm (Eisenia fetida) at 500 Hz. While SPR-W5 WATERGATE (Figure 3.3A) is effective at suppressing the central water transition, it leads to partial cancelation of the sample peaks resulting in a distorted line shape. A similar result is obtained with ES, albeit the sample peaks are distorted slightly less along with more signal from the residual water. This is further demonstrated on a standard sample of 5 mM sucrose (supporting Figures B-1 and B-2) which shows signals are lost in both SPR-W5 WATERGATE and ES at 500 Hz. Presaturation performed reasonably well with all the signals from the worm sample remaining in phase (Figure 3.3). Unfortunately, the water sidebands cover a large part of the spectrum making it inadequate for general use, a conclusion also supported by other studies [17].

Given the potential of presaturation, a related approach termed, presaturation utilizing relaxation gradients and echoes (PURGE), was explored [29]. PURGE suppressed the water centerband and sidebands across the spectrum, while the sample signals largely remain unaffected. Interestingly while PURGE is not designed to cancel sidebands from the sample, these sidebands are attenuated somewhat by the sequence (seen most clearly for signals highlighted with a bracket in Figure 3.3 (0 to -1 ppm)). This likely arises as the stimulated echoes in PURGE behave as refocusing blocks leading to phase altered spinning sidebands that are then partially cancelled by the complex phase cycle in PURGE. Indeed, this is similar to the basis of common sideband suppression approaches such as PASS [48] and is a fortuitous side effect of the PURGE sequence. Unfortunately, however,
sidebands are not completely suppressed and concatenation with a dedicated sideband suppression module is still required for spectra completely free of both water and sidebands at low spinning speed.

**Figure 3.** Most common water suppression pulse sequences are compared on a rotor packed with a small *E. fetida* (earthworm) and spun at 500 Hz. The dotted area highlights the isotropic water resonance. The locations of sidebands are highlighted in pink while spectral distortions are indicated with arrows.
3.4.3 Sideband Suppression

The most commonly employed sideband suppression methods are TOtal Sideband Suppression (TOSS) [49,50] and Phase Adjusted Spinning Sidebands (PASS) [51]. PASS has proved to be the most effective technique for suppression of the sidebands in biological samples and has been applied more frequently. Previously, PASS has been applied to yield high resolution spectra of excised rat organs such as liver, heart and brain, as well as on human organs such as prostate [17]. It has also been applied to study living bacterial biofilms with spinning rates as low as 40 Hz [31]. These solid materials often have high susceptibility gradients that cause larger broadening effects than those observed in the (semi-solid) biological samples. PASS, a 2D experiment, consists of a rotor-synchronized sequence of pulses applied during a constant evolution period \( \omega / 2 \pi \) = spinning rate in Hz). As shown in Figure 3.4A, it consists of five 180° pulses separated by an inter-pulse delay \( t_2 - t_6 \) which differ for each increment, with \( t \) being the rotor period. Since spinning sidebands oscillate with different frequencies, in PASS they can be delayed and thus separated from the centerband by the order of \( \pm n \) (frequency of rotation), while the isotropic portion \((0n)\) of the chemical shift is refocused [52]. As a result, in an experiment with \( n \) orders of visible sidebands at least \( n + l \) separate experiments need to be performed. The anisotropic portion of information is retained and can be extracted to provide additional biomedical insight where needed. Acquisition starts after one rotor period, so as the spin rate is lowered (hence increasing the rotor period) the amplitude of the signal is also reduced as a result of decay in the magnetization (mainly \( T_2 \) relaxation). In PASS, with the signal intensity distributed among the centerband and the \( n-l \) resulting sideband spectra, loss in the signal intensity is unavoidable. Hence, it is recommended to spin at the maximum allowable speed in order to reduce the evolution increments, also the number of sidebands.

The original TOSS sequence uses four 180° pulses with inter-pulse delays applied subsequent to the establishment of transverse magnetization [43]. The TOSS experiment was based on Dixon’s original idea [50] and was later modified by Antzutkin [53]. However, during the last decades several studies have appeared with further modifications in order to enhance the resolution, signal intensity and the sideband suppression efficiency [52]. Levitt and co-workers developed a series of five evenly spaced pulses of only one rotor-period duration with relatively easier numerical solutions explained in detail by Antzutkin [52]. TOSS is both sensitive to RF pulse imperfections.
and the instabilities in the spinning rate, and any errors on the pulse width or displacements of the pulses result in signal loss or can lead to unsuccessful suppression of the rotational sidebands. An improved sequence using a 243-step phase cycle largely overcomes the influence of such pulse imperfections [53]. In this study, this version is referred to as TOSS.243. The most common sequences in use today are frequently referred to as TOSS-a (based on 2 rotor revolutions) and TOSS-b (based on 2.2412 rotor revolutions) and the delays 1-4 defined in detail, elsewhere [50]. Here, TOSS-a and TOSS-b are compared to the more advanced one rotor period $5\pi$ pulse version [54] with an extensive 243 phase cycle (TOSS.243) [52]. The main advantage of TOSS over the PASS sequence arises from the fact that it is a 1D experiment which makes both implementation and concatenation with other techniques much easier. For future concatenation of PASS in front of a 2D sequence such as HSQC, would result in a 3D experiment whereas TOSS-HSQC would remain a 2D experiment.
Figure 3.4 The RF pulse sequences contain two segments: water suppression (pre-saturation or PURGE) and sideband suppression (PASS or TOSS.243). PURGE includes two types of delays: $\tau_1$ and $\tau_2$. In this study $\tau_2$ delay was set to a minimal setting (20 $\mu$s) while the $\tau_{1A}$ and $\tau_{1B}$ are optimized as described in section 3.3.3. Phase cycling for PURGE in both PURGE-PASS and PURGE-TOSS.243 are provided in Table 3.1, for both the PASS and TOSS.243 segments is the same as described previously [52,55]. PASS applies five 180° pulses with equal time intervals ($t_1$-$t_6$) which are continuously varied during acquisition [51], whereas TOSS consists of five 180° pulses separated by time intervals ($t_1$-$t_6$). In addition, in challenging samples it may be advantageous to repeat the PURGE block 1-2 times. Using more than 2 loops would approach the duty cycle of a standard magic angle gradient coil and is not recommended.
3.4.4 Incorporating Water Suppression into the Sideband Suppression Sequences

In this study, both pre-saturation and PURGE are concatenated with TOSS-a, TOSS-b, TOSS.243 and PASS. The addition of presaturation (Figure 3.3A) during the recycle delay is trivial and can be done without further adjustment to the main pulse sequence. The concatenation of PURGE to TOSS-a and TOSS-b is also relatively simple as both these TOSS sequences have a short phase cycle that can be fully expanded for testing purposes. Conversely, joining PURGE and TOSS.243 is less trivial due to the 243-step phase cycle in the TOSS.243 segment. As PURGE uses a 16-step cycle full expansion would lead to $243 \times 16 = 3,888$ scans to complete the cycle which is cumbersome and prevents short experiments. Interestingly, it is possible to combine both PURGE and TOSS.243 by treating the cycling within each element independently, while ensuring magnetization of the correct phase is passed between the two modules. The read pulse in PURGE essentially replaces the first $^1$H pulse in TOSS. As the TOSS.243 phase cycling is highly complex, it is simpler to leave it unchanged and reorganize the PURGE phase cycle such that its performance is retained but produces magnetization along the correct (YY-Y-Y) vectors at the start of the $5\pi$ pulse TOSS module. Table 3.1 summarizes how this can be achieved. PURGE can be reorganized such that the full cycle is still preserved but the $\varphi_{read}$ aligns the magnetization along the correct vector for TOSS. The result is that instead of the water cancelling after every other scan (as in the original sequence), it tends to build for 8 scans and then cancels over the next 8 scans for full cancelation after every 16 scans, while the performance of both modules is preserved without increasing the total phase cycle. The same approach can be applied to PASS (Table 3.1).
Table 3. 1A. Phase cycling in the original PURGE. B. Modified phase cycling in PURGE in order to sync to TOSS. 243 C. Modified phase cycling in PURGE in order to sync to PASS.

A.

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<td></td>
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B.

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C.

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<td>( \Phi_4 )</td>
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<tr>
<td></td>
<td>( \Phi_{\text{read}} )</td>
<td>0000222211113333</td>
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</tbody>
</table>

| Magnetization at acquisition (Original PURGE) | \( \Phi_{\text{acquisition}} \) | 1331311320020220 |
3.4.5 High Biomass Samples (Worm Tissue)

In order to develop the best sequence with most effective suppression, the basic sequences were evaluated using a rotor packed with earthworm (*E. fetida*) tissue. When considered within the full gamut of biological and environmental samples, the sample can be classified among the “easier” samples to work with, given its relatively high biomass content and relatively low water content (when compared to, for example, small organisms swimming in pure water, see end of this study). Figure 3.5F shows that Presaturation alone is relatively effective for reducing the water resonance although the first and second order sidebands arise as humps centred around 3.9 and 2.9 ppm. These sidebands are suppressed using Presat and PURGE (Figure 3.5A-B) indicating PASS’s ability to suppress the water sidebands. TOSS-a and TOSS-b both provided excellent water suppression, however, PASS and TOSS.243 show slightly improved sideband cancelation and spectral resolution. The improved line shape in the TOSS.243 and PASS sequence is most obvious, by comparing the alanine resonance at ~1.48 ppm and the signals in the 0.5-1.5 ppm region across all spectra in the series. Further, when compared to PASS and TOSS.243, TOSS-a and TOSS-b give slightly reduced sideband suppression most obvious by the small inverted peaks at ~2.9 ppm. Given the excellent sideband suppression using PASS and TOSS.243 they were both selected and used in the remainder of this study.

3.4.6 Performance with Spinning Speed

Figures B-3 to B-6 demonstrate the limits of spinning rate of PURGE-PASS and PURGE-TOSS.243 performance on a rotor packed with an earthworm or a standard sample of sucrose. Predictably, as spinning is reduced there is a gradual decrease in intensity in the isotropic peaks signal as it is spread into the sidebands. However, it is clear that both PURGE-PASS and PURGE-TOSS.243 generally remain effective down to ~100 Hz. However, for the earthworm ~50% of the isotropic intensity is lost when reducing spinning from 500 to 300 Hz (Figures B-3 and B-4), and while this may be compounded in part due to rotor instabilities (see supporting discussion), the larger rotor periods (and subsequent delays) at lower spinning speeds will certainly lead to relaxation losses in complex samples. As such while the sequences will work down to 100 Hz,
faster rotational speeds, for example 500 Hz, would likely give better quality NMR results and studies at lower spinning speeds are only advised when faster spinning is not an alternative.

Figure 3. 5 Compares various sideband suppression sequences applied to a rotor packed with a small *Eisenia fetida*. All spectra are scaled to the methyl resonance at 0.96 ppm. The dotted region highlights the higher resolution obtained using sequences B and C. The arrows indicate residual sidebands of different order in the presaturation sequences. The efficiency of the water suppression in the 5.1-4.6 ppm region can be also compared among the spectra.
3.4.7 Low Biomass Sample (*D. Magna*)

*D. magna* contain very low biomass contents, with about 10 adults contributing to combined dry mass of ~0.4 mg [4]. To test the effectiveness of PURGE-PASS the metabolic profiling of 20 crushed *D. magna* in H₂O/D₂O (95:5, v/v) was performed. Due to the low biomass to water ratio it is considered a challenging sample that requires very effective water suppression. To further improve water suppression, the PURGE element of both PURGE-PASS and PURGE-TOSS.243 was looped 0-2 times (Figure 3.6). Repeating the PURGE element clearly improves water suppression providing a flat baseline especially close to the central water resonance. Unfortunately, such achievement is accompanied by a ~10-20% signal loss observed for each incremental PURGE loop, resulting from additional relaxation loses with each loop. This was further confirmed with 5 mM sucrose (see Figure B-7). As such, the PURGE loop is “optional” and recommended if metabolites close to the water peak are of considerable interest and suppression without loops is inadequate. With the exception of the region close to the main water signal the remainder of the aliphatic region is largely free of water and sideband artefacts, with just one slight distortion from the 2nd order sidebands at ~2.9 ppm (highlighted with an arrow in Figure 3.6). This is largely suppressed with 2 loops, especially in the TOSS.243 sequence (see inset Figure 3.6).
Figure 3.6 The effect of number of loops on the signal intensity is depicted for A) TOSS.243 (243 scans) and B) PASS sequences (32 scans over 8 slices). The inset (3.0-2.7 ppm) is expanded in order to highlight the artefacts due to second order sidebands and demonstrate how addition of loops affects these artefacts. The experiments were performed on a rotor packed with *D. magna*, representing challenging sample, still with higher biomass compared to an actual *in vivo* sample. PURGE is applied in all cases for water suppression.

3.4.8 *In vivo* Sample

Finally, the sequences were compared *in vivo* on *H. azteca*. These freshwater amphipods are among the most commonly used model organisms by many environmental organizations as they burrow in the sediments and can reflect both sediment and aquatic toxicity [39,56]. In this case, the signal from biomass is very weak, with almost 95% of the organism’s body consisting of water along with an intense signal from the surrounding water. Further, as the water is present in different environments (bound, free intracellular, extracellular etc.) it is very inhomogeneous giving rise to
Figure 3.7 Represents A) PURGE-TOSS.243 and B) PURGE-PASS spectra of *H. azteca in vivo*, spinning at 500 Hz. In both cases 2 loops of the PURGE block were required to permit acceptable water suppression. For TOSS.243 the water suppression is highly effective, and cancellation improves with each fully completed phase cycle (243 scans). For the PURGE-TOSS.243 experiment, 8262 scans were collected equating to 34 completed phase cycles. For all other figures in this study, the TOSS.243 was only completed once (243 scans).

A wide multi-component peak. Compounded with the magnetic susceptibility distortions (leading to weak and broad lines) from the intact organisms it is clear this represents one of the most challenging samples possible. Figure 3.7 shows that in both cases PURGE-PASS and PURGE-TOSS.243 can effectively suppress the water and sidebands, resulting in an aliphatic region that in large part is free of spectral distortions. Unfortunately, this is not observed for the aromatic
region which is distorted using both pulse programs. Signals from amides and proteins are present, although their discrimination from the spectral artefacts is challenging.

3.4.9 Comparison with the current, state of the art methods

Presaturation PASS is currently the most commonly employed approach to suppress both sidebands and water that has been used in many studies [21,30]. Figure 3.8 compares the effectiveness of PURGE-PASS introduced in this chapter and presaturation PASS across the studied samples. With the earthworm sample (relatively high biomass-to-water ratio) both sequences perform well (Figure 3.8A) and are relatively effective at suppressing sidebands and the water signals. In the more challenging *D. magna* sample the PURGE variant shows improved water suppression, leading to better discrimination of the metabolites close to the water, especially the 4-3 ppm region (Figure 3.8B). When switching to the most challenging sample, *in vivo*, the presaturation PASS is unable to suppress the water sufficiently and signals from the sample cannot be determined (Figure 3.8C). This is consistent with the previous literature reports that due to relatively broad water, cannot be tackled with presaturation alone. Conversely, the optimized $\tau_1$ delays in PURGE, along spoil gradients and comprehensive phase cycle [29] help cancel the water more effectively and producing usable data.

3.5 Conclusions

In summary, this chapter introduced two pulse sequences PURGE-PASS and PURGE-TOSS.243, that are capable of suppressing main water signal and sidebands down to 100 Hz in delicate *in vivo* and *in vitro* samples. Both sequences are effective even in the most challenging *in vivo* samples. When compared, both spectra result in similar signal-to-noise per unit time. While both sequences are effective, PURGE-TOSS.243 may be slightly more attractive given that: 1) it is a 1D experiment (PASS is 2D), which should make it easier to concatenate with other 2D experiments; 2) in practice it tends to be slightly more effective at lower spinning speeds (see appendix section);
and 3) it generally provides slightly improved water suppression. Both sequences are relatively simple to implement and capable to study delicate samples that cannot be spun fast in their native state and should find use in a wide range of biological, medical and environmental applications. The code for Bruker spectrometers is available from the corresponding author upon request.

**Figure 3.8** Top panel: Presat-PASS and bottom panel: PURGE-PASS: are applied and compared using different biological samples with different biomass ratios. A) *Ex vivo* on whole *E. fetida* (high biomass to water ratio) B) *Ex vivo* performed on a rotor of crushed *D. magna* (very low biomass but relatively sharp and “shimable water”) C) *In vivo* on 3 alive *H. azteca* (broad and weak sample signals and a broad distortion due to challenging water).
3.6 References


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Chapter 4

In vivo Comprehensive Multiphase NMR

This chapter is adapted from:

Yalda Liaghati Mobarhan, Ronald Soong, Daniel Lane and Andre J. Simpson; “In vivo Comprehensive Multiphase NMR” Magnetic Resonance in Chemistry, Accepted
4.1 Abstract

Traditionally, due to different hardware requirements NMR has developed as 2 separate fields, one dealing with solids, and one with solutions. Comprehensive Multiphase (CMP) NMR combines all electronics and hardware (magic angle spinning, gradients, high power RF handling, lock, susceptibility matching) into a universal probe that permits a comprehensive study of all phases (i.e., liquid, gel-like, semi-solid and solid), in intact samples. When applied in vivo it provides unique insight into the wide array of bonds in a living system from the most mobile liquids (blood, fluids), through gels (muscle, tissues) to the most rigid (exoskeleton, shell). In this chapter the practical aspects of in vivo CMP are discussed including; handling the organisms, rotor preparation, sample spinning, water suppression, editing experiments and finishes with a brief look at the potential of other hetero-nuclei (2H, 15N, 19F, 31P) for in vivo research. This chapter is aimed as a general resource for researchers interested in developing and applying MAS based approaches to living organisms. While the focus is CMP-NMR, many of the approaches can be adapted (or directly applied) using conventional HR-MAS and in some cases even standard solid state NMR probes.

4.2 In vivo NMR

Scientific questions are becoming increasingly complex and biological relevance is always at the forefront of discussion. As such it is clear that high resolution molecular-level in vivo methods must play a central role in the next generation of scientific research. NMR spectroscopy is arguably the only modern technique that can provide high resolution metabolic and structural molecular information in living organisms in a non-invasive manner [1]. To avoid confusion this chapter focuses on high-field in vivo NMR, defined here as small living organisms completely sustained within vertical bore high resolution NMR spectrometers (as opposed to more traditional horizontal MRI systems). In high-field NMR systems there are two main approaches, 1) Flow based NMR using solution state NMR probes and 2) Magic Angle Spinning (MAS) based approaches. Flow
based approaches provide a low stress environment in which the organisms can be maintained indefinitely [2-5] and is thus, ideal for looking at metabolic responses to external stimuli. MAS approaches are highly complementary to flow-based approaches and narrows the spectral line shape permitting more detailed assignments (especially from $^1$H NMR). CMP when applied to its full potential can provide a comprehensive overview as to all the components (liquids, gels, and solids) within a living organism.

