A Novel Approach for the Environmental Study of Organisms in their Natural State

Kimberly L. Colson[1], Martine Monette[1]

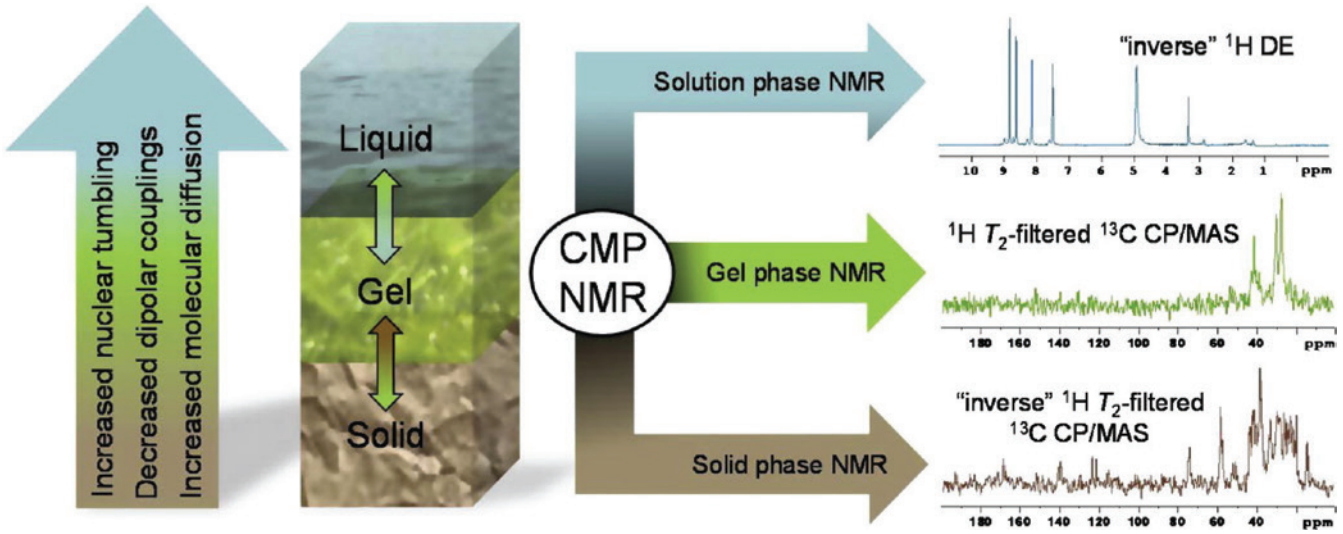
[1]Bruker BioSpin Corporation, Billerica, MA 01821 USA

A key aspect of environmental research relies on the ability to study organisms in their natural state in order to fully assess the consequence of exposure to toxins and an ever changing environment. However, typical analytical approaches rely on sample processing, such as extractions that provide only a selective window into the true natural state. Living systems are multifarious and it is the delicate synergism between physical organization, chemical reactivity, and biological process that gives rise to life. Organisms contain an array of different physical phases including liquid, gels and solid, therefore different analytical approaches are used. Each of these phases contains an array of different chemical components, each of which contribute to the organism's function. Often it is synergy between these phases that give rise to overall structure and chemical processes in natural organisms. Consider for example a plant. Its epidermis is covered by a waxy cuticular layer that acts as a water barrier. It also provides mechanical strength and protection to the plant. The vascular tissue provides structural support and contains xylem and phloem tissues that transport fluids and nutrients.

As such, a range of different structural components are present in the organism that in turn exhibit a range of different rigidities from true solids (e.g., cross-linked ligno-celluloses in the cell walls), 'gels' (various swollen biopolymers), to true solutions (liquids in vascular conduits). Indeed, the complex relationship between the chemical components as well the assimilations of components ultimately determines plant growth. Consequently, there is a great need in environmental research to study samples in their natural state and be able to extract and differentiate structure/interaction information from liquids, gels, and solids.

