Analytica Chimica Acta: X 6 (2020) 100051

Contents lists available at ScienceDirect

Analytica Chimica Acta: X

journal homepage: www.journals.elsevier.com/analytica-chimica-acta-x

Ex vivo Comprehensive Multiphase NMR of whole organisms: A complementary tool to *in vivo* NMR

Rajshree Ghosh Biswas [a], Blythe Fortier-McGill [a], Mohammad Akhter [a], Ronald Soong [a], Paris Ning [a], Monica Bastawrous [a], Amy Jenne [a], Daniel Schmidig [b], Peter De Castro [b], Stephan Graf [b], Till Kuehn [b], Falko Busse [c], Jochem Struppe [d], Michael Fey [d], Hermann Heumann [e], Holger Boenisch [e], Marcel Gundy [e], Myrna J. Simpson [a], André J. Simpson [a, *]

[a] University of Toronto Scarborough, Department of Physical & Environmental Sciences, 1265, Military Trail, M1C 1A4, ON, Canada
[b] Bruker Switzerland AG, Industriestrasse 26, 8117, Fällanden, Switzerland
[c] Bruker Biospin GmbH, Silberstreifen 4, 76287, Rheinstetten, Germany
[d] Bruker Corporation, 15 Fortune Drive, Billerica, MA, 01821-3991, USA
[e] Silantes GmbH, Gollierstrasse 70c, D-80339, München, Germany

ARTICLE INFO

Article history:
Received 2 April 2020
Received in revised form
29 May 2020
Accepted 23 June 2020
Available online 27 June 2020

Keywords:
Comprehensive multiphase nuclear magnetic resonance spectroscopy
Daphnia magna
Ex vivo
Multiphase editing
Intact samples

ABSTRACT

Nuclear Magnetic Resonance (NMR) spectroscopy is a non-invasive analytical technique which allows for the study of intact samples. Comprehensive Multiphase NMR (CMP-NMR) combines techniques and hardware from solution state and solid state NMR to allow for the holistic analysis of all phases (i.e. solutions, gels and solids) in unaltered samples. This study is the first to apply CMP-NMR to deceased, intact organisms and uses ^{13}C enriched *Daphnia magna* (water fleas) as an example. *D. magna* are commonly used model organisms for environmental toxicology studies. As primary consumers, they are responsible for the transfer of nutrients across trophic levels, and a decline in their population can potentially impact the entire freshwater aquatic ecosystem. Though *in vivo* research is the ultimate tool to understand an organism's most biologically relevant state, studies are limited by conditions (i.e. oxygen requirements, limited experiment time and reduced spinning speed) required to keep the organisms alive, which can negatively impact the quality of the data collected. In comparison, *ex vivo* CMP-NMR is beneficial in that; organisms do not need oxygen (eliminating air holes in rotor caps and subsequent evaporation); samples can be spun faster, leading to improved spectral resolution; more biomass per sample can be analyzed; and experiments can be run for longer. In turn, higher quality *ex vivo* NMR, can provide more comprehensive NMR assignments, which in many cases could be transferred to better understand less resolved *in vivo* signals. This manuscript is divided into three sections: 1) multiphase spectral editing techniques, 2) detailed metabolic assignments of 2D NMR of ^{13}C enriched *D. magna* and 3) multiphase biological changes over different life stages, ages and generations of *D. magna*. In summary, *ex vivo* CMP-NMR proves to be a very powerful approach to study whole organisms in a comprehensive manner and should provide very complementary information to *in vivo* based research.

1. Introduction

All living systems are carefully attuned to their environments [1]. Perturbations to their environment (i.e. changes in temperature, light, pH, contamination etc.) may bring about an array of metabolic responses within the organism. Environmental metabolomics often involves studying an organism's response to stressors such as toxins, heat, light etc., followed by detecting and interpreting these changes in the organism's metabolome [2–4]. By understanding the biochemical pathways impacted inside an organism, it is possible to understand these pathways further, thereby explaining why chemicals are toxic (i.e. toxic-mode-of-action). In the future, through understanding and categorizing complex stress

* Corresponding author.
E-mail address: Andre.simpson@utoronto.ca (A.J. Simpson).

https://doi.org/10.1016/j.acax.2020.100051

responses, it is hoped that these responses themselves can help identify the types of stressors impacting natural organisms and in turn help focus monitoring policies and remediation efforts.