4.2.1 HR-MAS vs CMP-NMR

HR-MAS NMR was introduced in 1996 [6], combines MAS, a lock (provides stability over time), susceptibility matched stators (further improves line shape) and pulse field gradients (permits diffusion measurements, improved 2D NMR and improved water suppression). MAS reduces the dipole-dipole, anisotropic interactions and averages magnetic susceptibility mismatches across a sample [7]. Therefore, when applied to a living organism, swollen components, including solutions (metabolites) and gels (mobile membranes, parts of tissue) become easy to observe and information rich $^1$H NMR profiles are obtained. As such HR-MAS has enjoyed wide applications to intact biological systems such as tissues or whole cells, as well as a range of living organisms including Drosophila melanogaster [8], Caenorhabditis elegans [9], Aporrectodea caliginosa [10], Calanus finmarchicus [11], Daphnia magna [12], Saccharomyces cervisiae cells [13] and mice [14].

Comprehensive Multiphase NMR (CMP) probes were introduced in 2012 [15] and have been commercially available from Bruker Biospin since 2015 [16]. The probes integrate all components of a HR-MAS probe with added benefit of the high-power RF handling required for the study of true solids (i.e. cross polarization and high-power decoupling). As such it allows the study of all components (liquids, gels and solids) in complex multiphase samples. When combined with spectral editing approaches (discussed later), various phases can be studied and differentiated in situ allowing complex processes such as the transport of molecules across phases [17] or swelling between phases to be monitored [18-20].
Traditionally, for NMR analysis, samples were dried for solid state analysis or extracted for solution state studies [21]. However, such invasive procedures can drastically change the chemistry and biology of the sample, with no better example than the impacts on a living organism. In many ways, CMP-NMR can be thought of as changing NMR technology to match natural samples, rather than changing the sample to match a specific NMR technique. CMP-NMR has permitted the study of structural interactions and processes in unaltered heterogeneous samples, including soil [17,18,20,22], swelling of rubber by biofuels [17,19,22-24] intact plants [25-27], and to follow biological processes (growing seeds) [26].

When applied to living system CMP-NMR allows the ability to study all phases, from the most solid (shell), through the gels (membranes, tissues, protein) to the liquid (blood, metabolites). The first application of CMP-NMR to a living organism was on H. azteca (freshwater shrimp) [28] and the technique showed great potential to link more rapid stress responses (manifested as metabolite fluxes in the solution phase) to longer term “structural” impacts (such as thinning of shells induced by long term contaminant exposure) [29,30]. This first paper concludes “The technique acts as the ideal “molecular interpreter” providing the desperately needed connection between the physical (for example environmental stress and disease) to the chemistry that ultimately defines these processes”. Since then CMP-NMR has also been applied to monitor D. magna [31,32] and seed growth [26] while its application to living systems is still very much in its infancy.

Given the considerable potential of in vivo CMP-NMR, it is likely that other groups may wish to adapt and apply this approach. This chapter aims to summarize the key requirements for CMP-NMR. The first section focuses on the in vivo specific parameters, including rotor preparation, spinning speed, and labelling protocols. The second section covers the experimental implementation including water suppression and spectral editing approaches. While the last section touches on future potential especially for nuclei such as $^{31}$P, $^{19}$F, $^2$H, $^{15}$N, that have yet to be studied using in vivo CMP-NMR.
4.2.2 SAMPLE PREPARATION, HANDLING AND SPINNING

4.2.2.1 Organisms

A number of commonly used model organisms in aquatic and sediment toxicity testing such as *H. azteca*, and *D. magna* are small enough into fit a 4 mm rotor. *H. azteca* (Figure 4.1A), is a sensitive organism to biotic and abiotic variables and a number of studies have been conducted on it to investigate sediment and aquatic toxicities [33-35]. *D. magna* (Figure 4.1B) is one of the most studied organisms for aquatic toxicity since it is a keystone species that connects the consumers and producers in aquatic ecosystems.
Figure 4. 1 A) Hyalella azteca, B) Daphnia magna. C) A plastic pipette is cut to provide a wide opening that can be used to pick up and transfer the organisms to the NMR rotor. D) Top view of an organism inside the rotor. E) A side view through a sapphire rotor showing the organisms inside.

4.2.2.2 $^{13}$C Isotopic Enrichment

Protocols for culturing of both D. magna and H. azteca are provided elsewhere3,34. Specifically for NMR studies, the organisms can be enriched by replacing their normal food source (algae) with $^{13}$C enriched algae. We find 2 mg/L per organism of 99% $^{13}$C Chlorella Vulgaris (Silantes GmbH) three times a week works well [36], as this strain has particularly high amount of soluble lipids [36]. In such studies, the organisms are separated from the adults after birth, with a constant supply of $^{13}$C enriched C. Vulgaris as the only source of food. In order to prevent cross contamination by airborne algae from other sources within the lab, the labelling tanks are cleaned regularly. This is performed by passing the organisms through a mesh and acid/base washing the empty containers and rinsing thoroughly. Isotopic enrichment is recommended from birth for at least 3-4 weeks prior to the studies [3]. It is important to remember that if the organisms have been recently fed their $^{13}$C enriched stomach contents will show up in the $^1$H-$^{13}$C NMR spectra. If this is undesirable then the guts can be purged using $^{12}$C algae as described by Dutta Majumdar et al., [3].

4.2.2.3 Sample Handling

Living organisms are very delicate and need to be handled with care. To ensure the organisms are not injured while loading the rotor, they are transferred using a plastic pipette with a cut tip to provide a wide opening as shown in video 1. A sapphire rotor can be used to observe the behavior of the organisms once inside the rotor, however these rotors tend to be more expensive than zirconia rotors. Depending on the aim of the experiment, a different number of H. azteca (from one large to seven smaller organisms) can be loaded into a 4 mm rotor.
Video 4.1 Shows a *H. azteca* being loaded into a sapphire rotor.

4.2.2.4 Spinning speed

Spinning at high speeds (> 6 KHz) is routinely applied to enhance the line shape in non-living samples [6,37]. However, for biological tissues cell damage has been reported above 3 KHz [38]. Although in some bacterial and plant cells, the cell walls may provide additional structural support against centripetal forces [39], while others may be easily damaged. For example, after 2h spinning at 3.5 KHz, larger lipid-laden adipocyte cells experienced higher forces with up to 19% loss in the cellular integrity, while smaller preadipocytes showed no visible signs of cell lysis under the same conditions [40]. This is consistent with findings from *in vivo* studies, which in general, identify ~2.5 KHz as the maximum speed organisms can be spun [12,28]. At 500 MHz, 2.5 KHz spinning is sufficient to locate the water sidebands outside the $^1$H spectral range of interest, as shown in Figure 4.2A-C.

The duration and spinning rate that organisms can survive depends on the species and needs to be investigated prior to each *in vivo* study. Figure 4.2D plots the survival over time for two species *D. magna* and *H. azteca*. *H. azteca* have a more robust exoskeleton which provides additional structural support. As such *H. azteca* can survive ~6 h (100% survival rate) at 20°C, while *D. magna* survival rate decreases after 4 h. Reducing the spinning rate from 2.5 KHz to 50 Hz, increases the 100% survivability window of *H. azteca* to over 48 h and to 24 h for *D. magna*. As such a conundrum exists, where lower spinning rates extend the longevity of the organism and hence the experimental duration, while faster spinning reduces overlapping sidebands (Figure 4.2) and increases chemical shift information. For $^1$H NMR Liaghati Mobaran et al. [32] introduced a combined water and sideband suppression approach which reduces sidebands *in vivo* for speeds down to 300 Hz. In other work, $^2$H-$^{13}$C enrichment and $^2$H-$^{13}$C HMQC detection offers promise for extracting rich metabolic profiles down to 50 Hz [31].
Figure 4. The impact of spinning rate on the location, number of the sidebands and the intensity of isotropic peak spinning at A) 2.5 KHz to B) 500 Hz and C) 300 Hz is demonstrated. (*) represent the sidebands. D) The survival percentage at spinning rates of 50, 500 and 2.5 KHz for *H. azteca* and at 50 and 2.5 KHz for *D. magna* is plotted over time [32]. Diamonds and circles respectively represent *H. azteca* and *D. magna*. The experiments are performed at 20 °C, in triplicate, with the standard deviation reported. Note the data are generated using a customized cap to permit oxygen exchange.
4.2.2.5 Oxygen Delivery

Traditionally, rotors are fitted with air tight seals to prevent liquids from leaking, however in the case of in vivo studies this prevents oxygen reaching the organisms inside the rotor. The earlier studies on D. magna used an anesthetic to put the organisms to sleep, which reduces their oxygen requirement. When anaesthetized, the organisms stayed alive for up to 2-3 h spinning at 2000 Hz [12]. The drawback of an anesthetic is that it may also impact metabolic processes that in many studies are of primary importance. An alternative approach was introduced by Liaghati Mobarhan et al., [28] and involves drilling a 500 µm hole into the cap (Figure 4.3A). Interestingly, spinning pushes the liquid to the edge of the rotor walls (sealed with viton O-ring) and forms a tiny vortex at the center that permits air exchange. Figure 4.3B shows the impact of the cap on the organism’s survival. In this study, in order to distinguish the impacts of anoxic stress from spinning stress, the rotor was not spun (Figure 4.3B). The authors recommend customized caps drilled with a hole for all in vivo studies. Note organisms can survive spinning >48 h (see Figure 4.2), the drive air blowing across the cap helps exchange oxygen in the rotor, which in turn sustains the organisms alive for longer periods.

To make the cap, we recommend O-ring seal 4mm rotor caps from Wilmad Scientific. Then place the cap into the rotor, and the rotor into a high-quality lathe. Place a high-quality drill bit (KT-0200-S from Performance Micro Tools works well) in the static collet and bring the static drill bit towards the rotating rotor (Figure 4.3C). The authors find this approach produces caps that spin well inside the NMR. All attempts using conventional drill presses failed and produced caps that would not spin, likely due to the holes being very slightly off center.
Figure 4. 3 A) Shows a customized cap B) Highlights the effect of utilization of the customized caps on *H. azteca* survival rates compared to a normal cap [28], C) The lathe drilling a hole in the center of the cap.

4.2.2.6 Temperature, Number of Organisms and Position

The number of organisms per rotor impacts survivability. Too many organisms lead to overcrowding and increased oxygen consumption, while fewer organisms give lower NMR signal. Figure 4.4A shows that 3 medium sized adults is the maximum *H. azteca* that can be studied, while retaining 100% survivability over a 1 h window, spinning at 2.5 KHz. Temperature also has a significant impact on organism longevity, in large part, as lowering the temperature can put aquatic
organisms into a more docile state which leads to reduced oxygen consumption [41]. If the temperature is reduced to 5 °C, full survival for up to 4 organisms can be maintained for *H. azteca* (Figure 4.4A). Additionally, *H. azteca* which are generally long and thin and unable to turn around inside the rotor, putting them head down towards the bottom of the rotor increases survival by keeping their gills submerged, even if some liquid is lost via the cap through evaporation. With their heads down, the organisms can survive for 12 h at 5 °C (Figure 4.4B).

![Figure 4.4](image)

**Figure 4.4** The impact of temperature and the position of the *H. azteca* inside the rotor on survival rates adapted from Liaghati Mobarhan *et al.*, [28].

4.2.2.7 Spectrometer Lock

A spectral lock is required for *in vivo* research, especially if longer 2D NMR experiments are implemented. The simplest solution is to perform *in vivo* MAS studies in 5% D₂O [28]. In many
organisms no signs of additional stress have been reported for up to 20% D₂O [36]. However, D₂O is known to be toxic to some organisms and could potentially impact metabolism even in small amounts [42]. As such if the goal of the MAS study is to follow metabolic responses or toxic impacts it may be preferable to put the D₂O in a sealed 0.5 mm capillary containing 5-10 µL D₂O placed in the bottom of the rotor as an external lock system. The details on how to make these lock capillaries have been provided elsewhere [22].

4.2.3 Experimental Considerations

4.2.3.1 Water suppression

Due to the wide range of water environments within a living system, the water peak in vivo is both intense and relatively broad even under MAS. The most effective way to suppress the water at reasonable spinning speeds (>1.5 KHz) is Shaped Presaturation W5-WATERGATE [43]. This technique simply uses a train of shaped presaturation pulses to narrow the water signal after which W5-WATERGATE [44] inverts the sample signals via W5 DANTE blocks and dephases the residual water using pulse field gradients. This sequence and an optimized version termed TWINS [22] have been tailored specifically for environmental samples with broad and intense water and are extremely efficient, in vivo. Figure 4.5 shows the approach on a rotor of H. azteca spinning at 2.5 KHz. Without water suppression no signals from the organisms can be seen even when the baseline is expanded 128 times. However, when using SPR-W5-WATERGATE, the water is effectively suppressed and ¹H signals from the organism are recovered. For all studies >1.5 KHz the authors recommend SPR-W5-WATERGATE, as it simple to implement and very robust.

Unfortunately, at slower spinning speeds, SPR-W5-WATERGATE performs very poorly and nearly all signal is lost below 1 KHz spinning speed. At these slower speeds, a combined sideband and water suppression approach is required. Recently, a new approach termed PURGE-TOSS.243 was developed specifically for water and sideband suppression for in vivo organisms spinning at lower speeds (down to 300 Hz). The approach is a 1D experiment that is simple to use and produces suppression superior to as presaturation-PASS [45] (a 2D approach) commonly used to separate isotropic and sideband overlap in complex samples [32].
Figure 4.6 compares $^1$H SPR-W5-WATERGATE and $^1$H PURGE-TOSS.243 on *H. azteca* spinning at only 500 Hz. While $^1$H SPR-W5-WATERGATE fails, the $^1$H PURGE-TOSS.243 recovers the $^1$H *in vivo* profile effectively. The main drawbacks for PURGE-TOSS.243 are; as a presaturation based approach, shimming the water to produce the narrowest line shape is required on each sample, and due to its complex phase cycle, 243 scans (486 recommended) are required. While this at first may seem highly prohibitive, it should be remembered that due to their complex and heterogeneous nature most signals from living organism are weak and require a reasonable number of scans for adequate signal-to-noise.
Figure 4.5 A) Without water suppression there are no observable signals from *H. azteca* B) After the spectrum is expanded vertically x 128 times without water suppression. C) With the addition of SPR-W5-WATERGATE the water is effectively suppressed and $^1$H signals from the organism are recovered. In addition, by expanding the baseline x64 times aromatic region shows signals from aromatic amino acids. Adapted from Liaghati Mobarhan *et al.*, [32].

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4.2.3.2 Spectral Editing Experiments

Living organisms contain a complete distribution of phases from true solids (shells and bone), gels (tissue, membranes) through solutions (blood, metabolites). The combination of CMP-NMR with spectral editing to isolate signals from the various phases is extremely powerful and gives a unique window as to the sub-components within a living organism. The spectral editing approaches discussed here were introduced by Courtier-Murias et al., along with the CMP probe [15] and readers interested in the full details and possibilities of spectral editing should refer to this work. Here the most important experiments for in vivo editing are reviewed. Similar to Courtier-Murias
et al., as the ideas are quite complex, the approaches are first introduced on a standard mixture before discussing the *in vivo* results.

Figure 4.7 depicts the $^1$H NMR of a mixture of Nicotinamide, Aminohexylamide Gel, and Cholesterol Acetate swollen in water. Nicotinamide is completely soluble in water and forms a solution, the Aminohexylamide is a swellable gel, while the Cholesterol Acetate is completely insoluble in water and remains as a crystalline solid. Using $^1$H detection both the Nicotinamide and Aminohexylamide Gel are easily detected. However, the Cholesterol Acetate is not visible due to the strong $^1$H-$^1$H dipole interactions leading to a spectral profile many KHz wide. While it is possible to selectively detect the solids via $^1$H Combined Rotation and Multiple Pulse Sequence (CRAMPS), see Courtier-Murias *et al.*, [15] for more information, the approach is challenging to implement and the spectral profile recovered is broad and lacks detailed chemical shift information. As such the most practical approach for spectral differentiation of all the phases is to use $^1$H detection for solutions and gels, and $^{13}$C cross polarization approaches for the solids and semi-solids.

![Figure 4.7](image_url)

**Figure 4.7** $^1$H NMR of a standard multiphase mixture of Nicotinamide, Aminohexylamide Gel, and Cholesterol Acetate used to demonstrate the spectral editing experiments. Adapted from Courtier-Murias *et al.*, [15].
4.2.3.2.1 $^1$H Sub-editing of dissolved molecules and gels

Solution state signals can be separated using both relaxation filters ($T_2$, CPMG) or diffusion-based approaches. For brevity only, the diffusion-based approaches are discussed here. In the authors experience both approaches work well, however, the diffusion based often provides more complete spectral editing. On the other hand, relaxation-based editing does not require gradients and thus opens the possibilities of performing basic editing on solid probes with no pulse field gradients. Readers interested in relaxation based editing should refer to Courtier-Murias et al., [15].

Figure 4.8A shows a conventional $^1$H spectrum. This is a BPPLED [46] sequence with all the delays and gradients set to zero. The result is a stimulated echo that gives a near identical result to the conventional single pulse $^1$H experiment. However, as later other BPPLED sequences will be subtracted from this reference, it is best practice to use a BPPLED sequence for the reference to ensure optimal subtractions. Figure 4.8B is a diffusion edited spectrum. In this case the diffusion gradients are turned on and the respective diffusion delays are set to appropriate values. The exact settings of these values depend on the goals of the project and can vary from sample to sample. We recommend strong diffusion editing such that mobile molecules are completely attenuated. This will generally involve setting the main diffusion delay between 100-200 ms, the gradient pulse (i.e. half the pulse pair to 1.25-2.5 ms) and the gradient strength to ~95% the maximum (normally ~40-50 gauss per cm depending on the probe). Readers should note that rotor synchronizing both the gradient lengths and the diffusion time to multiples of the rotor period will generally lead to better results. In Figure 4.8B, the solution components are completely attenuated and only signals from the gel, and the nicotinamide within the gel, remain. As such Figure 4.8B represents the molecules with restricted diffusion, which can be generally classified as “gels”. For example, these molecules do not physically diffuse, but are dynamic enough to survive the relatively long delays required for diffusion editing.

Figure 4.8C contains the molecules that are attenuated by the diffusion gradient and represents the molecules that are dynamic and can move within the sample (i.e. dissolved components). Figure 4.8D-F outlines how the spectrum in 4.8C is obtained. Figure 4.8D is a spectrum collected with the diffusion delays set to their appropriate values for diffusion editing, but with the strength of the diffusion encoding/decoding gradients set to zero. Figure 4.8E is identical, with the exception that the diffusion gradient is turned on, resulting in the dissolved molecules being attenuated. By
subtracting E from D, the “inverse diffusion editing spectrum” which contains only signals from the freely diffusing molecules is created in Figure 4.8F (same as Figure 4.8C).