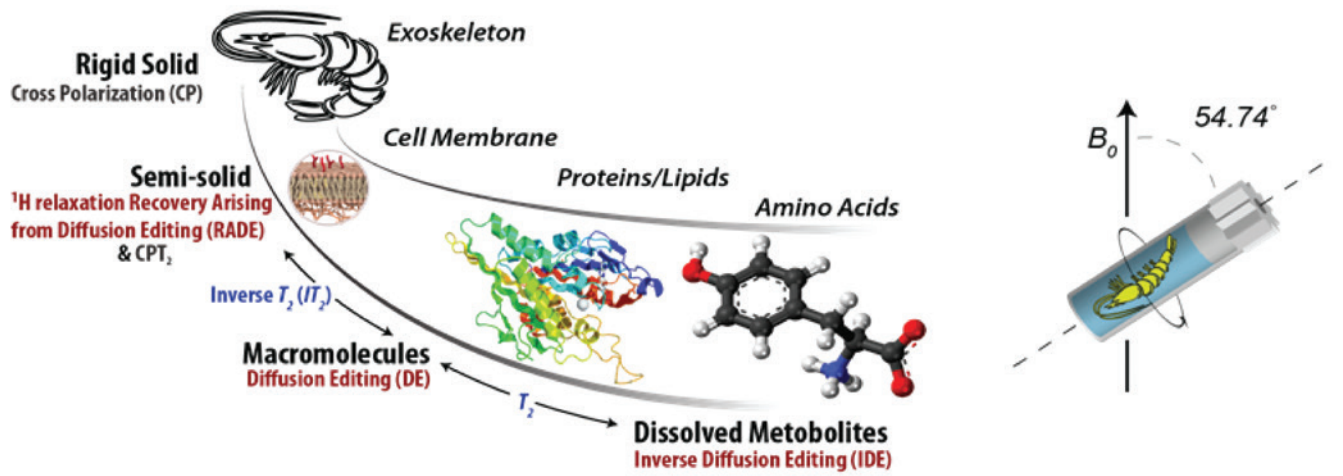
In recent years, NMR has proved extremely useful in unraveling structure and function in extremely complex systems, in turn providing unsurpassed molecular detail for heterogeneous samples [1–5]. Currently, most heterogeneous samples are studied according to their individual phases: in solution, in gel (HR-MAS) or in solid phase. However, studying each phase separately can potentially remove important chemical/physical information, making it impossible to fully understand complex samples holistically. The integration of concepts from different fields of NMR spectroscopy enables us to produce a novel comprehensive approach that can be used to study and differentiate between the various phases in a heterogeneous sample as well as identify the key structures and their molecular interactions within each phase. In this respect, there has been a collaboration between the Environmental NMR Centre (University of Toronto) and Bruker BioSpin over many years. The resulting hardware, the comprehensive multiphase (CMP)-NMR probe, permits the full range of solid-state, gel, and solution-state experiments to be performed on samples without pre-treatment or extraction. Using CMP-NMR technology, novel experiments can be designed that build upon advantages of both solution- and solid-state NMRs such as gradient-enhanced solid experiments or diffusion-weighted

Figure 1



Comprehensive Multi-Phase NMR combines the capabilities of solid-state, gel-state and liquid-state NMR into a single approach using a single sample and single CMP-NMR probe. This allows all components (solutions, liquids and gels) to be analyzed and fully differentiated in intact unaltered samples in their natural (fully swollen) state[9-15].

Figure 2



While spinning induces stress on the living organism, it permits the detection of contaminants in all phases *in-vivo*, allowing correlations to be established between the state of the contaminant (bound/free and binding receptor), metabolic change and also structural change (protein, lipid membrane disruption, thickness of shell etc.)[14] When combined with in-vivo solution-state flow NMR[11,16], metabolites can be measured in a low stress state (flow) and correlated all structural changes (CMP, solution/gel/solid) to provide an unprecedented window into the biological stress response.

CP-MAS. CMP-NMR changes NMR technology to match natural samples, rather than changing the sample to match a specific NMR technique. Figure 1 shows an example of the benefit gained: a comprehensive view leading to enormous insight into an unperturbed system.

The CMP technology can also be utilized on living organisms, as shown in Figure 2. *Hyalella azteca* is a species of freshwater shrimp which is native to Central America, the Caribbean and North America and is the most abundant amphipod in North American lakes.[6] Domesticated, it is widely used as a laboratory animal for testing ecotoxicity.[7-8] More specifically, it is an important indicator of water quality and plays a key role in the food chain. Studies performed on the complete living organism from exoskeleton to metabolites using the CMP-NMR spectroscopy enable the characterization of their feeding habits and the nutritional value of their diet. The metabolite mosaic can be correlated to the toxic stress of the organism, enabling us to understand better the effects of water contaminants.

Hyalella azteca rely on algae as a key food source for needed lipid and amino acid intake. Several key experiments performed on an algae sample using the CMP probe provide details about the nutritional value of the algae for the organism. As previously discussed, the use of CMP-NMR technology has considerable implications for understanding structure and interactions in plants and other organisms. This paper describes the use of a CMP probe and various NMR techniques applied to a sample of $^{13}C/^{15}N$ dried algae resuspended in D_2O. Identifying the metabolites and dissolved components within algal cells by NMR represents a first step, permitting future studies of carbon transfer between species and through the food chain. It will also provide a foundation to better understand the role of algae in the formation of dissolved organic matter and sequestration/transformation of carbon in the aquatic environment in general.

Materials and Methods

The CMP probe combines Magic Angle Spinning (MAS), solids and high resolution techniques. Unlike conventional HR-MAS probes, the CMP probe has high power handling capabilities, enabling techniques like cross polarization magic angle spinning (CPMAS) to be performed. The probe has a convenient and suitable sample volume (4 mm stator, 80 uL) for studies of multiple types of organisms for example. It also has excellent ^{1}H detection, high sensitivity for ^{13}C detection and also high stability for long experiments (lock channel). When needed, the probe is also capable of excellent water suppression using gradient techniques.

NMR experiments were performed using a Bruker AVANCE III HD NMR spectrometer with a ^{1}H frequency of 500 MHz equipped with a 4 mm four channel (^{1}H, ^{13}C, ^{15}N, ^{2}H) CMP probe fitted with a gradient. A D_2O lock was used for all experiments. Decoupling was used in all 1D and 2D experiments to remove ^{1}H-^{13}C coupling from the ^{13}C enriched sample, with the exception of Figure 8. Low power experiments were carried out using *garp* for proton observe and *waltz16* for carbon observe. High power ^{1}H decoupling was also used at 90 kHz for all solids-type experiments. All NMR spectra were processed via Bruker's Topspin 3.5.

The algae *Chlorella reinhardtii* 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 autothrophically and exclusively with $^{13}CO_2$ (98% enriched with stable Isotope ^{13}C) and $^{15}NH_4Cl$ ammonium chloride (99% enriched with stable isotope ^{15}N). The cultivation parameters including media, temperature, light intensity, and pH were adjusted to gain a maximum growth rate. The harvested algal biomass was freeze dried and stored at 20 °C prior to use.