**Figure 4.8** $^1$H NMR of standard multiphase mixture of Nicotinamide, Aminohexylamide Gel, and Cholesterol Acetate. A) Conventional $^1$H NMR which shows all components dynamic enough to be detected using $^1$H NMR. B) A diffusion filter highlights the “gel-like fraction”. C) The Inverse Diffusion Edited spectrum (IDE) which highlights the dissolved components. The IDE is created from spectra D and E as described in the main text [15]. Adapted from Courtier-Murias et al., [15].

4.2.3.2.2 Relaxation Recovery Arising from Diffusion Editing (RADE) ($^1$H detected semi-solids)

As demonstrated above, diffusion editing is excellent for highlighting both the dissolved and gel-like phases. However, using very long delays can lead to significant relaxation. If the signals that relax are not accounted for, then fast relaxing components could potentially be missed. Luckily,
these signals are easy to recover using an approach termed “Relaxation Recovery Arising from Diffusion Editing” (RADE) [15] depicted in Figure 4.9. Figure 4.9A is a BPPLED sequence with all delays and gradients set to zero and the gradient powers turned off, giving the same result as a single pulse $^1$H experiment. Figure 4.9B is the same experiment with the delays set to the values required for diffusion editing. The resulting spectra are quite different and signals from fast relaxing components (the gel (see oval in 4.9B) and the Nicotinamide within the gel (see * in 4.9B)) are preferentially lost. These are recovered by a weighted subtraction of B from A, resulting in the spectrum C highlighting the signals with fast relaxation that may be missed if diffusion editing were used alone. These rigid-gel or mobile solids in C, have enough dynamics to appear in $^1$H NMR but exhibit the fastest relaxation and represent the most “solid-like” components that can be detected using conventional “low power” $^1$H NMR (i.e. without resorting to advanced $^1$H narrowing approach such as CRAMPS).
Figure 4. $^1$H NMR of standard multiphase mixture of Nicotinamide, Aminohezylamide Gel and Cholesterol Acetate. A) Conventional $^1$H NMR which shows all components dynamic enough to be detected using $^1$H NMR. B) A diffusion editing experiment but with the diffusion encoding/decoding gradients set to zero power, but all the delays set appropriately for diffusion editing. C) RADE spectrum which is created by a weighted subtraction of B from A and represents the fastest relaxing $^1$H detectable components in the mixture, consistent with “semi-solids” or “rigid gels”. Adapted from Courtier-Murias et al., [15].

4.2.3.2.3 $^{13}$C NMR detection (Semi and Rigid Solids)
As discussed briefly, true rigid solids are difficult to detect using $^1$H detection and require approaches such as CRAMPS that produce broad lines and are hard to implement. As such solids
are best detected using $^{13}$C. However, as CMP-NMR probes are designed to handle the high power required for solid state experiments (i.e. of cross polarization and high-power decoupling), this can be simply achieved. For solid components in a multiphase sample, cross polarization can be used as a filter to select only carbons that form strong spatial dipole with protons in the sample. Molecules that exhibit tumbling or motion, average and modulate the dipole interaction making cross polarization extremely inefficient. This is summarized in Figure 4.10. Figure 4.10A shows the $^1$H-$^{13}$C cross polarization of the mixture of Nicotinamide, Aminohexylamide Gel, and Cholesterol Acetate swollen in water. Both signals from cholesterol acetate and Aminohexylamide Gel appear, but signals from nicotinamide do not appear, as it is too dynamic to efficiently cross polarize. The mobile solids can be selected by adding a very short $^1$H $T_2$ filter (2 CMPG echoes with an inter-pulse delay of 7.5 $\mu$s, to give a total filter of 30 $\mu$s) prior to cross polarization. This filter suppresses the signal from crystalline solids due to their very fast $^1$H $T_2$ and only selects the components from the more mobile solids, which in this case is the Aminohexylamide Gel (compare Figure 4.10B and 4.10E). The rigid solids are then recovered by subtracting 4.10B from 4.10A. A comparison of 4.10C and 4.10F shows that Cholesterol Acetate is selected. As described by Courtier-Murias et al. [15] the rigid solids can also be selected directly via T1$_o$ filter, however, this involves a high power spin lock of considerable length which is extremely demanding on the NMR probe. As such we strongly recommend using the $T_2$ filtered CP and recovering the rigid solids via difference.
Figure 4.10 $^{13}$C Cross polarization-based approaches used to select the solid components from the multiphase mixture. A) Standard cross polarization selects the Aminohexylamide Gel and Cholesterol Acetate but not the Nicotinamide. B) A T2 filtered CP selects the dynamics solids (Aminohexylamide Gel). C) The rigid solids are obtained via difference (A-B). D-F) $^{13}$C NMR spectra of Nicotinamide, Aminohexylamide Gel and Cholesterol Acetate standards for reference. Adapted from Courtier-Murias et al., [15].

4.2.4 Spectral Editing Applied In vivo

Figure 4.11 shows the spectral editing approaches described above applied to H. azteca, in vivo as reported by Liaghati Mobarhan et al., [28]. Figure 4.11A represents the IDE spectrum and highlights the most mobile components in the organism from which the majority of amino acids can be directly assigned. 4.11B highlights the components with restricted diffusion such as “gel-like” components which may include bound species, biopolymers, high molecular-weight macromolecules. Resonances consistent with omega-3 fatty acids along with triacylglycerides
(TAG) are amongst the most dominant components in this fraction [36]. Figure 4.11C represents the RADE spectrum, most consistent with “semi-solids” and strong contribution from lipids, likely due to faster relaxing components in membranes and cell walls [26]. In addition, signals from aromatic amino acids are noted, consistent with proteins existing within rigid tissues such as muscle. Such pools are important as the protein content can reflect the physiological status and energy reserves of a living organism [47,48]. 4.11D also selects semi-solids but in this case T2 filtered 13C CP NMR detection is used. The signals are consistent with long chain lipids (membranes), with the resonances at 70 ppm and 130 ppm respectively correlating to C-O-C and C=C entities in lipids. Finally, 4.11E shows the most rigid components which are consistent with chitin. Chitin [poly(β-(1-4)-N-acetyl-D-glucosamine] is the most rigid component and constitutes the exoskeleton. The 22.1 ppm correlates well with the CH₃ in acetamide group and resonances at 54.4, 59.6, 73.1, 74.7, 82.2, 103 ppm respectively belong to C2, C6, C3, C5, C4, C1 of the hexose ring and the 173 ppm predominantly correlates to C=O in the acetamide group [49]. Chitin in the exoskeleton mainly exists in the form of chitin–protein matrices that provide additional structural support. The overlapping peaks at 55–20 ppm are consistent with rigid protein residues in these matrices.
Figure 4.11 Spectral Editing techniques are used to spectroscopically classify the phases of *H. azteca* in five $^1$H and $^{13}$C NMR spectra spanning from the most dissolved (i.e. metabolites) through gel components (i.e. lipids, protein) and semi-solids (i.e. cell membranes) to the most rigid components (i.e. shell), reflected respectively in A-E. Adapted from Liaghati Mobarhan *et al.*, [28].
4.2.5 2D Experiments and Assignments

In addition, to spectral editing and conventional 1D, 2D NMR is extremely useful for more detailed assignment. Figure 4.12 shows a typical In-phase $^1$H-$^1$H COSY of living *H. azteca*. The In-Phase COSY experiment was introduced in 2005 by Xia *et al.*, [50], and gives considerable sensitivity enhancements especially in complex samples with fast relaxation as is the case *in vivo*. In Figure 4.12 it is concatenated with W5-WATERGATE water suppression, this version of the sequence is available from the authors of this manuscript on request. $^1$H-$^1$H COSY helps confirm the assignment of a number of metabolites and is especially useful as it can be applied to organisms without the need for isotopic enrichment.

![Figure 4.12 In-Phase $^1$H-$^1$H COSY experiment of *H. azteca* reproduced from Liaghati Mobarhan *et al.*, [28].](image)
Figure 4.13 shows a typical $^1\text{H}^{-13}\text{C}$ HSQC spectrum for *H. azteca*. By extending the spectra into a second dimension, the resolving power of NMR is significantly increased from a peak capacity of 3000 for 1D $^1\text{H}$ to ~2,000,000 reported for 2D $^1\text{H}^{-13}\text{C}$ HSQC [51]. This additional spectral dispersion is very useful in overcoming spectral overlap making assignments against standard metabolic databases such as the Bruker Bio-reference Databases [28] or Human Metabolome Database (http://www.hmdb.ca/statistics) much easier. While this experiment requires $^{13}\text{C}$ enrichment it permits a wider range of metabolic components to be assigned *in vivo*. As *in vivo* CMP-NMR of labelled organisms is still in its infancy there is still considerable potential for applying many other types of 2D and higher dimensional NMR experiments. At present one of the shortcomings in assignment is the limited number of metabolites with assigned 2D NMR. Currently there are ~700 assigned in the Bruker Bio-reference Databases (releases 2-0-0 through 2-0-5) and close to 900 metabolites with 2D spectra in the HMBD, with the majority targeting human metabolites. However, as the field of *in vivo* NMR and environmental NMR continues to grow, organism specific databases especially for commonly studied species such as *D. magna* will likely ensue in the near future.
Figure 4.13 A) the in vivo $^{1}$H-$^{13}$C HSQC of $^{13}$C isotopically enriched $H. azteca$ with main chemical shift regions labelled. B) About 40 metabolites are identified in the aliphatic region. C) aromatic region. D) list of color-coded assignments of the metabolites corresponding to the colored dots in B and C. (*) Represent the aromatic residues in proteins. Reproduced from Liaghati Mobarhan et al., [28].
4.3 Future Potential and Hetero-Nuclear NMR

CMP-NMR is still in its infancy and the final section of this chapter briefly investigates the potential application of hetero-nuclei that may be of interest for in vivo studies.

4.3.1 Carbon ($^{13}$C)

While 1D solid state $^{13}$C detection in vivo was discussed above, no “solution state” low power decoupled $^{13}$C NMR spectra were shown. Figure 4.14 considers the impacts of H. azteca feeding on $^{13}$C enriched algae as an example. Figure 4.14A shows a $^{13}$C experiment with low power decoupling of 99% for $^{13}$C enriched algae. The top panels (i + ii) compare the algae at the start (i) and the control (i.e. no H. azteca) after 6 h (ii). As can be seen few, if any, changes arise without the H. azteca present. In the presence of H. azteca (iii) the lipids decrease slightly while the signals from glucose increasing quite substantially. This is consistent with lipids from the algae being utilized by H. azteca as an energy source [47]. $^{13}$C NMR is a simple and effective approach to obtain metabolic information in vivo and does not require water suppression. In this case as only low-power decoupling was used, the experiment will bias $^{13}$C attached to protons with relatively narrow line shape (i.e. liquids and gels) and discriminate against true solids [26,27]. If absolute quantification is required with each phase being represented equally, then the recently published approach of stepped decoupling, specifically developed for $^{13}$C quantification in multiphase samples, should be employed [52].

4.3.2 Nitrogen ($^{15}$N)

Nitrogen is a highly versatile nucleus for the study of biological molecules including amino-acids, proteins and DNA [53]. In Figure 4.14B the organisms were raised on 99% $^{13}$C/$^{15}$N doubled labelled Chlorella Vulgaris (Silantes GmbH). Figure 4.14B(i) shows $^{15}$N CP-MAS NMR highlighting nitrogen containing components in the more rigid structures of living H. azteca, consistent with predominately amide in proteins. Sidebands are visible due to the slow spinning
speed of 2.5 KHz required for \textit{in vivo} analysis. The same organisms were later spun at 6.666 KHz (\textit{ex vivo}, scans increased 10 fold) (Figure 4.14B(ii) and the isotropic peak is significantly narrowed as the anisotropic and residual dipolar interactions are further averaged by the faster spinning [54]. Figure 4.14C(iii) depicts a complimentary experiment focusing on mobile metabolites. This experiment is a N-edited HSQC such that only H-C bonds directly bonded to $^{15}$N are observed [55]. In this case various small metabolites including amino acids and choline are detected \textit{in vivo}. The experiment demonstrates that $^{15}$N is a very interesting nucleus not just for detection but also as a useful X- filter for reducing spectral overlap.
Figure 4. 14 A) $^{13}$C NMR spectra of i) 99% $^{13}$C C. Vulgaris, ii) Same rotor of $^{13}$C C. Vulgaris, after 6 h in the absence of H. azteca. iii) $^{13}$C C. Vulgaris in the presence of H. azteca (natural abundance $^{13}$C) after 6 h. The pink region highlights the $\alpha$, $\beta$-glucose peaks, which intensifies in the presence of H. azteca in contact with the algae. $^{13}$C NMR spectra were acquired using 2048 scans at 1.1 KHz spinning at 15 °C. B) $^{15}$N NMR 1D $^{15}$N CP-MAS of $^{13}$C/$^{15}$N enriched H. azteca provides a general profile on the $^{15}$N content of the rigid components i) at 2.5 KHz in vivo ii) at 6.66 KHz ex vivo iii) $^{15}$N-edited $^1$H–$^{13}$C HSQC in vivo on H. azteca. $^{15}$N NMR i) was acquired with 10240 scans. ii) Was acquired with 102400 scans and 2D spectra iii) were acquired with 386 scans at 128 increments, spinning at 2.5 KHz. C) $^2$H NMR Compares $^1$H NMR and $^2$H NMR detection. i) $^1$H–$^{13}$C HSQC spectra of $^{13}$C isotopically enriched algae spinning at 50 Hz highly distorted crosspeaks and some information is masked by water resonance. ii) $^2$H–$^{13}$C HMQC of $^2$H/$^{13}$C isotopically enriched algae spinning at 50 Hz show full metabolic profiles without interference from the water sidebands (both experiments took 1 h to complete).

4.3.3 Deuterium ($^{2}$H)

Unlike its proton counterpart, $^2$H is a quadrupole nuclei, albeit with a relatively small CSA of only 770 Hz and smaller hetero-nuclear and homonuclear dipolar couplings, compared to that of $^1$H [56]. As such when a molecule containing $^2$H is completely dissolved, the rapid tumbling averages the quadrupole interaction to zero, leading to sharp lines, but if molecular tumbling is restricted the quadrupole interaction is quickly reintroduced and the signals are broadened up to hundreds of KHz [57]. Interestingly, per-deuterated algae gives well-resolved $^2$H–$^{13}$C correlations, as only the truly dissolved components are selectively detected [31] as shown in Figure 4.14C(ii) Particularly exciting is that while at 50 Hz nearly all information is lost from a $^1$H–$^{13}$C correlation, excellent correlations can still be obtained in the $^2$H–$^{13}$C experiment. As shown earlier the 100% survivability window of shrimp at 50 Hz is greater than 48 h (see Figure 4.2). This suggests $^2$H may be a very interesting nucleus for future in vivo metabolomics research and warrants further investigation [31].

$^2$H NMR also holds great potential for following the assimilation of molecules into an organism. Figure 4.15A shows a simple example where perdeuterated phenylalanine is added to H. azteca
and utilized over ~25 h. Over time the signal disappears, indicating that due to interaction with the organism, $^2$H phenylalanine becomes less mobile and transforms into a form not observable with narrow-windowed $^2$H detection. For example this could resemble incorporation into proteins [31]. Future work could utilize wideline $^2$H to investigate if binding information in the “solid state” can be monitored \textit{in vivo}. The wideline pattern of $^2$H is especially sensitive to molecular orientation [58] and represents an interesting source of potential information in future studies.

4.3.4 Fluorine ($^{19}$F)

The $^{19}$F isotope is 100\% natural abundant and yields high NMR sensitivity (83.3\% of proton) and a wide chemical shift range (~400 ppm) that provides high spectral dispersion. Up to 20\% of pharmaceuticals are fluorinated [59] and many end up contaminating water sources across North America [60]. Similarly, perfluorinated chemicals (PFCs) have been widely used in commercial applications and are emerging as group of environmental pollutants found globally; from tap water to animal tissues and far as the arctic where there are no sources of direct exposure [61]. Particularly, perfluorooctanesulfonate (PFOS) is of global concern due to its persistence and bioaccumulation in the environment. In 2006 Environment Canada concluded that PFOS is a toxic compound with potential immediate or long-term adverse impacts on the environment and living species [62]. Studies report that PFOS may cause serious effects on humans and wildlife; such as possible liver, kidney, thyroid and reproductive deficiencies and is potentially a carcinogen [63-65]. Containing a hydrophobic tail and hydrophilic head, PFOS behaves differently than traditional hydrophobic compounds. Hence, studies continue to unravel the poorly understood environmental fate of this pollutant. To demonstrate the feasibility of $^{19}$F NMR for \textit{in vivo} studies, \textit{H. azteca} was exposed to 23 mg/L PFOS slightly below the LC50 of 27.7 mg reported by Lee et al [66] for \textit{H. azteca}. In the presence of the \textit{H. azteca}, the signals from PFOS become broadened, suggesting a general interaction with the organisms. An additional peak appears around -120 ppm in the presence of \textit{H. azteca}, however, this is most likely just inorganic fluoride in the organism’s biomass [67]. Two peaks labelled with “?” appear to increase in intensity. The peaks also exist in the control PFOS sample and are likely secondary products from manufacturing. It is important to note that the standard (Figure 4.14B(III) was acquired using 2400 scans (100mg/L), the \textit{in vivo}
spectrum was acquired using 10 times more scans (24,000 scans). As such, likely the secondary products (labelled with "?") are relatively consistent while the PFOS is decreasing. This is consistent with the fact that $^{19}$F has a relatively large chemical shift anisotropy (CSA) especially at higher fields (>200 MHz) and requires fast spinning for optimal observation in the solid state [54]. As such slow spinning at 500 Hz (used to enhance the survivability of the organisms), is likely not enough for efficiently detecting the PFOS once it enters the organism and becomes bound in the less dynamic state. While the loss of signal overtime could be of use to follow the loss of PFOS from the solution state, faster spinning would be required to detect the bound component. This is a considerable drawback of very slow spinning and a consideration for heteronuclei such as $^{19}$F that have a relatively large CSA. In summary, the observation that new peaks do not form in the presence of $H. azteca$ further supports the general understanding that PFOS does not easily undergo biotransformation [68].
Figure 4. 15 A) $^2$H NMR. (i-ii), D-Phenyl-d5-alanine (10g/L) dissolved in culture tank water. Each spectrum was taken 11 h apart and demonstrate that D-Phenyl-d5-alanine remains stable in solution without the presence of shrimp. (iii-vi) respectively acquired at 2.46 h, 5.30 h, 11h and 25 h after the introduction of *H. azteca*. Experiments are performed at 500 Hz and 15 C°. B) $^{19}$F NMR (i) PFOS solution, dissolved in culture tank water (2400 scans). (ii) PFOS in the presence of *H. azteca* acquired over 18 h (24,000 scans). Experiments are performed at 500 Hz and 15 C°.

C) $^{31}$P NMR. Left panel shows changes in the $^{31}$P chemical shift and diffusion of glyphosate (0.045mg/mL) in the presence of *H. azteca*. Each 1D spectrum is 512 scans and 6 min long. The right panel shows projection of the diffusion dimension from the corresponding DOSY spectra. 256 scans in 32 increments were used for DOSY. All $^{31}$P experiments were performed at 5° C and spinning rate of 2.5 KHz.

4.3.5 Phosphorous ($^{31}$P)

Phosphorous is a key component of molecules related to energy transfer, such as ATP (adenosine triphosphate), as well as, nucleotides and phospholipids. As $^{31}$P NMR has been so well-documented for metabolic based *in vivo* research [4,69,70] here we chose to exemplify the use of $^{31}$P to follow the fate of glyphosate, the world's most commonly used herbicide [71], within *H. azteca*. Figure 4.15C(i) shows the $^{31}$P peak from glyphosate and the corresponding diffusion profile projected from a DOSY experiment. The diffusion distribution is relatively tight centering around -9.1 log(m$^2$/s) which is consistent for free glyphosate. After another 6 h, (Figure 4.15C(ii)) the $^{31}$P NMR chemical shift remains similar while the diffusion takes on a distinct asymmetry with tailing to the right. This is consistent with a fraction of the $^{31}$P not being attenuated by the stronger gradients and thus exhibiting very slow diffusion as would be the case if a fraction becomes associated with the *H. azteca* biomass and exhibits slow to no diffusion. After 21 h the $^{31}$P peak becomes broad and shifts ~2 ppm. Meanwhile, a drastic shift in the diffusion profile consistent with a wide range of restricted chemical environments is observed (likely due to glyphosate entrapped within the organism’s biomass) in 4.15C(iii). This time point marks organism’s death. After 36 h the chemical shift of the $^{31}$P moves slightly to higher field, while the diffusion profile suggests the molecules become more dynamic. This trend continues during the final time point (42
h), and while the diffusion increases, it never returns to that of the free form. One explanation could be that after the organisms die their cells rupture and the glyphosate becomes more dynamic, but the failure to return to the free diffusion co-efficient suggest the glyphosate is binding with or in exchange with various cell components. The actual diffusion measured at the end of the experiment is likely the average of the free and bounds states [72]. Of particular interest here, is that by combining conventional chemical shift information with a physical NMR measurement (i.e. diffusion) a much better overall understanding of the process is obtained compared to the spectroscopy alone.

4.4 Conclusions

CMP-NMR provides a unique and comprehensive overview with the potential to study all the organic bonds inside a living organism. Studies focusing on the metabolic profile provide information on rapid stress responses, while studies on shell and bone provide the ability to assess “structural” impacts (such as thinning of shells induced by long term contaminant exposure). The use of hetero-nuclear NMR such as $^2$H and $^{19}$F provides a route to monitor the fate, binding, bioaccumulation and bioconversion of xenobiotics inside organisms. $^{13}$C and $^{15}$N enrichment combined with 2D NMR greatly enhance the range of metabolites that can be identified. However, MAS is able to narrow the $^1$H spectrum line shape so that a range of metabolites can be directly identified without the need for labelling. Arguably this is one of the greatest advantages of CMP and HR-MAS NMR that small organisms can be taken directly from the environment and studied as is. A considerable drawback of MAS is that only small organisms that fit inside 4 mm rotors can be studied. While this will be alleviated somewhat by 7 mm CMP-NMR probes, currently in development [73], the approach will likely always be restricted to relatively small animals. In large part due to the fact that as the diameter of the rotor increases so does the centripetal forces on the organism/organisms inside, which in turn, lowers the maximum spinning rate that the organisms can survive. However, for small aquatic organisms such as D. magna, H. azteca and C. elegans, which are used extensively in soil and water toxicity testing, CMP-NMR will likely prove a powerful and essential tool for explaining the mechanisms and molecular impacts of contaminants
on living systems, and ultimately providing a better understanding as to which contaminant combinations are most problematic for human and ecosystem health.
4.5 References


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5 Chapter 5

In vivo Ultra Slow MAS $^2$H-$^{13}$C NMR Emphasizes Metabolites in Dynamic Flux

This chapter is adapted from:

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5.1 Abstract

NMR Spectroscopy is a powerful tool for metabolite screening and due to its non-invasive nature can be applied \textit{in vivo}. However, the magnetic susceptibility mismatches within the intact organisms, lead to broad signals and loss of spectral information. Magic angle spinning (MAS) is the most robust way to reduce these distortions, enhancing line shapes and providing a wealth of metabolic information, \textit{in vivo}. Unfortunately, mainly due to overlapping water sidebands, relatively fast spinning is required (~2500 Hz) which induces stress on the organism, leading to mortality within a relatively short time frame. Here, a novel approach is introduced utilizing $^2$H/$^{13}$C isotopic enrichment that demonstrates the following advantages: 1) $^2$H is a quadrupolar nucleus hence, in $^2$H-$^{13}$C 2D NMR only the most dynamic molecules (mobile metabolites) are observed \textit{in vivo}, while structural components are broadened beyond detection. In turn, this results in a well-resolved and unique window into the dynamic metabolite pool that includes newly released or synthesized molecules correlated to a biological response/process. 2) $^2$H shares the same chemical shift window as $^1$H making assignment relatively easy. 3) $^2$H detection reduces problems associated with the water peak and as the dynamic molecules are selectively detected, no sidebands are observed. 4) As such samples can be spun slowly (50 Hz), reducing the stress and increasing 100% survivability to 24 h for \textit{Daphnia magna} and 48 h for \textit{Hyalella azteca}. To our knowledge this represents the only MAS based NMR approach that can provide high resolution 2D NMR \textit{in vivo} metabolic fingerprint at slow spinning rates.

5.2 Introduction

NMR is a highly versatile analytical technique with applications spanning from the medical to environmental fields [1,2]. In recent decades, NMR has made enormous impact in the field of metabolomics, giving scientists a rare glimpse into the metabolome of individuals and providing a detailed molecular-level understanding of biological processes [1], disease biomarkers [3] and toxic responses [4]. Recently there has been considerable interest in \textit{in vivo} metabolomic research
[5,6], for which NMR is the ideal tool due to its non-destructive nature, high reproducibility and rich molecular information. Both solution state (i.e. organisms non-spinning within a flow system) [7] and HR-MAS NMR (i.e. organisms spinning at the magic angle) [5,8] can be applied to study small organisms in high-field vertical NMR systems. HR-MAS has the distinct advantage that magnetic susceptibility distortions arising from intact organisms are averaged by magic angle spinning, resulting in excellent line shape even in living samples. As such numerous studies have applied HR-MAS NMR over the last decade to study living organisms with examples including *Drosophila melanogaster* [9], *Caenorhabditis elegans* [10], *Aporrectodea caliginosa* [8], *Calanus finmarchicus* [11], *Daphnia magna* [12] and *Saccharomyces cervisiae* cells [13]. Arguably one of the most impressive applications was that of slow turning of whole mice. Unfortunately, due to the low sensitivity of the experiment only the largest peaks from lipids could be detected and metabolites desrimination was not possible.

Liaghati Mobarhan et al. showed that by drilling a hole in the rotor cap, oxygen could be supplied to organisms under MAS, overcoming hypoxia, and leaving the stress caused by the fast spinning of the rotor as the main drawback of MAS approaches [14]. $^1$H HR-MAS *in vivo* NMR has been most commonly applied at high spinning rates ($\sim$2.5 KHz). This is largely to prevent spinning sidebands (mainly from water peak) masking the isotropic information. For example, on a 500 MHz NMR spinning at 2.5 KHz shifts the first order sidebands to $\sim$0 and 10 ppm, just outside the main $^1$H chemical shift window. Unfortunately, 2.5 KHz is the upper speed limit organisms can tolerate [12] with cell rupture starting around 3 KHz [15]. At slower spinning rates sidebands obscure the majority of spectral information. Although recent progress has been made in both water and sideband suppression techniques that permit *in vivo* studies to be performed at spinning rates down to 500 Hz [16], this is only applicable to $^1$H 1D NMR as the complex phase cycle involves 243 steps which would make 2D NMR extremely inefficient. Furthermore, sidebands are known to be reduced by using tiny 12 µL sealed spherical inserts that have all air bubbles removed [17]. Unfortunately, such sealed inserts are incompatible with organisms such as *H. azteca* which are too large to fit and require access to the atmosphere in the form of a vented cap to breath.

2D NMR $^1$H-$^{13}$C NMR is essentially required for assignment and metabolite discrimination *in vivo* [14].
In this manuscript, a novel approach is investigated that involves fully $^2\text{H}/^{13}\text{C}$ enriching organisms. The substitution of $^1\text{H}$ with $^2\text{H}$ is routinely performed to enhance the sensitivity of NMR experiments, particularly in the field of structural biology [18]. Unlike its proton counterpart, $^2\text{H}$ is a quadrupole nuclei, albeit with a relatively small CSA of only 770 Hz and smaller heteronuclear and homonuclear dipolar couplings, compared to that of $^1\text{H}$ [19]. As such when a molecule containing $^2\text{H}$ is completely dissolved the rapid tumbling averages the quadrupole interaction to zero, leading to sharp lines. However, if the molecular tumbling is restricted in anyway, the quadrupole interaction is quickly reintroduced and the signals are broadened considerably up to hundreds of KHz [20]. Theoretically, if $^2\text{H}/^{13}\text{C}$ experiments are performed in vivo only the very mobile fraction would be detected. This is arguably very advantageous for metabolomics as the freely tumbling molecules are likely those in dynamic flux (recently synthesized or released) and thus of most biological relevance in terms of representing the biochemical responses to external stimuli. Furthermore, $^2\text{H}/^{13}\text{C}$ or $^{13}\text{C}/^2\text{H}$ detection should overcome complications due to the intense water signal. 2D $^1\text{H}/^{13}\text{C}$ correlation experiments under slow spinning conditions are impeded due to sidebands of water which obscure the chemical shift information of other species. However, the substitution of $^1\text{H}$ with $^2\text{H}$ nuclei, allows for the acquisition of $^2\text{H}/^{13}\text{C}$ correlation NMR spectrum without an intense water signal and the associated complications from its sidebands. Interestingly, while one may expect the $^2\text{H}$ nucleus to lead to line broadening via quadrupolar relaxation, the impacts observed for the dynamic fraction, which is selectively detected by the 2D $^2\text{H}/^{13}\text{C}$ correlations here, are less than the natural linewidth recorded for in vivo samples. For example, Liaghati Mobarhan et al., [14] showed that even spinning at 2500 Hz, 2D $^1\text{H}/^{13}\text{C}$ produced line widths that averaged 0.2 ppm (or 100Hz at 500 MHz) in the $^1\text{H}$ dimension. However, in this study, spinning at only 50Hz, the same organisms produced $^2\text{H}$ lines between 0.1 and 0.2 ppm (50Hz-100Hz at 500 MHz) in 2D $^2\text{H}/^{13}\text{C}$ and thus more resolved spectral profiles that are easier to assign. Additionally, the $^2\text{H}/^{13}\text{C}$ coupling is only $\sim$25 Hz compared to its $^1\text{H}/^{13}\text{C}$ counterpart (145 Hz), and the longer evolution delays required act as a relaxation filter to remove unwanted broad resonances. This can be thought of as both advantageous and disadvantageous. A disadvantage in that the longer delays make observing the more rigid components very difficult, yet an advantage in that the approach naturally selects only the dynamic components in vivo as demonstrated here.

Here, ultralow spinning speed $^2\text{H}/^{13}\text{C}$ in vivo NMR is explored for the first time. In order to validate the approach, two aquatic invertebrates, *H. azteca* (freshwater shrimp) and *D. magna* (water flea),
were used as model organisms. These organisms are keystone species commonly used in aquatic toxicity assessments [21,22]. Two types of studies are performed to monitor the changes in metabolomes under MAS conditions, in vivo. The first monitors the organisms feeding on $^2$H/$^{13}$C enriched algae and the second follows the metabolism in fully enriched $^2$H/$^{13}$C organisms, themselves.

5.3 Results and Discussions

5.3.1 Increased Survivability at Low Spinning Rate

A previous study indicated that under anesthesia D. magna can survive 4 h of MAS conditions at 2.5 KHz [12] in a sealed rotor. In another study on H. azteca, after using rotor caps customized with a drilled hole to permit oxygen exchange, the organisms survived for ~6 h at room temperature without anesthetics at the same spinning speed [14]. Unfortunately, fast spinning induces metabolic stress on the organisms and tissues, therefore, metabolic profiling at lower spinning rates is highly desirable for the analysis of such delicate samples. In Figure 5.1 the percentage survival rate of D. magna (represented by circles) and H. azteca (represented by diamonds) at both spinning rates of 50 Hz and 2.5 KHz are plotted versus time. Note in all cases samples are spun at 20 °C using a cap with a hole and without the use of anesthetic. Figure 5.1 depicts that reducing spinning from 2.5 KHz down to 50 Hz extends the survivability threshold of D. magna from 85% at 4 h to 100% for 24 h, while for H. azteca the 100% survivability threshold increases from 6 h [14] to over 48 h. This is primarily due to the reduced centripetal forces induced on the organism as a result of lower spinning rate. Another important factor contributing to the higher longevity at 50 Hz is the reduced water evaporation through the 500 µm hole on the cap, due to lower gas pressures required for rotor rotation. The larger survival time window of >48 h in H. azteca compared to 24 h in D. magna is attributed to their more robust exoskeleton. These time windows are particularly valuable in toxicity assessment protocols which are often performed over either 24 h or 48 h exposure times [23].
Figure 5.1 Survival rate percentage plots for *H. azteca* and *D. magna* at spinning speeds of 50 Hz and 2.5 KHz. Ultra-slow spinning rate of 50 Hz improves the survivability threshold of *D. magna* from almost 85% (red circles) after 2 h spinning at 2.5 KHz to 100% survivability for 24 h (grey circles). At 50 Hz the survival rate threshold is greatly enhanced to 48 h for *H. azteca* (blue diamonds) compared to previous studies at 2.5 KHz with 100% survival rate limited to 6 h at 20°C. All measurements were performed in triplicate with the standard deviation reported.

5.3.2 1H MAS NMR at Low Spinning Rate

While lowering the spinning speed enhances organism’s survival rate, the quality of the 1H NMR data is greatly impaired. To demonstrate this, Figure 5.2A-D shows the effect of reducing the spinning speed, using algae as an example. In the 1D spectra at 50 Hz (Figure 5.2A) the isotropic information is lost under the overlapping water sidebands, while at an increased spinning rate of 2.5 KHz (Figure 5.2B), the 1H spectrum contains a wealth of information and the sidebands fall outside the chemical shift range of proton (0-10 ppm). Similarly, for 2D 1H-13C HSQC (Figure 5.2D), at 2.5 KHz a range of correlations are observed. However, when the spinning rate is reduced to 50Hz (Figure 5.2C) the majority of contours disappear, and the remaining cross-peaks are highly distorted, making extraction of chemical information challenging. As such, a conundrum exists where the organisms require slow spinning to reduce cellular stress and increase survival, yet, 1H
detection provides little information at these slow spinning speeds. To address this issue $^2$H NMR detection at slower spinning rates is introduced here.

### 5.3.3 $^2$H MAS NMR at Low Spinning Rate

$^2$H is an NMR-active, quadrupolar nucleus with a very small quadrupole moment [24]. Therefore, the rapid tumbling in dissolved small molecules averages out the quadrupole interaction, giving rise to relatively sharp lines. However, motional restrictions lead to the quadrupole interaction being reintroduced and broadening signals up to hundreds of KHz [20]. Considering this fact, it may be expected that a whole fully $^2$H enriched organism would result in broad NMR spectra due to the quadrupolar broadening. Figure 5.2E-F show the $^2$H NMR spectra for $^2$H/$^{13}$C labelled algae at 2.5 KHz and 50 Hz, both showing a roughly similar spectral profile, albeit with lower spectral resolution compared to $^1$H NMR spectra at 2.5 KHz (Figure 5.2B). This is expected given the reduced bandwidth of $^2$H (1ppm $^2$H is $\sim$76 Hz, compared to 1ppm $^1$H $\sim$500 Hz at 11.7 Tesla) and the fact that $^2$H is a spin 1 quadrupolar nucleus [25]. Interestingly, even at 50 Hz the chemical shift information from the algae remains intact, with no interference from the sidebands. In part, this is due to the absence of a dominant water peak (i.e. water sidebands do not swamp the sample as observed in $^1$H detection), along with the fact that solid-like materials (more likely to produce sidebands) are considerably broadened due to the quadrupole moment and have minimal contributions to the 0-10 ppm ($\sim$750 Hz) spectral window. When 2D $^2$H-$^{13}$C experiment is performed the dynamic components are further emphasized due to relaxation during the $^2$H-$^{13}$C evolution delays ($1/2 J = 25$ ms) in the experiment. In the 2D $^2$H-$^{13}$C HMQC spectra in Figure 5.2G-H, despite the $\sim$12% reduction in the total signal intensity at 50 Hz compared to 2.5 KHz, a surprisingly well-resolved NMR profile containing metabolite signals [26] is obtained at both spinning speeds. The data in this study are acquired using $^2$H-$^{13}$C HMQC, however $^{13}$C-$^2$H HETCOR and $^2$H-$^{13}$C HSQC were also considered. In the supplementary information all three experiments are compared both experimentally and theoretically, concluding that in the case of deuterium-carbon correlations, HMQC is the most effective approach.
Figure 5. 2\textsuperscript{1}H NMR spectra of \textsuperscript{13}C enriched algae at spinning rates of (A) 50 Hz sidebands dominate the spectrum (B) 2.5 KHz resulting in sideband free spectrum in the 0-10 ppm chemical shift range. \textsuperscript{1}H-\textsuperscript{13}C HSQC at spinning rates of (C) 50 Hz and (D) 2.5 KHz. \textsuperscript{2}H NMR spectra of \textsuperscript{2}H/\textsuperscript{13}C isotopically enriched algae representing the 1D profiles at (E) 50 Hz and (F) 2.5 KHz. The 2D \textsuperscript{2}H-\textsuperscript{13}C HMQC at spinning rates of (G) 50 Hz and (H) 2.5 KHz show similar profiles at both spinning rates. All spectra are scaled just above the noise floor and all took 1 h to acquire.
5.3.4 *In vivo* Studies

In order to investigate the feasibility of $^2\text{H}$ NMR in monitoring *in vivo* metabolic processes, two different *in vivo* studies were performed on both *H. azteca* and *D. magna*. The first approach studies the utilization of $^2\text{H}/^{13}\text{C}$ enriched algae by the organisms and traces the fate and transformation of the food molecules. Such studies can reveal the impact of environmental conditions at the metabolic level and provide information on the uptake and conversion of nutritional or contaminant sources, *in vivo*. The second approach is *in vivo* NMR investigation of fully $^2\text{H}/^{13}\text{C}$ enriched organisms to study the real-time metabolomic fluxes under starvation (i.e. changes in the organism’s biomass studied inside the NMR without food) and demonstrate the ability to follow metabolic processes within the organisms themselves.