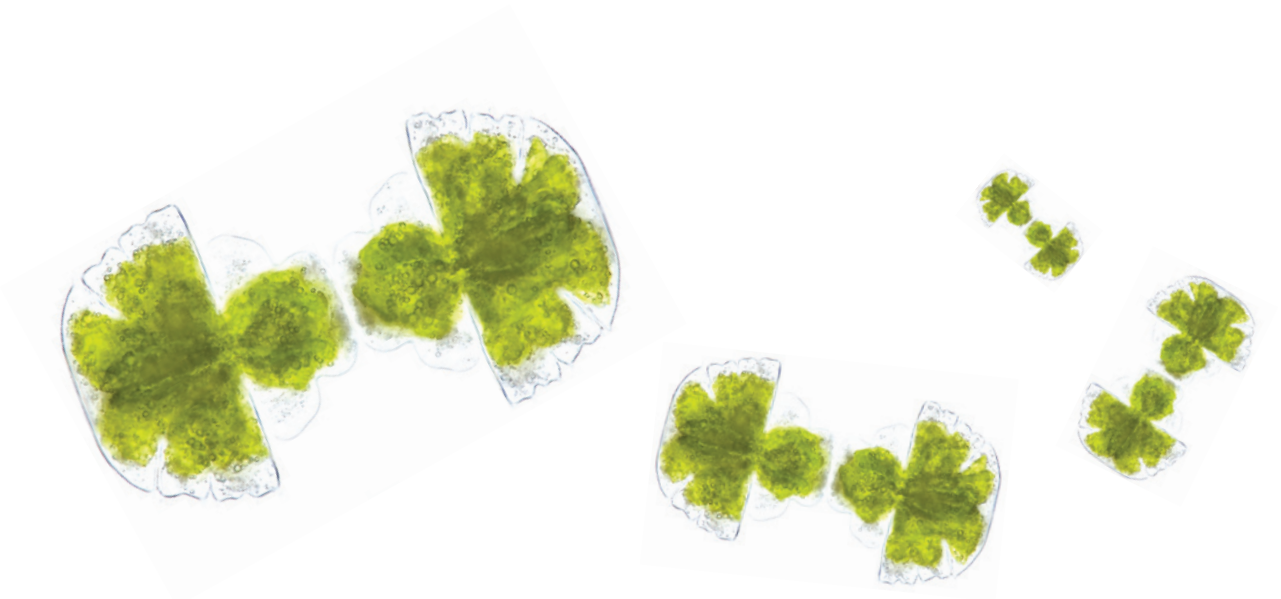
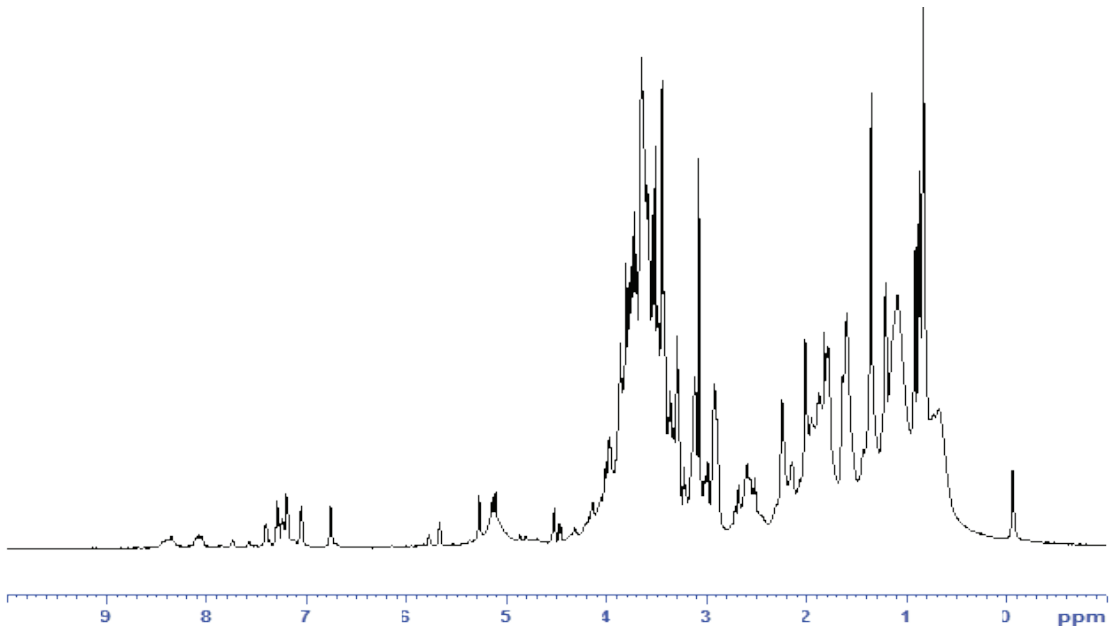


Figure 3



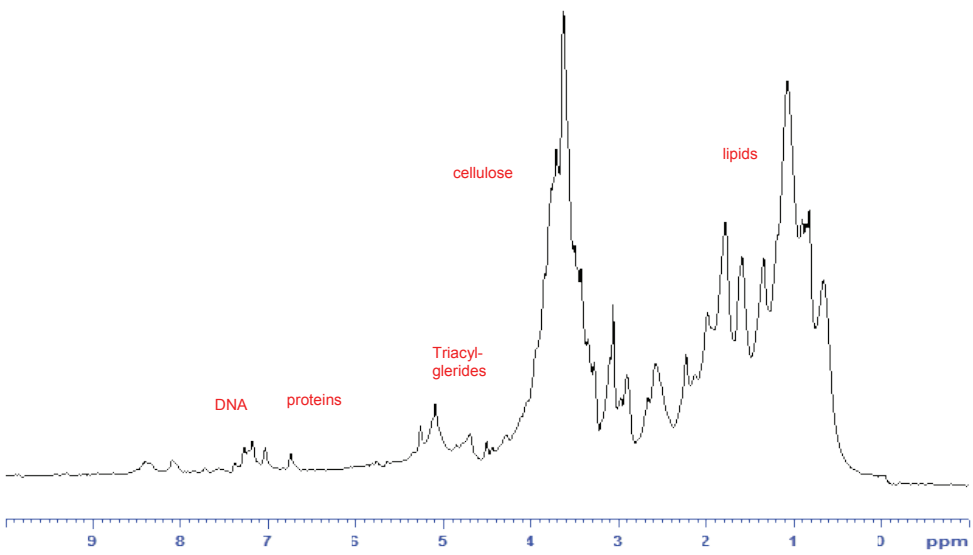
1D ^{1}H HRMAS of algae sample suspended in D_2O, using presaturation and ^{13}C decoupling.

Results

All NMR experiments were performed on $^{13}C/^{15}N$ enriched algae suspended in D_2O. One dimensional NMR experiments were acquired using the presaturation method for suppression of residual HOD signal, and ^{13}C decoupling. The 1D ^{1}H spectrum on Figure 3 shows resonances that are consistent with signals for structural and metabolic components including lipids, carbohydrates and amino acids. However, due to considerable overlap, further assignment is difficult.

In order to differentiate signals from solution, gel, and solid components, editing approaches use filters that exploit various properties inherent in different phases (nuclear tumbling, dipolar interactions, and molecular diffusion). The resulting data provide physical-phase edited NMR spectra mainly targeting basic structural information. Figure 4 shows the use of such a filter with the RADE[9] experiment: recovery of relaxation losses arising from diffusion editing. RADE is discussed

Figure 4



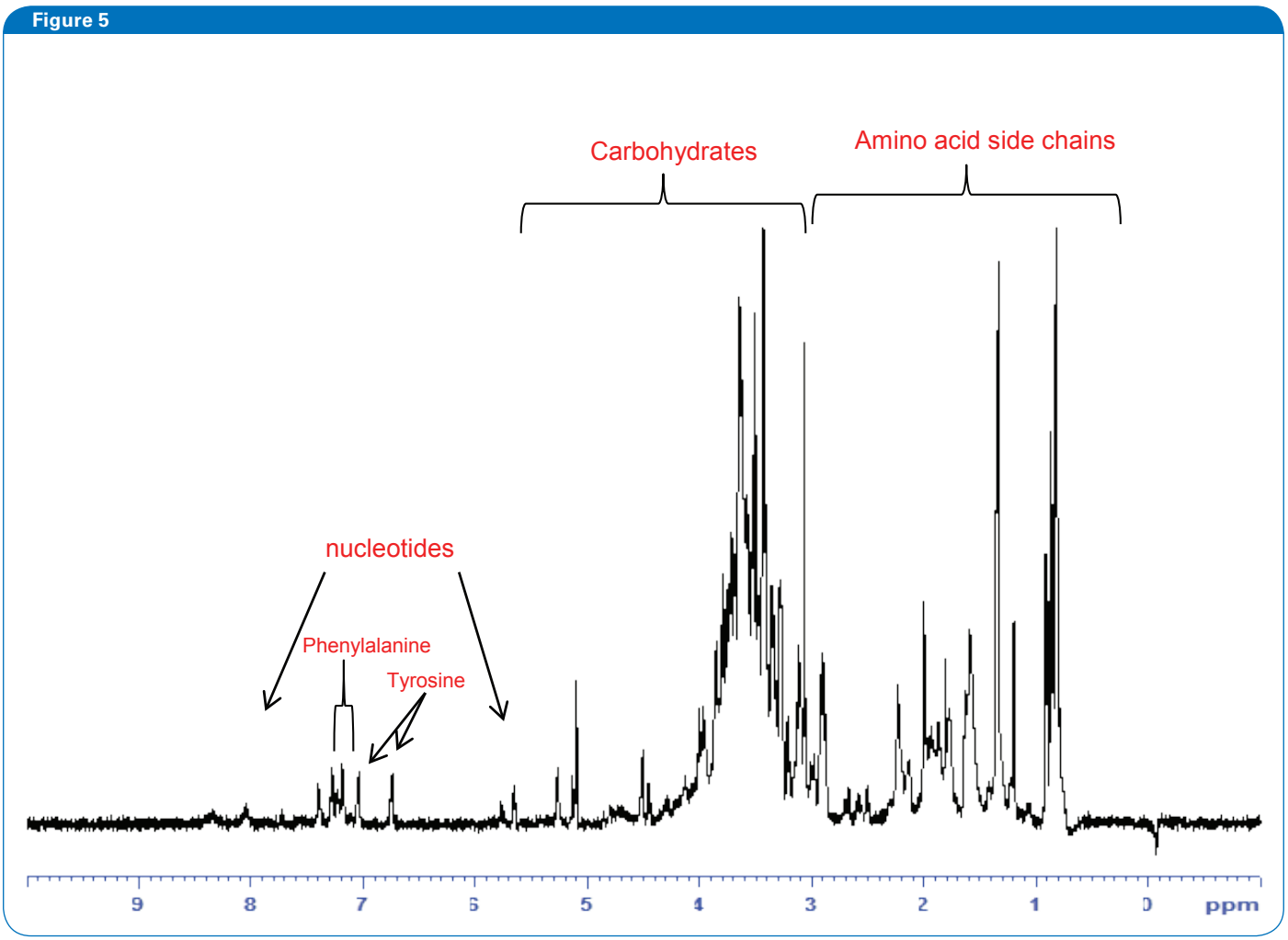
RADE experiment showing fast relaxing semi solids: lipids and cellulose