5.3.4.1 Utilization of $^2\text{H}/^{13}\text{C}$ Enriched Algae by the Organisms

In the following sections $^2\text{H}-^{13}\text{C}$ NMR is used to investigate $^2\text{H}/^{13}\text{C}$ enriched algae utilization by non-enriched *H. azteca* and *D. magna*.

5.3.4.2 *H. azteca*

Non-enriched *H. azteca* were monitored in the presence of $^2\text{H}/^{13}\text{C}$ isotopically labelled *C. Vulgaris* with $^2\text{H}$ NMR data collected in 3.5 h increments over a 28-h period. Figure 5.3A and 5.3E respectively represent the 1D and corresponding 2D NMR for the fully labelled swollen algae collected prior to loading the organisms. As the algae has been freeze dried it constitutes dead cells and its profile remains relatively constant over time in the absence of crustaceans (see supporting Figure C-2). Therefore, the observed changes in the metabolic profiles in the presence of organisms are essentially correlated to the metabolic activity of the crustaceans on the algae. Note as the crustaceans are not $^2\text{H}$ or $^{13}\text{C}$ enriched they show no contribution to the signals at $^2\text{H}-^{13}\text{C}$ spectra. Given that $^2\text{H}$ and $^1\text{H}$ nuclei share the same chemical shifts, standard NMR databases can be used to make the $^2\text{H}$ NMR assignments. The signals that arise from the algae itself (Figure 5.3A and 5.3E) are consistent with the most dynamic entities in betaine, $\text{CD}_3$ from lipids (18 ppm),
carbohydrates (~65 ppm) and CD₃ in threonine along with the three amino acids (alanine, proline, glutamic acid) with high abundant concentrations. Methyl groups are preferentially detected due to their rotational motion that averages the ²H quadruple interactions.

Figure 5.3B-D show the 1D ¹³C NMR projections of the corresponding 2D experiments shown in 3F-H, with the most significant variations highlighted. After 3.5 h of feeding the spectral profile is dominated by both essential (leucine, valine, and threonine) and non-essential (proline, alanine and glutamic acid) amino acids, likely produced from the breakdown of algal proteins. Beyond forming structural proteins, amino acids also act as precursors for other metabolites (such as enzymes, neurotransmitters) and are involved in the vast majority of biological processes, making them valuable molecular descriptors for monitoring in vivo changes. This approach enables the direct observation of the amino acid fluxes. In Figure 5.3B threonine and leucine are identified by their mobile (CD₃) groups. Both free glutamate and alanine are known to be abundant in the nervous systems of marine crustaceans [27] and (along with other free amino acids) act as osmoregulators in marine invertebrates. Alanine, leucine, glutamate and valine play important intermediary roles in tricarboxylic acid (TCA) cycle [28]. Proline is known to act against oxidative stress in plants and other organisms such as D. magna [29]. Also, high levels of proline have been found in the free amino acid pool in the hemolymph in insects [30].

Within 3.5 h of the commencement of feeding, methylene groups from carbohydrates appear at ~65 ppm along with signals characteristic of glucose at 72 ppm. Carbohydrates are the primary “fuel” essential to a wide range of cellular processes. It is not clear whether they have been synthesized de novo using algal precursors or simply released from available storage molecules such as starch existing within the algal biomass, that cannot be observed in the intact macromolecular form using the ²H-¹³C experiments. 4-aminobutyric acid (GABA), the major inhibitory transmitter at invertebrate synapses is also observed at this time point. GABA receptors play a critical role in regulating neuronal activities in the nervous system of invertebrates. Studies have shown that during exposure to some xenobiotics their activities are mimicked, causing up or down regulation of GABA. Therefore, GABA has been proposed as a biomarker for exposure to certain pesticides in D. magna [31]. As for the lipids after 3.5 h, the mobile terminal groups (CD₃-CD₂-) resonances become more pronounced possibly due to release from the algal biomass for likely use as an energy source. Interestingly, while the CD₃ groups from lipids become more
visible, other broad lipid resonances (for example the main chains (CH$_2$)$_n$) that dominate the $^1$H-$^{13}$C spectra of organisms (algae or $H$. azteca) [26] are missing in the $^2$H-$^{13}$C HMQC spectra. This indicates they do not possess the local dynamics required to average the quadrupole interactions for $^2$H-$^{13}$C detection. This fact is arguably advantageous, since in organisms (with high fat contents, such as $Daphnia$) lipids dominate the $^1$H NMR spectra, masking other metabolites of interest [7,32,33]. The intense resonance at $\sim$56 ppm is consistent with the dynamic methyl groups in betaine [34]. Betaines are usually present in free state and act as osmolytes in some species of aquatic invertebrates, fish [35] and marine algae [34]. The tertiary N-butyl group peak grows overtime with approximately same rate as the second betaine peak ($^1$H 3.8 ppm, $^{13}$C 69 ppm). However, the N-butyl group appears more intense in part due to the higher local motions which average out the quadrupolar interactions and therefore, enable more sensitive detection. It is clear that the quadrupole interactions have a huge impact on peak intensity and absolute quantification from the $^2$H-$^{13}$C experiment may be highly complex.

After 7 h, contributing signals from glycine, lysine, arginine and isoleucine appear. With the greater dispersion provided in the corresponding 2D spectra, methionine is identified in Figure 5.3G. Interestingly, a slight decrease in glutamate and concurrent appearance of glutamine resonances, suggest that glutamate to glutamine cycle is active [28]. Through this cycle, glutamate and glutamine are interchangeably converted, to deliver the essential glutamate for neural activities in both invertebrates and vertebrates. The resonance that appears at (3.5 ppm, 69 ppm) belongs to choline which plays important structural and signaling roles in organisms. Choline has also been identified as biomarker in Alzheimer's and many cancer types and thus, in future $^2$H-$^{13}$C NMR may provide useful insights to understand the molecular events associated with such diseases [36].

After 28 h, while a slight increase is observed in valine, other metabolites such as lysine and GABA increase preferentially. In contrast, alanine becomes somewhat suppressed (best observed in the projections Figure 5.3C-D). An interesting observation is noted for betaine which demonstrates the potential of this technique in detecting the structural changes in the metabolites. While the signal from the tertiary N-butyl group remains similar in both panels C and D, the second betaine peak ($^1$H 3.8 ppm, $^{13}$C 69 ppm) decreases in intensity (best depicted in the 2D spectra). A possible explanation is that betaine either becomes less hydrated or binds to cellular components thus reducing its molecular mobility. In turn, this has lower impact on the tertiary N-butyl group with
many degrees of rotational freedom but lead to preferential broadening and signal loss for the methylene shift which is adjacent to the positively charged nitrogen and carboxyl group, both of which would likely be involved in the binding mechanism.
Figure 5. 3 Time course following the metabolic changes of $^2$H/$^{13}$C isotopically labelled algae being consumed by *H. azteca* spinning at 50 Hz. (A-D) 1D $^{13}$C NMR projections and (E-H) 2D $^2$H-$^{13}$C HMOC spectra.
5.3.4.3  *D. magna*

To further investigate food utilization behaviors of the organisms using $^2$H NMR, a second species, *D. magna* was also studied. Natural abundant *D. magna* were fed $^2$H/$^{13}$C isotopically labelled *C. Vulgaris* and monitored using $^2$H HR-MAS NMR at 50 Hz. The metabolomic profile of blank algae is shown in Figure 5.4A and D, as a reference. Figure 5.4B-C show the 1D $^{13}$C NMR profiles of *D. magna*, with the areas consisting of major changes during 3.5-14 h highlighted. After 3.5 h in comparison to the blank algae, proline, alanine, betaine and glutamic acid become more pronounced while new signals from leucine, valine and GABA arise. This increase in the amino acids is likely an indication that algal proteins are digested. In Figure 5.4E the appearance of glucose after 3.5 h is likely due to main metabolic pathways TCA cycle, glycogenosis and glycolysis for energy production. After 14 h, signals continue to grow across the chemical shift region as a result of the *D. magna* activity in catabolizing the algal proteins into their amino acids constituents. Particularly considerable increases in glycine and the branched chain amino acids valine, leucine and isoleucine are observed, consistent with their release from algal biomass. These residues have a primary role in protein synthesis and account for up to 25% of most dietary proteins. Also, there is a slight increase observed with alanine. Interestingly in comparison to *H. azteca*, lipids do not accumulate in *D. magna* (see supporting Figure C-3) which may be due to the fundamental difference in the *D. magna*’s ability to synthesize and process lipids. The differences in metabolism among these two species are addressed in more detail in the following section.
**Figure 5.4** Time course following metabolic changes of $^{2}$H/$^{13}$C isotopically labelled algae being consumed by *D. magna* spinning at 50 Hz. (A-C) 1D $^{13}$C NMR projections and (D-F) 2D $^{2}$H-$^{13}$C HMQC spectra.
5.3.5 Comparison of \textit{H. azteca} and \textit{D. magna} in the Utilization of Algal Biomass.

As observed in Figure C-3, both \textit{H. azteca} and \textit{D. magna} produce similar spectral profile after feeding on algal biomass. The main differences appear to be the ratio of $\alpha/\beta$ glucose and the amount of accessible lipids. After 14 h in \textit{D. magna} an $\alpha/\beta$ glucose ratio of $\sim$38:62 is observed that is consistent with the equilibrium found in free solution. Interestingly, in \textit{H. azteca} this ratio is $\sim$50:50 which suggest that the glucose is not completely free to interconvert between the 2 forms and thus re-establish the $\alpha/\beta$ equilibrium ratio, this could be due to weak binding, slow dynamics, or the presence of starch fragments (i.e. $\alpha$-glucose). In addition, the lipid profiles are noticeably different in these two species. This is most clearly observed from their terminal CD$_3$ group which is the most easily detected moiety. In the $^{13}$C NMR of \textit{H. azteca} the lipids are the 3$^{rd}$ most intense peak, when compared with \textit{D. magna}, this same characteristic peak is significantly suppressed. With \textit{H. azteca} (see Figure 5.3A-D) the lipid signal appears after 3.5 h consistent with their release into solution as the algal cellular matrix is digested. In contrast, in \textit{D. magna}, following the initial increase in the lipid content, their attenuation after 14 h suggests their rapid utilization. This is consistent with the species inability to synthesize most lipids and instead assimilate them from their die. In fact, while studies show less than 2% of the accumulated lipids in \textit{D. magna} are synthesized \textit{de novo}, biosynthesis of $\omega$3 and $\omega$6 fatty acids in the brine shrimp has been reported [37]. Studies on \textit{D. magna} confirm that their lipid fraction is more important compared to their carbohydrate reserves [38,39] and if available organisms consume lipids with higher rates.

5.3.5.1 \textit{In vivo} NMR Investigation of Fully $^2$H/$^{13}$C Enriched Organisms

In order to further explore what information can be extracted using $^2$H-$^{13}$C \textit{in vivo} NMR, the metabolic profiles of fully $^2$H/$^{13}$C enriched \textit{H. azteca} and \textit{D. magna} were traced in pure water. Note the organisms guts were purged with non-enriched algae prior to analysis [7]. As such the only source of $^2$H-$^{13}$C is the organism’s biomass and the signals that appear over time result from the utilization of the biomass reserves for metabolic processes.
5.3.5.2 $^{2}\text{H}/^{13}\text{C} H. azteca$

$^{2}\text{H}/^{13}\text{C}$ enriched $H. azteca$ were monitored under starvation for a period of 49 h. After 3.5 h inside the rotor and spinning at 50 Hz (i.e. the time for the first $^{2}\text{H}/^{13}\text{C}$ HMQC to complete), in Figure 5.5A very few peaks are identified in fully enriched organisms. 2D $^{2}\text{H}/^{13}\text{C}$ HMQC emphasizes more dynamic components, therefore, structural macromolecules such as proteins and lipids are severely broadened due to the $^{2}\text{H}$ quadrupole moment. The general scarcity of cross peaks suggests that the majority of the $^{2}\text{H}/^{13}\text{C}$ bonds exist in a motional restricted environment, consistent with storage and structural components. The few peaks that are initially detected represent flexible end units of lipids, along with the isobutyl group in leucine, the tertiary butyl group in betaine and the methyl group in threonine, all of which have noticeable rotational dynamics. In Figure 5.5D-I, the spectral regions reflecting the most remarkable changes during 3.5-28 h, are highlighted in blue and the chemical shift areas with subsequent changes from 28-49 h are highlighted in pink.
**Figure 5.5** (A-C) 2D $^2$H-$^{13}$C HMOC spectra of $^2$H/$^{13}$C enriched *H. azteca* at a spinning rate of 50 Hz in pure water, acquired after 3.5 h, 28 h and 49 h of starvation. Green rectangles emphasize the major changes, the insets in B and C show the aromatic region and the phenylalanine peaks that appear after 49h. (D-F) Corresponding 1D $^{13}$C NMR projections. (G-I) Corresponding 1D $^2$H NMR projections. The spectral regions with significant changes during 3.5-28 h and 28-49 h time points are respectively highlighted in blue and pink.
Living organisms store energy mainly in the form of lipids, carbohydrates or proteins. Under nutritionally deprived conditions such as here, the organism relies on using its own biomass [7,40,41]. Appearance of metabolite signals overtime may imply either the breakdown of previously undetectable (i.e. polymeric or bound) macromolecules to a freely dissolved detectable form or de novo biosynthesis. After 28 h, consistent with protein degradation, additional amino acid resonances particularly from alanine, proline, leucine, isoleucine, glycine, valine, methionine, glutamate, lysine are identified. Proline, alanine, glycine and glutamate exist in free form and serve as osmoregulators [42]. Proline and glycine constitute the main amino acid pool in the hemolymph in insects [30]. Alanine is the most abundant free amino acid and the second most abundant amino acid constituting protein sequences [43]. Glycine [44], alanine [45] and GABA [46] have been repeatedly connected to stress responses and likely triggered due to starvation for 28 h.

After 49 h, as tissues undergo further proteolysis, a slight increasing trend in most amino acid levels is observed, with valine and isoleucine (best observed for methyl peak at 13.5 ppm) increasing preferentially. The bioaccumulation of isoleucine and valine may also be an indication of the disruption in the pathways leading to acetyl coA (a main precursor in TCA cycle) production [28]. In addition, resonances from methionine and phenylalanine are observed for the first time. These hydrophobic residues often hidden within the internal hydrophobic core of the globular proteins and thus, more amenable to release once the 3D shape of the protein is altered [47]. Methionine does not normally exist in the free form and excess methionine is usually stored as an energy reserve by crustaceans [48] which may explain its release after prolonged starvation. Phenylalanine serves as a substrate for DOPA and dopamine enzymes that regulate behavioural activities such as reproduction [49]. Phenylalanine may be observed as a result of inhibition of such non-essential pathways while the organism struggles to direct the limited energy reserves towards combating the starvation. While the general increasing trend in amino acids supports proteolysis, the diminished levels of glycine and glutamic acid, suggest their uptake into a non-detectable form or transformation into other metabolites. Glycine is an essential precursor for synthesis of proteins, nucleic acids and lipids and many methylation reactions [50]. Likewise, glycine depletion may be a response to compensate for elevation of other free amino acids in order to regulate osmotic balance. Glutamic acid participates in multiple regulatory and energy production process and may be converted to other metabolites depending on the organisms metabolic demand [64].
Lipids show an interesting signature. At the starting point, the terminal CD$_3$ groups are exclusively observed due to their rotational freedom. Subsequently, after 28h the (CD$_3$-CD$_2$-(CD$_2$)$_n$) groups show a strong contribution at 22-25 ppm, suggesting that the lipids have become less “rigid” and perhaps have been released into a more dynamic state, however, the main chain (CD$_2$)$_n$ peak is hardly visible. This suggests only the terminal groups exhibit dynamics, with the main chains having less freedom consistent with lipids associated in emulsions, rather than free lipids fully dissolved in water. It is likely due to their very low solubility in water that the lipids show a somewhat separate phase but their change in dynamics is consistent with conversion into more readily accessible form for energy production. These signals show slight increase from 28 to 48 h suggesting their continuous release for energy metabolism.

Although carbohydrate resonances are initially absent, after 28 h signals from glucose along with generic resonance from CD$_2$ in carbohydrates at 65 ppm are observed. This suggests the release of bound carbohydrates (such as glycogen) possibly from the hepatopancreas into hemolymph. Eventually, after 49 h there is a complete depletion in the glucose level, possibly due to enhanced glycolysis [51], supporting glucose role as most readily useable energy reserve. Previously, increased glycolysis along with glucose attenuation in invertebrates has been reported as a corresponding sign of stress [29]. In the event of shortage in carbohydrate reserves, glucose can be biosynthesized de novo from lipids, glycogenic amino acids or lactate. It has been stated that under stress conditions such as extreme temperatures, hypoxia or contaminant exposure, crustacean’s energy consumption is elevated or under anoxic conditions such as low tides or burrowing in reduced substrata with insufficient oxygen concentration they may switch to anaerobic metabolism pathways [52,53]. It can be concluded here, that starvation has led to increased metabolism and higher oxygen consumption which in turn, triggers alternative metabolism. The presence of lactate and slight depletion in alanine (after 49 h) can be interpreted due to gluconeogenesis or anaerobic glycolysis, where alanine and lactate can both regulate gluconeogenesis of pyruvate to glucose [54]. Lactate is also a key component in Cori cycle through which the lactate produced during anaerobic glycolysis is converted to glucose [55].
5.3.5.3 $^{2}\text{H}/^{13}\text{C} \textit{D. magna}$

$^{2}\text{H}/^{13}\text{C}$ enriched \textit{D. magna} were monitored under starvation conditions for up to 21 h (within their maximum 100% survival window, see Figure 5.1). After 3.5 h, only few distinct signals arising from most mobile entities of the lipids and betaine are detected (Figure 5.6). Following up the metabolism after 21 h, several amino acid peaks including proline, valine, leucine, threonine and glutamic acid are identified. Branched amino acids have been reported as useful biomarkers for protein synthesis and degradation, and their increase in the fasting state is associated with protein degradation [56]. The lipid profiles in \textit{D. magna} are especially interesting, as many essential lipids cannot be synthesized \textit{de novo} and must be taken through their diet. As with \textit{H. azteca} the lipid signals elevate over time. However, an interesting observation in \textit{D. magna} is the more intense main chain (CD$_{2}$)$_{n}$ peak, compared to the CD$_{3}$ and CD$_{3}$-CD$_{2}$-(CD$_{2}$)$_{n}$ end units. This suggests the lipids are much more dynamic (possibly more dissolved) than in \textit{H. azteca}. It has been found that abundant lipids are accumulated in the form of oil drops by Cladocera [57]. This could be related to the fact that \textit{D. magna} have adapted to assimilate these essential molecules from their food more efficiently. As previously observed in labelled \textit{H. azteca}, at the end of the experimental time window there is no detectable contribution from glucose resonances, suggesting that if formed they are utilized quickly. However, interestingly the CD$_{2}$ resonance from carbohydrates is observed, suggesting that glucose (or another carbohydrate) may be present just below the detection limit. GABA [58] and lactate are also observed. GABA serves as starvation biomarker in some invertebrates and is known to correlate to stress response [44-46]. Lactate is a by-product of anaerobic metabolism under cellular hypoxia. Lactate allows glycolysis to proceed, however with minimal ATP production with respect to the oxidative pathways [59]. Overall, the lower signal to noise ratio in \textit{D. magna} experiments is attributed to lower body mass of only $\sim$40 µg per adult reported (total of 400-600 µg for 10-15 organisms in the rotor), while the mean dry weight in \textit{H. azteca} spans from 600-1000 µg per organism [7,60]. In addition, \textit{D. magna} have higher energy demand due to constant reproduction.
5.3.6 *In vivo* comparison of *H. azteca* and *D. magna* metabolism

Generally, both *H. azteca* and *D. magna* show similar metabolomic profiles during starvation. The signal to noise ratio is lower in the *D. magna* experiments in large part as they contain up to 95% water and very low biomass. The most interesting difference is the stronger (CD$_2$)$_n$ lipids peak in *D. magna*, while this peak barely detectable in *H. azteca* (see Figure 5.5 vs Figure 5.6). This suggests that while lipids are present in both species (indicated by the terminal CD$_3$-CD$_2$- groups) the main chains are more easily detected in *Daphnia* as the lipids are more dynamic (possibly more solubilized inside *D. magna* vs *H. azteca*) and consistent with oil droplets. This is particularly promising as it shows that the potential of $^2$H-$^{13}$C *in vivo* NMR may go beyond identification of metabolites in real-time and potentially also provides information on site specific molecular dynamics which could prove useful for better understanding molecular processes and mechanisms.