in detail by Courtier-Murias et al.,[9] but essentially accounts for any components that preferentially relax during diffusion-based experiments and fully recovers the signals. In simple terms RADE will select the most rigid components observable by low power ^{1}H-detect approaches and represents, rigid-gels/semi-solids.

Conversely, it is possible to use similar filters combined with post-processing difference spectroscopy of diffusion weighted spectra (see Ref. 9 for further details). The result is only components that have unrestricted self-diffusion (i.e., truly soluble components) that are retained in the final edited spectrum (inverse diffusion editing IDE), as shown on Figure 5. The downfield region shows the presence of aromatic metabolites (Figure 5). Due to less overlap and characteristic patterns from common metabolites, some specific assignments can be performed.[9] In particular, signals corresponding to the aromatic amino acids tyrosine were observed along with aromatic nucleotide bases (6.0 ppm).

Two dimensional NMR spectroscopy techniques are ideal for the identification of individual metabolites in complex heterogeneous samples as it increases spectral dispersion and provides coupling information allowing individual metabolites to be assigned. Through ^{1}H-^{13}C 2D HMQC spectra, overlapping chemical shifts in 1D ^{1}H NMR can be resolved in the ^{13}C dimension. Metabolites in the HMQC spectrum can be identified by matching against AMIX Bruker Bio-reference spectral database of HSQC data.[17] Many metabolites including a range of amino acids and amino acid derivatives have been confirmed thanks to HMQC spectra (unpublished results). In particular the aromatic amino acids phenylalanine and tyrosine, which showed weak signals in ^{1}H NMR, can clearly be identified in the HMQC spectra. In the same aromatic region, resonances from nucleosides, nucleotides and their constituents can be identified. The anomeric region contains resonances from several mono and di-saccharides. A wide range of carbohydrates can be observed also in the aliphatic region although there is still considerable overlap (Simpson et al, unpublished results).