*Figure 5. 6* 2D $^2$H-$^{13}$C HMQC spectra of $^2$H/$^{13}$C enriched *D. magna* at a spinning rate of 50 Hz in pure water, acquired after A) 3.5 h and B) 21 h of starvation.
5.3.7 Comparison of the Fed and Starved Populations of *H. azteca*

A comparative analysis on the metabolomic profiles of the two population of *H. azteca* (natural abundant organisms consuming $^2$H/$^{13}$C labelled food and $^2$H/$^{13}$C enriched organisms under starvation), reveals some interesting differences. While feeding on algae the organisms continue to produce excess amount of glucose from the algal biomass, conversely under starvation the produced glucose is quickly depleted forcing the body to utilize proteins and lipids as alternative sources of energy. Further, in the feeding population methionine appears after 7 h (most likely readily obtained from the algal protein), while in the starved population being generated from the organisms biomass it is only observed after 49 h. In crustaceans methionine is stored to be used as energy reserve during molting and its production under starvation conditions is likely an indicator of energetic stress [48]. Moreover, in the feeding population the non-essential amino acid arginine is observed after 7 h and slightly increases over time (released from algal protein degradation), whereas during starvation limited nitrogen supply may have prohibited its synthesis. This is in agreement with previous studies where in organisms grown on limited nitrogen cultures the synthesis of amino acids containing more than one amino group was suppressed [62]. Another significant difference is the absence of lactate during 28 h in the fed organisms and its appearance in the starved population within the first 21 h of the experiment. This suggests alternative energy pathways such as gluconeogenesis or anaerobic glycolysis have initiated in which alanine and lactate can regulate gluconeogenesis of pyruvate to glucose.

5.4 Conclusions

This work aimed to introduce the *in vivo* 2D $^2$H-$^{13}$C NMR of living organisms. The approach selectively detects the dissolved metabolites, while molecules with restricted motion are extensively broadened by the $^2$H quadrupole interactions and not detected. As only the dissolved component is observed, under slow MAS sidebands are not problematic. Conversely with $^1$H-$^{13}$C NMR at very slow spinning speeds (50 Hz), the vast majority of metabolic information is masked by water and sideband peaks. At 50 Hz organism survival increases from 4 h to $>24$ h for *D. magna*.
and from 6 h to > 48 h for *H. azteca*. As such the $^{2}$H-$^{13}$C arguably represents the only approach that affords detailed molecular fingerprints at ultra-slow spinning speeds *in vivo*.

Despite the great potential of this technique, numerous considerations must be taken into consideration for future work. First the kinetic isotopic effects of $^{2}$H is quite substantial and organisms prefer $^{1}$H substrates if available [61,62]. However, despite the kinetic isotopic effects of $^{2}$H, as demonstrated in this study, it is still possible to culture fully $^{2}$H labelled living organisms, which could be considerably useful in kinetic isotope effect studies. For example it has recently been shown that due to slower reaction rates of heavy atoms, enriched organisms show longer life spans [62] this is currently an area of great research interest. Arguably, complete $^{2}$H enrichment of an organism, as performed here, is the most extreme case and in future the introduction of selectively $^{2}$H/$^{13}$C labelled components into organisms could be highly informative. For example, enriched proteins, would not be observed by $^{2}$H-$^{13}$C 2D NMR and are only detected after being broken down into small molecules used for bioconversion or energy production. Conversely, if $^{2}$H/$^{13}$C enriched metabolites/nutrients/drugs were introduced their fate and conversion could be selectively monitored. A further complication of the $^{2}$H-$^{13}$C HMQC is that the signal intensity observed appears to be dependent on both the concentration of the molecular unit, as well as its dynamics. For example, the methyl groups in betaine are preferentially detected due to local rotational dynamics. This clearly complicates absolute quantification of metabolites using a $^{2}$H detected approach. On the other hand, in carefully designed studies this additional dynamic information could be a significant source of information that holds potential in elucidating reactions mechanism (in free solution, or enzyme bound) and binding orientations. In summary, $^{2}$H-$^{13}$C NMR holds great promise to understand *in vivo* mechanisms under ultra-slow spinning regime where more conventional NMR approaches fail, as well as, serve as complementary tool combined with other NMR and non-NMR based metabolomics techniques.
5.5 Experimental

5.5.1 Algae and Organism Preparations

5.5.1.1 $^2$H/$^{13}$C Algae Enrichment

$^2$H/$^{13}$C doubly isotopically enriched *C. Vulgaris* was supplied by Silantes GmbH. $^{13}$C/$^2$H labeling of *C. vulgaris* (wt, SAG culture collection Goettingen) was achieved by growing the cells in $^{13}$CO$_2$ and D$_2$O (99% enrichment for both isotopes; Silantes GmbH) for 340 h in a photobioreactor (PBR) constructed by Silantes GmbH. The PBR is built as a closed system avoiding loss of the stable isotope labelled $^{13}$CO$_2$ and airlift driven having 25 L operating volume, permitting circulation of the media with 1 vvm. The algae were cultivated in regular Behrens media (+ KNO$_3$), as described previously [63], using the following parameters: pH 7.4 - 7.8 (pH-probe InPro®3253I/Sg, Mettler Toledo), temperature 30 °C (InPro®3253I), light intensity 1300 μmol/(m$^2$·s) (quantum detector LI-250A, Li-Cor Biosciences GmbH). A computer-controlled gas management system (Silantes based on Labview 10.1, National Instruments) was incorporated to keep the $^{13}$CO$_2$ content (pCO$_2$-probe InPro®5000, Mettler Toledo) at a concentration of 2%. The nitrogen carrier gas was allowed to vary between 65% and 85% corresponding to the oxygen content which increased during autotrophic growth of algae between 15% and 30%. If the oxygen content reached a value of 30% (pO$_2$-probe Visiferm Do Arc., Hamilton) an N$_2$ purging step was introduced in order to reduce the O$_2$ content to the initial value of 15%. To avoid loss of $^{13}$CO$_2$ during N$_2$ purging, the $^{13}$CO$_2$ addition was stopped at the end of a growth cycle until a value of 0.5% was reached by metabolic depletion. If the oxygen reached the initial value of 15% due to N$_2$-purging, the computer-controlled valves (Valve 221606, Buerkert) were closed and another growth cycle was resumed. The $^{13}$C-content of the algae biomass was determined by analysing the enrichment in “C18” fatty acids of isolated algae biomass by GCMS (Thermo Quest Polaris Q MS/ Trace GC2000, Thermo Fisher). The $^2$H-content was determined by Shanghai Research Institute of Chem. Ind. Both the $^2$H and $^{13}$C contents of the algae were determined to be >98%. After freeze-drying the algae biomass was stored at room temperature.
5.5.1.2  \textit{H. azteca} Culturing

\textit{H. azteca} were originally provided by Environment Canada, then cultured in house at the University of Toronto. Culturing conditions were controlled according to protocols provided by Environment Canada [23]. The organisms were kept in 2 L plastic beakers filled with dechlorinated (API tap-water conditioning agent), aged tap water and hardness of 124 mg/L at 24°C. Organisms were exposed to 16:8 h light to dark photoperiod using a fluorescence commercial lamp. 20% of the overlaying water was changed 2 times a week and prior to feeding. Feeding sources were selected based on the nature of the studies as mentioned in the following section.

5.5.1.3  \textit{D. magna} Culturing

\textit{D. magna} were originally provided from an established culture maintained at the Ontario Ministry of the Environment (Toronto, Ontario) and further maintained in house at the University of Toronto according to protocols provided by Environment Canada [7]. Organisms were cultured in 4 L glass vessels consisting of aged, aerated, dechlorinated tap water, at pH 7.5–8.5, hardness of 124 mg/L and at 20°C. The culture medium was changed 3 times a week prior to feeding. The organisms were exposed to 16:8h light to dark photoperiod. Feeding sources were selected based on the nature of the studies as mentioned in the following section.

5.5.2  Sample preparation for NMR

5.5.2.1  Algae Studies

$^1$H NMR data acquisition (Figure 5.2A-D) on algae were performed on a rotor filled with 35 mg of lyophilized and ground $^{13}$C labelled \textit{C. Vulgaris} suspended in 200 $\mu$L of H$_2$O/D$_2$O (95:5, v/v), and 80 $\mu$L of the resulting mixture was used to fill the rotor. For $^2$HNMR experiments (Figure 5.2E-H), $^2$H/$^{13}$C enriched \textit{C. Vulgaris} samples were prepared identically with the exception of 100% H$_2$O used for swelling.
5.5.2.2 Feeding Studies

Feeding studies were performed on natural abundance organisms raised on natural abundant algae and fed with $^{2}$H/$^{13}$C enriched algae during the NMR acquisition.

*H. azteca* - Figure 5.3 shows a time series of data for *H. azteca* feeding on $^{2}$H/$^{13}$C enriched *C. Vulgaris*. At the start of the experiment ~5 mg of $^{2}$H/$^{13}$C enriched *C. Vulgaris* was swollen in 200 µL of H$_2$O, subsequently the rotor was filled using 80 µL of the mixture. A spectrum of the algae was collected for 3.5 h (as blank) after which the spinning was stopped and 5-7 large (>5 mm) *H. azteca*, were let to swim from the tip of a plastic pipette into the rotor. Subsequently the metabolomic profiles in the presence of the enriched algae was monitored by $^{2}$H-$^{13}$C HMQC acquired every 3.5 h for up to 28 h.

*D. magna* - Figure 5.4 shows a time series of data for *D. magna* feeding on $^{2}$H/$^{13}$C enriched *C. Vulgaris*. At the start of the experiment ~5 mg of $^{2}$H/$^{13}$C labelled *C. Vulgaris* was swollen in 200 µL of H$_2$O then the rotor was filled using 80 µL of the suspended algae. A spectrum of the blank algae was collected for 3.5 h and subsequently the spinning was stopped and 10-15, adult (mid-size), non-enriched *D. magna* were allowed to swim from the tip of a plastic pipette into the rotor. Subsequently $^{2}$H-$^{13}$C HMQC experiments were collected every 3.5 h for 14 h.

5.5.3 $^{2}$H/$^{13}$C Enriched Organism Studies

Figures 5.5-5.6 follow the metabolism of both organisms enriched and raised using $^{2}$H/$^{13}$C isotopically enriched *C. Vulgaris*.

*H. azteca* - *H. azteca* were enriched from birth (for at least 4 weeks) by feeding on $^{2}$H/$^{13}$C isotopically enriched *C. Vulgaris* (2 mg per *H. azteca*) as their sole food source. 24 h prior to the experiments, 5-7 *H. azteca* were separated and fed on non-enriched algae to purge their gut from residual isotopically enriched algae as previously described [64]. The organisms were subsequently loaded into a rotor filled with water and monitored for up to 49 h, with $^{2}$H-$^{13}$C HMQC data acquired every 3.5 h (Figure 5.5).
D. magna - D. magna were enriched from birth (for at least 3 weeks) by feeding on $^2$H/$^{13}$C isotopically enriched C. Vulgaris (∼15 mg/100 daphnids/per day [32]) as their sole food source. 24 h prior to the experiments, 10-15 adult labelled daphnids were separated and fed on non-enriched algae to purge their gut from residual isotopically enriched algae as previously described [64]. The organisms were subsequently loaded into a rotor filled with water and monitored for up to 21 h, with $^2$H-$^{13}$C HMQC data acquired every 3.5 h (Figure 5.6). In all cases the rotor was sealed using a Kel-F cap with an o-ring seal customized with a hole as described previously [14].

5.5.4 NMR Spectroscopy

All NMR spectra were acquired using a 500 MHz Bruker Avance III Spectrometer with a $^1$H Larmor frequency of 500.13 MHz, fitted with a prototype CMP MAS 4 mm $^1$H–$^{13}$C–X probe and actively shielded Z gradient (Bruker BioSpin). X on this probe is tunable to $^{14}$N, $^{15}$N or $^2$H using exchangeable inserts and is a fully featured high power channel rather than a lock channel. In the case of $^1$H or $^1$H–$^{13}$C 2D the $^2$H channel was available for locking, while $^2$H–$^{13}$C and $^2$H experiments had to be acquired without a lock. Sample temperature was kept at 15±1 °C using a variable temperature unit (Bruker), consistent with average temperature of the natural habitat for fresh water organisms in northern lakes. Experiments were performed at spinning rate of 50 Hz unless otherwise stated. More detailed experimental parameters are as follows:

5.5.5 Survivability Tests

The viability experiments were performed in triplicates using a CMP probe external to the magnet. 5-7 large H. azteca with the size of >5 mm was used for each trial. For D. magna 10-15 adult organisms were used for each trial. For consistency the survivability experiments were performed at 20 °C, with no observable differences at 20°C and 15°C at spinning rate of 50 Hz (data at 15 °C not shown).
5.5.6 $^1$H NMR of Algae

For Figure 5.2A-D the following parameters were used. 1D $^1$H spectra were acquired using presaturation for water suppression, 2048 scans (1 h) and GARP-4 $^{13}$C decoupling. $^1$H 1D spectra were processed using an exponential decay corresponding to a line broadening of 2 Hz in the transformed spectrum. 2D $^1$H–$^{13}$C HSQC spectra were collected in phase sensitive mode (echo-antiecho), using 145Hz $^1$H–$^{13}$C $^1$J, garp4 decoupling, 48 transients, 128 increments, recycled at 1.3 $T_1$ for maximum sensitivity [65] resulting in an experimental time of ~1 h. 2D spectra were processed using a sine squared function phase shifted by a $\pi/2$ in both dimensions and a zero filling factor of 2.

5.5.7 $^2$H NMR of Algae

For Figure 5.2E-H the following parameters were used. 1D $^2$H NMR experiments were performed using presaturation for water suppression and 2048 scans (1 h) and GARP-4 $^{13}$C decoupling. The $T_1$ of $^2$H was very short (~100 ms). In theory this should lead to the 2D recycled very rapidly, potentially further increasing the signal to noise ratio. Unfortunately, the acquisition time required to collect 1K for $^2$H was 333 ms restricting the recycle time to slightly over 3 x $T_1$. Still, even with limitation it was possible to collect 2D $^2$H–$^{13}$C using 56 scans in 192 increments 25 Hz $^1$H–$^{13}$C $^1$J (optimized empirically for max signal) within a 1h period. 2D spectra were processed using a sine squared function phase shifted by a $\pi/2$ in both dimensions and a zero-filling factor of 2.

5.5.8 Sensitivity Comparison for 2D $^2$H NMR

In order to compare the sensitivity in the three most common $^2$H–$^{13}$C 2D experiments ($^{13}$C–$^2$H HETCOR, $^2$H–$^{13}$C HSQC, $^2$H–$^{13}$C HMQC) shown in Figure C-1, all spectra were acquired with 56 transients and 192 increments. 1k time domain points were collected in F2. All experiments used basic States-TPPI phase cycling which resulted in higher signal compared to gradients for coherence selection. The $^2$H–$^{13}$C coupling constant was determined empirically to give maximum signal at 25 Hz. Hard pulses were used throughout with the exception of the inversion pulse in
HETCOR that used a Broadband Inversion Pulse (Bip720,100,10.1 [66]). 2D spectra were processed using a sine squared function phase shifted by a \( \pi/2 \) in both dimensions, a zero filling factor of 2 and plotted just above the noise floor such that direct comparison between the spectra can be made.

5.5.9 In vivo and Feeding Studies

In all in vivo studies (Figures 5.2-6) \(^2\text{H}\)-\(^{13}\text{C}\) HMQC was acquired using 128 scans and 256 increments in the indirect dimension (3.5 h) and 1K time domain points. All other parameters were as described above.

5.5.10 Spectral Assignments

Compound identification and assignment was done using AMIX (Analysis of MIXtures software package, version 3.9.15, Bruker BioSpin, in combination with the Bruker Bio-reference NMR databases version 2-0-0 through 2-0-5. Spectra were calibrated against the Bruker Bio-reference NMR databases using proline and D-glucose resonances for reference. Assignment was performed using a procedure previously described [14,67]. Briefly, compounds with a greater than 80% match resulting from an automated search were chosen for manual inspection. Compounds were only assigned when all peaks in the sample overlapped with all peaks for the compound of interest in the database. Further, the chemical shifts of the identified compounds were plotted against the database values (\( r^2 \geq 0.99 \)) to confirm matching, and any compounds not meeting these requirements were discarded. Finally, the assignments were crossed-checked against other in vivo research in the literature [7,14,68].
5.6 References


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6 Chapter 6

Conclusions and Future Work
The outcome of this dissertation will help to propel scientific knowledge of biological mechanisms in the organisms, especially those concerning a response to natural and anthropogenic environmental stressors to a new level. *In vivo* CMP-NMR, introduced here permits the detection of environmental contaminants in all phases, with huge potential to detect the established correlations between the state of the contaminant (bound/free and binding receptor), metabolic change and any subsequent structural changes (protein synthesis, shell thickness). The capability to monitor longer recovery from exposure with high temporal resolution provides a promising key tool for use in risk assessment protocols. Furthermore, the methods developed here will greatly impact other fields of science, the use of CMP-NMR technology has considerable implications for understanding structure and interactions in the organisms.

### 6.1 Summary

The main objective of this dissertation is to extend the application of the newly developed CMP-NMR technology further, to observe ongoing biological processes inside an intact living organism, as is. This goal was successfully achieved in Chapter 2.

Through the technique developed in Chapter 2, in Chapter 3, the main drawback of the technique, that is, spinning speed that exerts additional stress on the organism and eventually translates to limitation in the experimental time window were addressed by improving experimental conditions such as slower spinning.

In Chapters 4 and 5 a range of applications that highlight the feasibility of using intact whole organisms were explored using hetero-nuclear NMR. Chapter 4 focused on $^{19}$F NMR and $^{31}$P NMR detection to study interactions with anthropogenic contaminants and $^{15}$N NMR to identify the total solid $^{15}$N-containing components, and hetero-nuclear correlation sequences that were developed and used. In Chapter 5 an ultra-slow spinning method (at only 50 Hz) using $^{2}$H-$^{13}$C 2D NMR was introduced and applied to study feeding behavior and stress mechanisms in *H. azteca* and *D. magna*. 

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6.2 Method development (Chapters 2 and 3)

Environmental metabolomics investigates the impact of a stressor on a host species by monitoring metabolite fluxes due to exposure. A comprehensive understanding of environmental toxicity requires molecular-level insight into an organism’s response. This may manifest itself across a range of media and phases including solutions (metabolites), gels (tissue) and solids (exoskeleton/shell). NMR possesses unique capabilities for in vivo and in situ research making it an indispensable analytical tool in environmental research. This fact along with the development of the recent CMP-NMR technology that enables a holistic perspective regarding an organism’s physiological status in response to environmental stimuli, were the basic motivation behind this dissertation.

Prior to CMP probe development, biological samples were either studied by solid state NMR which was limited to the analysis of dried solid material, or the preferred solution state NMR which requires harsh pre-treatment protocols to extract hydrophilic and lipophilic metabolites into completely dissolved phases [1]. Although, the solution state techniques are still quite popular in metabolomics studies, the extracts only provide a snapshot of the physical status of the organisms while the important molecular and structural information existing within the phases have been removed. High-resolution magic angle spinning (HR-MAS) [2] introduced later with the ability to observe swollen or semi-solid entities in intact samples. Thereafter, successful applications were reported with biological or clinical samples, and even whole organisms where the physical integrity of each specimen was crucial [3,4], however, limitations remained due to the inability to analyze the solid phase components.

Comprehensive multiphase NMR (CMP-NMR) [5], challenged these previous NMR techniques by combining pulse field gradients, magnetic susceptibility matching and lock channel applied in HR-MAS with the additional benefit of solid state high-power handling, all in a single probe to match any sample type. When combined with spectral editing and 2D NMR approaches, CMP-NMR overcomes the sample heterogeneity in a single experimental configuration. In addition, by using spectral editing, all components can be separated spectroscopically based on their rigidity, diffusion, and relaxation without physical or chemical isolation of the phases. When applied in vivo, CMP-NMR allows for analysis of H. azteca, such that all phases from the exoskeleton to the
dissolved metabolites were detected. Meanwhile, 2D NMR allows the assignment of more than 40 metabolites in the living organism. This approach, \textit{in vivo} CMP-NMR, will therefore establish a new platform in environmental metabolomics through an improved understanding of biochemical processes in the organisms.

On the other hand, \textit{in vivo} CMP-NMR, permits the study of solid components (such as shell, bone), as well as gels (membranes, membrane proteins) offers the possibility to directly correlate metabolic responses to structural and physical changes (for example shell thickness) and provides a holistic understanding of toxicity and environmental stress. Real-time monitoring of such changes provides information regarding the persistency of the elevated stress response within an organism. This improves the fundamental understanding of aquatic toxicity by suggesting a model for post-stress recovery and identifying biomarkers that can be used as “early warning” indictors of environmental stress.

By simultaneous monitoring of the contaminant fate in parallel to the metabolic stress response from the organism, it should become possible to develop a more comprehensive explanation of the poorly understood environmental fate, potency and toxicity of anthropogenic chemicals (or mixtures). This may eventually lead to a better understanding of the mode of actions, information that currently is desperately required to establish the most effective environmental policies. In summary, CMP-NMR \textit{in vivo} analysis provides a tool to examine the bioaccumulation, biotransformation, and excretion of the contaminants and drugs with real-time correlation to an organism’s biochemical response.

Chapter 3 addresses the major drawback of HR-MAS and CMP-NMR techniques, that is, the stress brought about in the live specimen by rapid sample spinning. In this chapter, a slow spinning technique was developed leading to high resolution spectra similar to the results obtained from Chapter 2. Although low spinning speeds are beneficial in preserving the organisms, inherit complications due to the inefficient water suppression and interference of its sidebands pose analytical challenges. Among the numerous pulse sequences reported for sideband suppression [6] many suffer poor resolution, or weak signals and long experimental times. As such we found a distinct lack of efficient high resolution approaches with minimal sensitivity losses at slow spinning and feasible application to delicate \textit{in vivo} samples. Therefore, two of the most efficient sideband suppression sequences were concatenated with an optimal water suppression technique
to produce high resolution 1D $^1$H NMR spectra in the most delicate in vivo samples down to 300 Hz. These techniques are relatively robust to study delicate samples in their native state and should be applicable in a wide range of biological, medical and environmental studies.

### 6.3 Applications (Chapters 4 and 5)

The following research chapters of this dissertation set out to investigate contaminant interactions with *H. azteca* and explore some common biological activities such as feeding and starvation in both *H. azteca* and *D. magna* while reducing the spinning speed down to only 50 Hz.

$^1$H NMR has excellent sensitivity; however, it suffers from low spectral dispersion rendering identification and assignment of the signals difficult. A valuable tool for high resolution structural determination and metabolic pathway elucidation by NMR is the use of partially or fully stable isotope enriched biomolecules to act as probes. In order to highlight the feasibility and investigate the extensive range of potential applications, $^2$H, $^{13}$C, $^{19}$F, $^{15}$N and $^{31}$P incorporation into the organisms, using nutritional and/or contaminant sources were investigated in chapters 4 and 5.

Chapter 4 introduces a range of 1D (directly detected $^{31}$P NMR and $^{19}$F NMR) and 2D correlation experiments (i.e., $^1$H-$^{13}$C, $^1$H-$^{15}$N, $^2$H-$^{13}$C) applied to follow nutritional/contaminant impacts on the organism (i.e. biomolecular responses), or the fate of the stressor (i.e. biotransformation, bioaccumulation and excretion of the molecules themselves).

Finally, Chapter 5 builds on the most promising aspect of the hetero-nuclear work identified in Chapter 4, namely $^2$H-$^{13}$C NMR. Chapter 5 marks the first high resolution MAS based technique, that permits rich metabolic fingerprints to be extracted at ultra-low spinning speed (50 Hz). The slow spinning speed allows the organisms to be maintained for over 48 h increasing the potential for use in toxicity assessments which are commonly performed over similar timeframes.
6.4 Limitations

There are numerous hurdles in performing an \textit{in vivo} CMP-NMR experiment, arising from the shortcomings in the technology or physical constraints specific to living samples. \textit{In vivo} NMR using a flow system was initially explored in 1981 with the promise of applications in toxicity research [7]. However, it has not yet become routine analysis tool envisioned at first with only few examples to date reported on HR-MAS based and flow based \textit{in vivo} NMR [3,6,8-10] This mainly originates from the complications due to oxygen/food requirements, intense water peaks, broad signals, and low sensitivity [11]. Living samples are in constant need of oxygen and although this was alleviated somewhat by using a customized rotor cap in as described in Chapter 2, future work investigating different size openings in the cap while monitoring oxygen levels \textit{in vivo} would help to optimize oxygen delivery. During the recent years improvements in water suppression techniques [12,13], isotopic enrichment [14], solution state cryoprobes [15] and 2D NMR [8,14,16], have greatly contributed to numerous [8,17] and with further developments \textit{in vivo} NMR has the potential to reignite the utility of this powerful technique with an unprecedented level of information [1].

6.4.1 Spin rate

Faster spinning speeds lead to sharper lines in phases where anisotropic averaging cannot occur through motion. Therefore, HR-MAS NMR commonly benefits from spinning samples at the same time this poses a great problem for \textit{in vivo} studies [4]. In Chapters 3 and 5 of this thesis the focus was on minimizing the spinning rate and the frequency of spinning at 2.5 KHz was decreased down to 50 Hz. It is worth mentioning that the techniques developed in chapter 3 are only applicable to 1D NMR and the method developed in chapter 5 requires $^2$H-$^{13}$C enrichment. As such no techniques exist that allow ultra-slow spinning 2D NMR without isotopic enrichment and future work should focus to improve robust slow spinning methodologies that can be interfaced with other 2D correlation studies.
6.4.2 Low Sensitivity

NMR techniques inherently suffer from low sensitivity which roots in small difference between spin population based on Boltzmann distribution. Therefore, only a small fraction of the total spins go through transitions and can be observed at each scan.

NMR signal is directly related to
1) Number of Nuclei (N)
2) Gyromagnetic ratio ($\gamma^3$)
3) Inversely to temperature
4) External magnetic field ($B_o^{2/3}$, field homogeneity)
5) Excitation field strength $B_1^2$ (RF pulse)

New technology development in NMR hardware in large part focuses on sensitivity and this is major impediment to new applications. Specifically, CMP-NMR suffers from additional sensitivity loses due to probe design and the nature of the (in vivo) samples. The first CMP-NMR probe with 4 channels ($^1H$, $^{13}C$, $^{19}F$, $^2H$,) suffers 40% lower sensitivity on the $^1H$ and $^{13}C$ channels due to the additional tunings [3]. Sensitivity is paramount for in vivo studies, particularly in vivo aquatic toxicity-based analysis performed in water. For example, in conventional solution state in vitro NMR, samples are extracted, concentrated and often consist of a total of more than 100 organisms equivalent to 10’s of ~mg of pure body mass. In contrast, intact living samples contain a high natural abundance water and the biomass only constituting a small percentage of the total mass leads to much lower concentration of detectable metabolites.

6.4.3 Metabolomic databases

While standard NMR spectra libraries and metabolomic databases including HMDB [2], BMRB [15], and COLMAR [10], are growing rapidly still, they only consist of about 2000 metabolites, which represent a small fraction of the total metabolites in a living system. Specifically, there are very limited number of entries for $^{13}C$ and other hetero-nuclei containing metabolites, and non-specific to environmental organisms such as D. magna and H. azteca. Ongoing research is needed to alleviate issues related to the global identification of metabolites in subject species relevant to
toxicity risk assessments. In addition, many metabolomic pathways specific to invertebrates remain unknown and current limitations make discovering new pathways more challenging.

6.5 Future Work

In vivo NMR is at its infancy and to ensure it realizes its full potential, ongoing development is needed, both in hardware design, as well as the range of pulse sequences based on different type of correlations.

6.5.1 CMP-NMR as a Complementary Technique

CMP-NMR can be used in combination with other techniques such as in vitro or in vivo solution state NMR. As complementary approach to in vivo flow NMR, the dissolved metabolomic fluxes of the organisms can be initially studied in a low stress setting (i.e. static flow-based NMR) to identify particular time points of interest (for example an unusual stress response). At this point, the organisms can be transferred to CMP-NMR for comprehensive studies of all phases and components to provide an in-depth understanding of the biomolecular processes involved.

Although an ample amount of information can be obtained from CMP-NMR and flow-based NMR, a high capital and operation cost, NMR is currently not accessible in many regulatory settings and is mainly considered a discovery tool. Although it is predicted that NMR is likely to become a key setting to the most effective future environmental risk assessment policies, in short term, technology transfer from the discovery-based NMR findings to other commonly accessible analytical platform such as mass spectrometry may provide government agencies the basis to develop new paradigms in toxicology assessments aimed at detecting sub-lethal toxicity. One way to achieve this may be to use CMP and flow-based NMR to detect and unravel in vivo stress processes, and subsequently search for any excreted metabolites in supernatants from the rotors (CMP-NMR) and in the flow system (solution NMR) using MS. If MS detectable metabolites can be correlated directly to the stress processes inside the organisms, they would be very useful
biomarkers that could be detected by environmental agencies using current MS based detection approaches.

6.5.2 CMP-NMR; Findings Appeal to other Scientific Fields

While this dissertation has successfully illustrated the application of CMP-NMR to aquatic organisms such as H. azteca and D. magna, its potential applications can eventually expand to any other biologically relevant field of science spanning from structural biology to biomedical fields leading to novel scientific discoveries. In biological sciences the technique should prove extremely versatile as it provides unprecedented levels of insight into both molecular structure and interactions which ultimately drive all biological processes, such as growth, death, aging, response and reproduction. In biomedical research the approach provides the framework to better diagnosis, therapeutics and drug development. In studies on different types of cancer, inborn metabolomic defects, diabetes, where currently HR-MAS is extensively deployed, CMP-NMR’s unique ability to study all components nothing will allow nothing to go unseen.

6.5.3 Selective Isotopic Enrichment

Uniform isotopic enrichments (2H, 13C and 15N) of the organisms have been used throughout this thesis to increase the signal in the 2D NMR experiments. This is an excellent approach as it makes metabolites in organisms easier to detect and ideal for non-targeted analysis.

However, if questions concerning specific pathways (i.e. Krebs cycle) are of interest, selective enrichment holds great potential. For example, to target the Kreb’s cycle precursor molecules, such as malate, fumarate can be introduced into the diet, which could provide unique insight into specific processes in the living organisms. The advantage of CMP-NMR lies in the ability in identifying the molecules synthesized using these tracers, as well as providing information to the mechanisms of formation by following how various 13C-12C are brought together in a molecular structure. In addition, the full solids capabilities of CMP enable to investigate rigid carbon stores such as structural carbohydrates and their assimilation under stress conditions such as starvation. Carbohydrates are the main fueling precursors in many anabolic pathways and may be selectively
studied to follow such processes. Combined this information provides a unique window into the metabolic pathways, carbon transfer and growth processes in vivo.

A potential application can be amino acid specific information in biochemical pathways obtained by the incorporation of doubly enriched amino acids into the organisms, then using CMP MAS experiments presented in Chapter 4 to study $^1$H-$^{13}$C-$^{15}$N correlations or other experiments that have been successfully applied to flow-state in vivo NMR to trace the $^{13}$C/$^{15}$N correlations on the specific isotopic enriched amino acid can be traced in the organism.

Another area of research with potential widespread application, is the study of contaminants containing hetero-nuclei. For example, $^2$H can replace $^1$H in practically any organic structure (contaminants and nutrients) with minimal impacts on the chemistry of the molecule and represents a molecular marker to follow the fate, biotransformation and bioaccumulation of tracer molecules. Other than the glyphosate studied here, various other pesticides and fertilizers containing $^{31}$P can be directly studied by $^{31}$P NMR methods depicted in Chapter 4. There are recent reports on emerging contamination in waterbodies due to insufficient filtration of pharmaceuticals and their by-products with unknown fates [18]. While many of these drugs contain $^{19}$F, they can be traced using $^{19}$F NMR to indicate possible by-products and discover new ways to remove them from wastewater.

6.5.4 7 mm Probes

NMR methodology commonly suffers from a low signal to noise ratio, and future technologies aiming to enhance sensitivity are critically needed in the future of in vivo NMR.

In the present thesis, CMP probes designed for 4 mm rotors, with 80 μl volume were used. The signal to noise ratio in in vivo studies is further limited by the number of the organisms packed into a 4mm rotor and their tiny size, with each adult H. azteca having a dry mass of less than 1 mg [19]. Therefore, longer experimental times are required to compensate for the low biomass of the organisms, which, in turn is dependent on the full survivability under MAS conditions.

NMR is a mass dependent tool, therefore, increasing the sample size (rotor size and number of the organisms) enhances the signal to noise ratio. The recently developed CMP probes using 7mm
rotors (400 μl volume) would be poised to improve sensitivity with a gain of up to 5 times. As the signal to noise is proportional to the square root of the number of scans, increasing the mass by 5 will reduce the time needed by 25 times. In addition, 7 mm probes can accommodate a wider range of organisms, potentially larger in size and without the need for isotopic enrichment. This has great implications as the organisms can be directly collected from the environment and studied in their natural state under experimental conditions that closely mimic the environment.

This could potentially become a key component of a risk assessment framework, however, currently with larger sample coil diameters, the field homogeneity decreases requiring stronger RF fields for excitation. The 7 mm probes also suffer from slower and inconsistent spinning and further development is needed in this area.

### 6.5.5 E-free probes

The interaction between the electric field and the sample can lead to excessive sample heating specially in biological samples were high proton decoupling powers and CP are needed. In recently developed E-free probes this problem has been alleviated by using a low inductance proton RF coil to reduce the electric field near the sample, resulting in a substantial signal to noise increase. Thus far, this approach has been applied extensively to study protein structure in the solid state, but to date no E-free CMP probes have been built. Such probes substantially reduce the RF heating in living organisms which results in increased sensitivity and possibly extended survival time inside the NMR for living samples.

### 6.5.6 CMP Cryoprobe

Cryoprobes offer a dramatic increase in signal to noise ratio by reducing the thermal noise in the NMR coil assembly and its electronics. In an NMR probe, coils are located close to the sample and can be kept at 4 K using cryogenic helium gas, while the sample temperature can be defined and stabilized by the user at any temperature. Cryoprobes have been developed and commonly used for solution state NMR, while in 2018 Bruker BioSpin, announced the release of first solid state cryoprobe. In the future cryoprobe CMP-NMR may be possible although considerable
complications, including how and where to mount the magic angle gradient would have to be overcome.

6.5.7 Multi-Receiver approaches

In *in vivo* NMR there is only a limited time window to observe the changes. Multiple receiver approaches in NMR, can substantially increase the efficiency of spectrometer by simultaneous acquisition from several nuclear species and extending the information content [20,21]. For example, it has been shown that numerous $^1$H, $^{13}$C and $^{19}$F experiments can be collected at the same time [22], if applied *in vivo* the $^{19}$F experiments could be used to trace the stressor molecule itself, while the $^1$H and $^{13}$C could be used to traces the response from the organism itself.
6.6 References


Appendices
Appendix A: Supplementary of Chapter 2

Comprehensive Multiphase NMR Applied to a Living Organism

A-1: Supporting Discussion and Methods

It has been demonstrated that the organisms could be kept alive and fully recover from up to 14 h of spinning at 2.5 KHz at 5° C. However, while this simplifies the NMR aspects of the study, the process itself will likely induce some stress. Techniques developed for slow magic angle spinning such as PHORMAT [1] can theoretically be applied and should eventually permit the extraction of chemical shift information, at spinning speeds as low as 1 Hz, and permit animals potentially as large as rats to be studied.