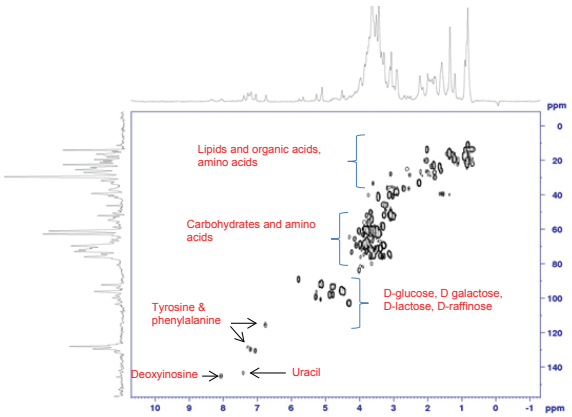
Figure 5



IDE experiment showing dissolved metabolites and amino acids

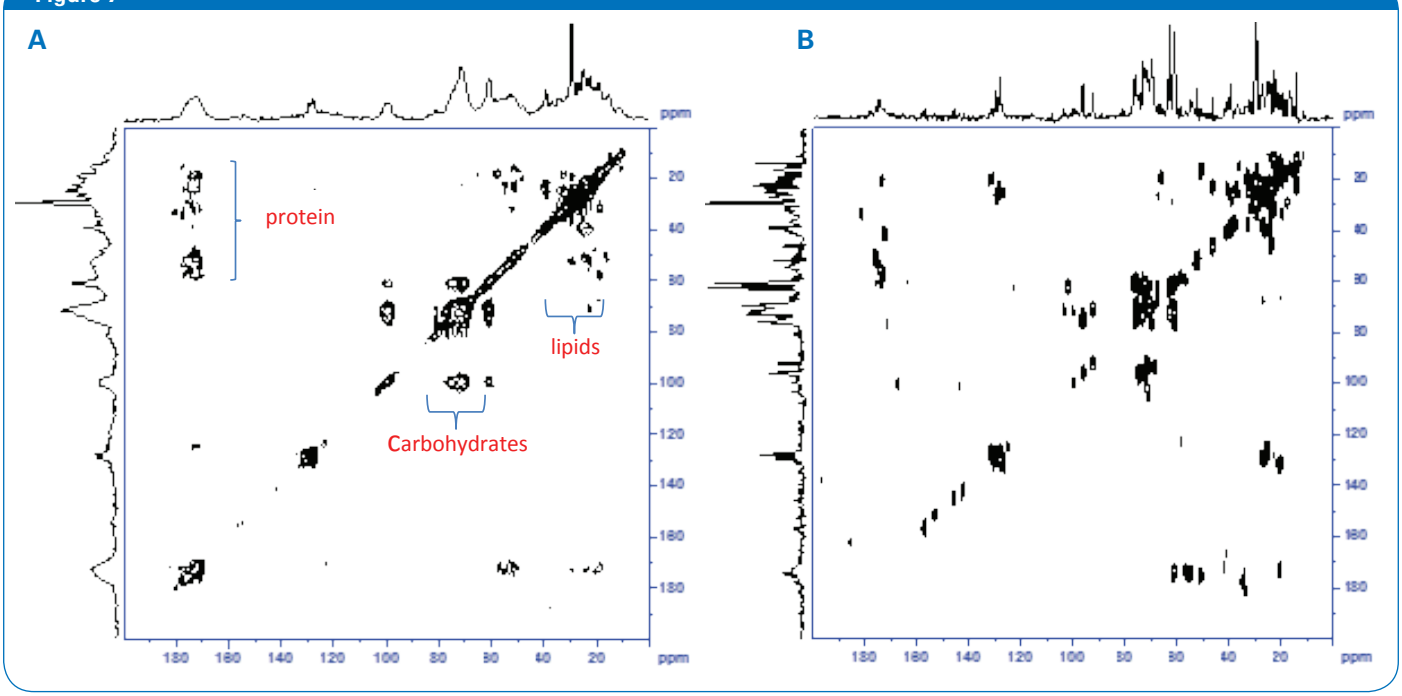
Similarly, ^{13}C one dimensional experiments can benefit from enhanced dispersion using a second ^{13}C dimension, as shown in Figure 7. Figure 7a shows ^{13}C-^{13}C correlations via dipolar coupling, arising from molecules that are present in the non-soluble components of the algae sample. On the other hand, Figure 8b shows ^{13}C-^{13}C correlations via scalar coupling, present in small and rapidly tumbling molecules in the mobile components of the algae. Both spectra have for projections the corresponding ^{13}C spectra acquired with the cross polarization method for the DARR spectrum and IDE method for the ^{13}C-^{13}C CT- COSY. As the DARR approach employs ^{1}H-^{13}C cross-polarization rigid bonds that efficiently undergo CP are preferentially selected. As such the experiment provides structural information from the solid part of the algae sample: proteins and lipids signal likely arise from membranes and membrane proteins, whereas carbohydrate signals originate

Figure 6



^{1}H-^{13}C HMQC experiment of uniformly labeled $^{13}C/^{15}N$ dried algae resuspended in D_2O

Figure 7



^{1}H-^{13}C HMQC experiment of uniformly labeled $^{13}C/^{15}N$ dried algae resuspended in D_2O

from cell walls. The constant time ^{13}C-^{13}C COSY does not have dedicated filter like the inverse diffusion editing experiment, but does contain a lengthy constant time period. Consequently, this experiment may show some signal from the less mobile components but will mainly select signals from the most dynamic components. As such the overall resolution of the COSY is significantly higher and matches very well the IDE ^{13}C spectrum as shown on Figure 7b.

So far, using magic angle spinning (MAS) NMR spectroscopy on a plant sample, it has been possible to resolve structural details otherwise unobservable by other biophysical measurements. Examples of both high resolution liquids methods and solids methods have applied back to back on the same sample to probe various structural components. These examples underscore the convenience of being able to switch back and forth between standard liquids NMR methods and solids method using the same sample and probe setup. This is not the only advantage of the CMP probe.