Interpretation of the spectra at present is challenging, as discussed previously this is in part due to the fact that NMR databases are still somewhat in their infancy, albeit they are evolving at a rapid rate in parallel with the growth of NMR based metabolomics in general. The main remaining hurdle then becomes the spectral overlap which hampers both interpretation and subsequent quantification. The easiest quantitative information could be accessed from the basic 1D NMR in combination with Electronic Reference To access In vivo Concentrations (ERETIC), a method developed to provide quantification from MRI, without the need for internal standards [2]. Unfortunately, the overlap in basic 1D NMR makes this challenging for most species. Theoretically quantification can be done via the spectral editing approaches (Figure 5.3), but additional ERETIC based protocols will be required that account for signal fraction during the weighted subtractions required for editing. ERETIC is possible in multidimensional NMR, [3] and when combined with special data acquisition techniques can be completely quantitative [4]. The sub-fraction of signals in the multidimensional dataset can be related to the total signal from the
whole organism, which can theoretically be achieved through spin counting [5]. While considerable research in this area is required, given that NMR (if acquired appropriately) is highly quantitative and that the fundamental tools to quantify in vivo without standards already exist, providing absolute quantitation should be feasible in the near future. As such the complexity of the whole organisms and the resulting spectral overlap remains the key challenge. One of the easiest ways to further reduce overlap (along with providing more connectivity information for assignment) would be to integrate other hetero-nuclear information into the NMR experiments to further provide spectral dispersion. Consider for example the peak capacity of a $^1$H-$^{13}$C HSQC dataset (employed here) is generally considered to be $\sim$2,000,000, while the peak capacity of 3D NMR is $\sim$100,000,000 [6]. The most obvious choice would be the incorporation of both a $^{13}$C and $^{15}$N isotopic label into the food source (i.e. $^{13}$C/$^{15}$N doubled labelled algae), giving rise to double labelled organisms. In this case, the great wealth of experiments to study biomolecules can be applied and it would be relatively easy to use $^{15}$N editing to extract sub-spectra that contain only amino acids, or even spectra for specific amino acids or target metabolites. $^{31}$P would be complimentary and could provide direct evidence relating to nucleotide bases and key energy molecules such as ATP and ADP. $^{14}$Si represents a very important nucleus with diatoms representing key contributors to the global fixation of carbon. A recent review concluded that the lack of molecular knowledge regarding silicon sequestration into diatoms (the major limiting nutrient in diatom growth) is limiting progress in this field of research [7]. The ability of the CMP probe to study all phases would allow researchers to follow how soluble silica is absorbed, transported by organic molecules and precipitated as solid cell walls in vivo. Beyond structure elucidation, in vivo CMP-NMR is ideal to study molecular interactions. It has the ability to follow in vivo binding, transformation, sequestration, bioaccumulation and excretion of the contaminants and drugs. In this light $^{19}$F common in many pharmaceuticals and environmental contaminants [8] provides a unique handle to selective view xenobiotics, and to elucidate their dynamics and kinetics [9,10]. If $^{19}$F is not naturally present $^2$H provides the potential to introduce an NMR active nucleus into pretty much any organic structure with little alteration to the molecular chemistry [11].

$^{13}$C labeling of the algae
13C,15N-labeling of the algae *Chlorella reinhardtii* (wt, SAG culture collection Goettingen, Germany) was achieved by growing the cells in 13CO2 and 15NH4Cl (enrichment in both cases 99%; purchased from Sigma Aldrich Isotec, St Louis, Missouri) for 150 h in a photobioreactor (PBR) constructed by Silantes GmbH (Munich Germany). The PBR is built as a closed system avoiding loss of the stable isotope labeled 13CO2. It is an airlift driven external loop tubular fermenter having 20 L operating volume, permitting circulation of the media volume with 1 vvm. The algae were cultivated in regular TP media (Tris-Phosphate w/o Acetate + 15NH4Cl), as previously described [7], using the following parameters: pH 7.0 - 7.4 (pH-probe InPro®3253I/Sg, Mettler Toledo), temperature 30°C (InPro®3253I), light intensity 1300 μmol/(m²s) (quantum detector LI-250A, Li-Cor Biosciences GmbH). A computer-controlled gas management system (Labview 10.1, National Instruments) was incorporated to keep the 13CO2 content (pCO2-probe InPro®5000, Mettler Toledo) at a concentration of 2%. The nitrogen carrier gas was allowed to vary between 65% and 85% corresponding to the oxygen content which increased during autotrophic growth of algae between 15% and 30%. If the oxygen content reached a value of 30% (pO2-probe Visiferm Do Arc., Hamilton) an N2 purging step was introduced in order to reduce the O2 content to the initial value of 15%. To avoid loss of [7] CO2 during N2 purging, the 13CO2 addition was stopped at the end of a growth cycle until a value of 0.5% was reached by metabolic depletion. If the oxygen reached the initial value of 15% due to N2-purging, the computer-controlled valves (Valve 221606, Buerkert) were closed and another growth cycle was resumed. The 13C-content of the algae biomass was determined by analyzing the enrichment in “C18” fatty acids of isolated algae biomass by GCMS (Thermo Quest Polaris Q MS / Trace GC2000, Thermo Fisher). The 15N-content was determined by Shanghai Research Institute of Chemical Industry stable isotope laboratory. Isotopic enrichment of 98-99% 13C and 15N were achieved in the algae biomass.
A-2: Supporting Figures and Table

**Figure A-1** $^1$H NMR data collected for a single shrimp using A. 256 and B. 4096 scans. While little information is lost in the aliphatic region, the lower signal-to-noise in the aromatic region reduces the information content in this region.
Figure A-2 $^1$H Spectra acquired on a single shrimp over a 20 h period. Note spectra A, D, E were collected using 256 scans, and spectra B and C using 4096 scans. The alanine signal increases over the 20 h period and may arise from spinning stress.
Figure A- 3 (A-C) compare the spectra from 3 different adult *H. azteca* (~7 mm length each). The spectral profiles are similar indicating the approach has a good degree of reproducibility. In contrast panel D shows the spectrum for 3 medium sized *H. azteca* (~3 mm length each) placed in the same rotor. A contribution from the doublet of lactate (2) on the left shoulder of the (CH$_2$)$_n$ resonance is an indication of anaerobic stress [13]. While alanine (1) has been reported as an indicator of stress in general in aquatic organisms [14]. Interestingly these younger *H. azteca* have strong contribution in regions (3) and (4) which have been assigned to omega-3’s and an overlapping region where DHA (docosahexaenoic acid) resonates [15]. This would be consistent with these compounds being essential to invertebrate early growth [16] and indicates that CMP-NMR could be a useful tool in general to study chemical physiology and dynamics.
Figure A- 4 Overlaid spectra of a $^1$H–$^{13}$C CP before (solid line) and after (dotted line) denoising using Singular-value decomposition.
Figure A-5 2D In-Phase-COSY experiment used to further confirm the assignments reported in the main section of the thesis.


Table A-1 Summary of experimental parameters.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sample</th>
<th>Rep. Rate</th>
<th>NS</th>
<th>Experiment Time</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>²¹H</td>
<td>1 Large shrimp</td>
<td>2.4 s</td>
<td>256* or 4096</td>
<td>10.5 min or 2 h 47 min</td>
<td>The longest ¹H T₁ for metabolites were estimated at 400 ms (inverse recovery experiment) leading to a repetition rate of 2.4 s (2 s delay + 0.4 s acquisition time). The relatively short relaxation times arise due to the fact the sample is a whole organism and it is uniformly ¹³C labelled (¹H relax via ¹³C interactions).</td>
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<tr>
<td>²¹H T₂</td>
<td>1 Large shrimp</td>
<td>2.4 s</td>
<td>4096</td>
<td>2 h 47 min</td>
<td></td>
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<tr>
<td>Diffusion Ref. (no delays/no gradient)</td>
<td>1 Large shrimp</td>
<td>2.4 s</td>
<td>4096</td>
<td>2 h.47 min</td>
<td></td>
</tr>
<tr>
<td>Run 1 Delays/no gradient (needed for RADE)</td>
<td>1 Large shrimp</td>
<td>2.4 s</td>
<td>4096</td>
<td>2 h 47 min</td>
<td>Due to the lower signal in diffusion editing 4096 scans were required in this experiment. For consistency 4096 scans were also collected for the other ¹H data shown in the main paper. However, fewer scans still provide useful data.</td>
</tr>
<tr>
<td>Run</td>
<td>Sequence</td>
<td>Sample Description</td>
<td>Delay (s)</td>
<td>Gradient (s)</td>
<td>Total Time (h min)</td>
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<tr>
<td>Run 2</td>
<td>13C (zgig)</td>
<td>1 Large Shrimp</td>
<td>5.16</td>
<td>5000</td>
<td>7 h 13 min</td>
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<tr>
<td>Run 2</td>
<td>CP</td>
<td>1 Large Shrimp</td>
<td>0.5</td>
<td>30720</td>
<td>4 h 27 min</td>
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<td>Run 3</td>
<td>COSY</td>
<td>1 Large Shrimp</td>
<td>0.5</td>
<td>NS=128, Increments=196</td>
<td>6 h 42 min</td>
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<tr>
<td>Run 4</td>
<td>HSQC</td>
<td>1 Large Shrimp</td>
<td>0.5</td>
<td>NS=600, Increments=128</td>
<td>14 h 41 min</td>
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A-3: Supporting Videos

Video A-1 Loading a large adult *H. azteca* heads in first, into a sapphire rotor.

Video A-2 Showing the *H. azteca* used to collect the majority of data for this study 3 weeks after nearly to 14 h of MAS (2.5 KHz, modified cap, 5 °C) NMR experiments
Appendix B: Supplementary of Chapter 3

B-1: Selection of the Most Effective Pulse Sequence

Figure B-1 Comparison of the different pulse sequences at 2.5 KHz spinning speed on a standard sample of 5 mM sucrose in H₂O/D₂O (95:5, v/v).
Figure B-2 Comparison of different pulse sequences at 500 Hz spinning speed on a standard sample of 5 mM sucrose in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (95:5, v/v).

B-2: Performance with slower spinning speed

In order to investigate the lower spinning limits of the two water/sideband suppression sequences they were tested first on a whole worm sample and then on a solution of 5 mM sucrose.

Figure B-3 highlights spectra for a whole worm. Unfortunately, due to the very heterogeneous nature of the sample, which made the rotor unbalanced, it was not possible to stably spin the sample below 300 Hz using our probe MAS controller combination. Even at 300 Hz there were considerable spin fluctuations during the runs and the sample did not remain spin locked throughout. While Figures B-3 and B-4 clearly show the sequence performs well at 400 Hz, it is not clear whether the degradation in spectral quality at 300 Hz and below is due to the unstable spinning or the sequence itself.
To further investigate this a rotor of 5 mM sucrose was used. In this case the even distribution of the solution lead to stable spinning even down to 50 Hz. Figures B-5 and B-6 Show that both the TOSS.243 and PASS variants work well, down to 100 Hz but fail at spinning speeds lower than 100 Hz. Closer inspection of the peaks at 4.05 ppm and 4.25 ppm show some modulation with spinning speed. This is clearly more apparent in the PASS where the ratio between these peaks varies with spinning speed while TOSS.243 in comparison is more reproducible. Previously it was believed that TOSS can only work at spinning rates higher than 1 KHz [1-3] where in this work efficient sideband suppression was observed down to spinning rates as low as 100 Hz using the 5 pulse, 243 variants.
Figure B-3 PURGE-PASS sequences applied at different spinning speeds on a rotor containing a whole intact juvenile earthworm. The spinning rate became unstable at 300 Hz and would not spin lock below 300 Hz.
Figure B-4 PURGE-TOSS.243 sequence applied at different spinning speeds on a rotor containing a whole intact juvenile earthworm. The spinning rate became unstable at 300 Hz and would not spin lock below 300 Hz.
**Figure B-5** PURGE-PASS at different spinning speeds on a 5 mM standard solution of sucrose in H₂O/D₂O (95:5, v/v).
**Figure B- 6** PURGE-TOSS.243 at different spinning speeds on a 5 mM standard solution of sucrose in H₂O/D₂O (95:5, v/v).

**Figure B- 7** Depicts the spectra acquired using different number of PURGE loops (blue 0, red 1, green 2) on a rotor of 5 mM sucrose in H₂O/D₂O (95:5, v/v) using: a. PURGE TOSS.243 and b. PURGE PASS. For clarity just, the anomeric signal is shown. About 20% of the signal is lost per PURGE loop (please see Chapter 3 for more details).
Appendix C: Supplementary of Chapter 5

C-1: Experimental Considerations for $^2$H-$^{13}$C experiments

Given the considerable promise of 2D $^2$H based NMR for metabolite detection in complex samples, the three main 2D correlation sequences; $^{13}$C-$^2$H HETCOR, $^2$H-$^{13}$C HSQC and $^2$H-$^{13}$C HMQC were briefly explored to acquire the highest sensitivity. As the gyromagnetic ratio of carbon is higher than that of deuterium it would be expected that $^{13}$C detected experiment would be the most sensitive. Further as HSQC only permits $^{13}$C magnetization to evolve ($^2$H J coupling has no influence during the evolution period), the $^2$H dimension should in theory be sharper in HSQC giving rise to stronger cross peaks when compared to HMQC. As such, conventional logic would predict a hierarchy of $^{13}$C-$^2$H HETCOR > $^2$H-$^{13}$C HSQC > $^2$H-$^{13}$C HMQC in terms of sensitivity. However, our results indicate the exact opposite is true. Figure C-1 compares the acquired HETCOR, HSQC and HMQC, on a sample of $^{13}$C/$^2$H isotopically enriched algae, with the corresponding $^{13}$C NMR projections added to illustrate the signal to noise ratios. As observed the sensitivity of HMQC experiment outperforms that of HSQC by a significant margin under identical conditions. This can be explained theoretically (please see supplemental section 2 for a further discussion) which demonstrates single quantum transfers fail to properly refocus the $^2$H-$^{13}$C J-coupling interactions. Importantly, this also explains the failure of HETCOR to provide an adequate 2D spectrum. HETCOR is based on a single quantum approach, which suffers the same problems as HSQC. As such, in practice, a simple $^2$H detection experiment based on multiple quantum approach yields better sensitivity compared to single quantum based methods due to the inadequate refocusing of multiple quantum terms under HSQC and HECTOR [1,2].

C-2: Theoretical Considerations for $^2$H-$^{13}$C experiments

Considering the enormous body of literature on 2D $^1$H-$^{13}$C correlation experiments, only a brief description will be given here. $^1$H-$^{13}$C correlation 2D NMR experiments comes in two flavors:
Hetero-nuclear Single Quantum Coherence (HSQC) and Hetero-nuclear Multiple Quantum Coherence (HMQC). Although these two experiments provide the same $^1\text{H}-^{13}\text{C}$ correlation, the pathway in which the coherences are transferred is different: HSQC uses a single quantum pathway while HMQC uses a multiple quantum approach for coherence transfer. Unlike the $^1\text{H}$ nuclei, $^2\text{H}$ is a quadrupole nucleus with spin = 1. Therefore, the transfer of magnetization between $^2\text{H}$ and its directly bonded $^{13}\text{C}$ will be different compared to its $^1\text{H}-^{13}\text{C}$ spin pair counterpart. Under the influence of $^2\text{H}$ nuclei, the observed $^{13}\text{C}$ NMR signal of a $^2\text{H}-^{13}\text{C}$ spin pair is split into a triplet, indicating the manifestation of multiple quadrupole term in the Hamiltonian. In fact, the heteronuclear zero and double quantum coherences do evolve under the first order spin-spin couplings for a $^2\text{H}-^{13}\text{C}$ spin pair. Therefore, these unwanted quantum coherences will have an impact on the transfer of magnetization from $^{13}\text{C}$ to $^2\text{H}$ for detection, which may result in the loss of signal intensity. The question remains - during the $^{13}\text{C}$ evolution period of both HSQC and HMQC, can $^2\text{H}-^{13}\text{C}$ J-couplings be effectively refocused via a 180 degrees pulse on the $^2\text{H}$ channel? In order to understand the effect of an 180$^0$ pulse on refocusing of $^2\text{H}-^{13}\text{C}$ J-couplings evolution, we have to invoke the product operator formulism.

HMQC

For the case of HMQC, the $^{13}\text{C}$ chemical shift evolves under a multiple quantum term:

$I_y S_x$  \hspace{1cm} I = $^{13}\text{C}$ and S = $^2\text{H}$

Under the evolution of $^2\text{H}-^{13}\text{C}$ J-coupling at $1/2t_1$, we have the following term:

$I_y S_x \cos(J\pi t_1) - I_x [S_x, S_z] + \sin(J\pi t_1)$

(1)

After the application of an 180$^0$S$_x$ on $^2\text{H}$ channel and under the influence of $^2\text{H}-^{13}\text{C}$ J-coupling at $1/2t_1$, equation (1) becomes:

$I_y S_x \cos^2(J\pi t_1) - I_x [S_x, S_z] + \sin(J\pi t_1) \cos(J\pi t_1) + I_y S_x \sin^2(J\pi t_1) - I_x [S_x, S_z] + \sin(J\pi t_1) \cos(J\pi t_1)$

(2)

$= I_y S_x \cos^2(J\pi t_1) + I_y S_x \sin^2(J\pi t_1)$

Since $\cos^2(J\pi t_1) + \sin^2(J\pi t_1) = 1$, then equation (2) simplifies to:

$I_y S_x$
This illustrates that under HMQC, $^2H-^{13}C$ J couplings are effectively refocused, allowing the $^{13}C$ to evolve during the $t_1$ encoding period and transfer back to single quantum for detection.

**HSQC**

On the other hand, for HSQC the $^{13}C$ chemical shift evolves under an antiphase term after the first INEPT period:

After the application of a $180_{\gamma}$ on $^2H$ channel following by an evolution of the $^2H-^{13}C$ J-coupling Hamiltonian we have the following equation:

$$I_zS_z (\cos^2(J\pi t_1) - \sin^2(J\pi t_1)) + 2I_xS_z^2 \sin^2(J\pi t_1) \cos^2(J\pi t_1)$$

(3)

Based on equation (3), the $^2H-^{13}C$ J-coupling fails to refocus via the $180_{\gamma}$ pulse on $^2H$. Therefore, the transfer of magnetization in the subsequent reversed INEPT will be ineffective, which leads to lower sensitivity for HSQC.

According to the product operator analysis, the performance of HSQC will be inferior compared to that of HMQC due to its failure to properly refocus the $^2H-^{13}C$ J-coupling interactions.
C-3: Supporting Figures

**Figure C-1** (A-C) $^2$H NMR projections of the corresponding 2D experiments in (D-F) 2D $^{13}$C-$^2$H HETCOR, $^2$H-$^{13}$C HSQC and $^2$H-$^{13}$C HMQC pulse sequences at 50 Hz performed on $^2$H/$^{13}$C enriched algae are compared. Note in this study the entire rotor was filled with algae (35 mg), thus, signals are easy to detect in comparison to the feeding studies where lower amount (~5 mg) is used for feeding.
**Figure C-2** $^2$H spectra of the $^2$H/$^{13}$C enriched algae A) at the start point (3.5 h experimental acquisition time) and B) after a 24 h period. No significant changes are observed in the absence of the organisms.

**Figure C-3** Figure S3. $^{13}$C NMR projections of the metabolites produced by A) *D. magna* and B) *H. azteca* in the presence of $^2$H/$^{13}$C enriched algae at the beginning and after C) 14 h feeding of *D. magna* and D) 24 h of feeding for *H. azteca*. 
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