Recently, the CMP probe technology was used in a recoupling NMR experiments to obtain detail at an atomic level, on a non-frozen, non-isotopically labeled amyloid Aβ1-40 oligomer. Using ^{1}H-^{1}H dipolar couplings as a NMR spectral filter to

remove both high and low molecular weight species, atomic-level characterization of a non-fibrillar aggregation product of the Aβ1-40 peptide was reported for the first time[18]. It is well known that high-resolution structural features of high molecular weight oligomers are difficult to characterize directly by ^{1}H solution NMR. However, the RFDR-based (radio frequency driven dipolar recoupling) 2D $^{1}H/^{1}H$ experiment performed using the CMP probe enables the specific detection of oligomeric species due to the line-narrowing afforded by magic angle spinning and the reintroduction of residual ^{1}H-^{1}H dipolar couplings by RFDR[18].

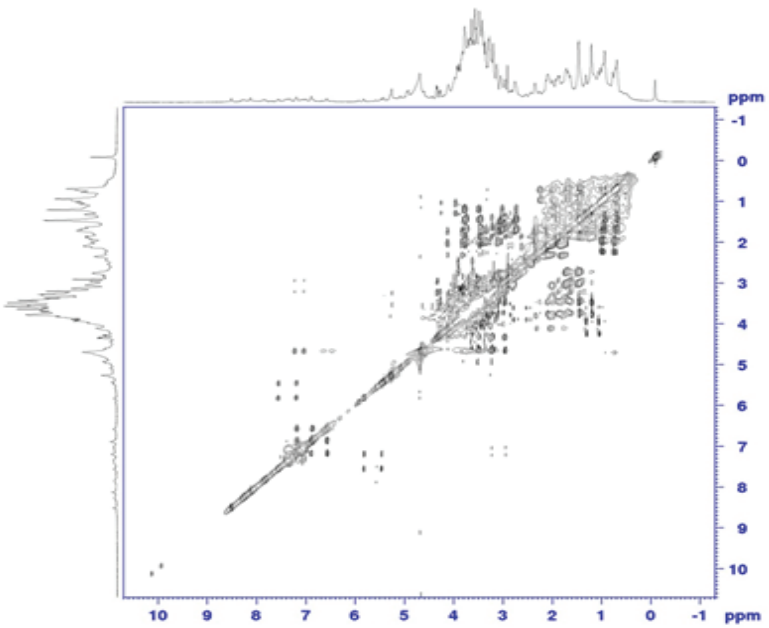
Figure 8 shows the RFDR based 2D $^{1}H/^{1}H$ chemical shift correlation experiment acquired on the algae sample. The resulting spectrum shows ^{1}H-^{1}H correlations through space via dipolar coupling using a strong RFDR pulse scheme during the mixing time. The experiment also utilizes gradients for efficient water suppression. The RFDR-based 2D $^{1}H/^{1}H$ experiment therefore acts as an efficient spectral filter for intermediate sized species; mobile molecules exhibit few cross-peaks because of inefficient dipolar recoupling while the linewidth from species in the solid phase is too large to generate a detectable signal. In this particular example ^{13}C decoupling was not applied such that the ^{1}H-^{13}C coupling is encoded into the spectral permitting easy extraction of coupling constants.

Since the RFDR pulse sequence utilizes the transfer of proton magnetization via coherent ^{1}H-^{1}H dipolar couplings, we would expect the RFDR pulse sequence to be more sensitive to larger molecules than the traditional NOESY experiment utilizing only NOE cross-relaxation. Accordingly, cross-peaks were not observed in 2D NOESY spectra obtained under MAS conditions (data show shown), indicating the dipolar interaction among protons dominates the transfer of magnetization between nuclei in the algae sample. These results suggest that the RFDR experiment combined with the capabilities of the CMP probe may provide a general method to study intermediate size particles in plant samples.

Summary

The CMP probe offers new technology that allows for a comprehensive analysis of all components (liquids, gels, solids) in whole samples in their natural state. In the case of living intact systems such as *Hyalella azteca* all phases from exoskeleton to metabolites can be observed. This breakthrough technology enables NMR to evaluate an intact organism to gain tremendous insights and assess the function and environmental effects on the organism's substructures. With this NMR approach, one can study intact biological and environmental organisms including soil, plants, tissues, cells, bone, and seeds. Complex process such as drug transport across phases, biological growth, contaminant sequestration, melting, flocculation and drying can be now followed in real time by monitoring every physical phase in an organism.

Figure 8



^{13}C-^{13}C correlation spectra a) DARR experiment and b) CT-COSY experiment

Acknowledgements and References

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