Nuclear magnetic resonance (NMR) spectroscopy is one of the most powerful and versatile analytical techniques used in the field of research. It has unprecedented ability to solve chemical structures and study molecular interactions *in situ* and *in vivo* [5–7]. Traditionally, NMR has developed as two fields: solution and solid state. Both require their own instrumentation, experiments, and samples need to be either dried or dissolved for analysis. However, such sample preparation can remove important information as to the natural swollen biological state, for example, loss of conformation, water accessible interfaces, or interactions between the different phases [8]. For instance, by studying the liquid-gel interface it is possible to determine how drugs move and bind in the body, how pollutants travel and sequester in soil [9], and even look into the metabolic processes of living organisms [10,11].

From an environmental toxicology perspective, *in vivo* research is the ultimate biological goal to fully comprehend the impacts of pollutants and toxins on life. In a sense, living organisms become the ultimate biosensors responding, in real-time, to environmental stressors, with their metabolic profile providing vital information that describes how the contaminants impact the organisms at the molecular level [12–14]. However, living organisms are heterogeneous multi-phase mixtures of solutions (blood, fluids), gels (lipids, tissue) and solids (bones, shells), which require an NMR approach that can study all phases in a sample holistically. Comprehensive Multiphase NMR (CMP-NMR) is a novel technique, introduced in 2012, combining the study of solution, gel and solid state NMR techniques into one probe [15]. CMP-NMR combines the hardware from both High Resolution–Magic Angle Spinning (HR-MAS) probes (i.e. lock, magic angle, gradient and susceptibility matched stator, to observe swellable components) and solid state probes (i.e. high power RF handling for high power decoupling and cross-polarization, to study non-swellable components) [15]. CMP-NMR allows for the observation and differentiation (via editing experiments) of all phases (solids, solutions and gels) and their interactions, at the molecular level, in unaltered samples [8–11,15–22].

To date, there have been only a few studies using CMP for *in vivo* samples [10,11,16,17], and its potential to study *ex vivo* organisms has not yet been explored. While using live samples may represent the ideal biological state, there are many factors that must be considered: A) Living organisms require oxygen, thus holes need to be drilled into the rotor caps which can lead to evaporation while spinning [10,11]. This reduces the number and duration of NMR experiments that can be performed. B) Living organisms need more room as space needs to be given for them to swim around and enough water to breathe [10,11]. This in turn leads to less biomass for analysis. C) Rotors can only be spun relatively slowly (far below the optimal speed for spectroscopy) in order to keep the organisms alive [10,11,18,19] leading to the appearance of spectral side bands, reduced resolution and reduced signal-to-noise [19]. D) Organisms survive better at lower temperature [10,11] requiring strict temperature control (not available in all labs). E) Dead organisms are easier to transport between labs (less border/customs issues) in the case of international collaborations. F) *In vivo* NMR is difficult to schedule, especially if organisms are being brought in from the environment (with the goal to observe their environmental state) as they need to be run in the instrument immediately. Whereas, deceased organisms (*ex vivo*) can be frozen and stored until analysis.

Conversely, using *ex vivo* organisms can be extremely beneficial as the experiments are not constrained by the limitations described above. As a result, more in-depth analysis can be performed with high resolution and a greater range of NMR experiments. Such work would not only lead to comprehensive NMR assignments (which in many cases could be transferred to better understand the less resolved *in vivo* signals) but depending on the study, could be beneficial over *in vivo* research. For example, if the research goal is to measure real-time response of an organism to a toxin, then *in vivo* research is ideal. However, if the research is asking: "can NMR tell the difference between male and females?" or "over their life span, did organisms at site A and B show any biochemical differences?" then the more in-depth information from *ex vivo* NMR may be more informative, and arguably more appropriate if the real-time aspects of *in vivo* analysis are not essential. As a result, *ex vivo* CMP studies can be highly complementary to that of *in vivo* and warrant further investigation.

In this study, *ex vivo* CMP-NMR was used to observe *Daphnia magna* (water fleas), over a variety of life stages and generations. *Daphnia magna* are freshwater, keystone species and are model organisms for environmental toxicology studies. As they are primary consumers, they play a major role in freshwater ecosystems and are responsible for the transfer of nutrients and toxins across trophic levels [20,21]. *Daphnia* are highly susceptible to stressors and thus can provide key insight into the toxic-mode-of-action of various environmental toxins [12,14]. As they can reproduce asexually in ideal conditions [22] (i.e., lab setting), the progenies are all genetically identical to their parents. The reduction of genetic variability leads to less variations in their metabolome, allowing changes from biological processes (i.e. growth, reproduction) or responses to toxins to be more easily discerned. Furthermore, the organisms have a relatively short lifespan [23], making them easy to follow on a generational scale. As a result, *Daphnia* are one of the most commonly studied species for aquatic toxicity testing on a global scale [24], making them an ideal subject to demonstrate the applications of CMP-NMR described here.

This study introduces *ex vivo* CMP-NMR, demonstrates ^{1}H and ^{13}C spectral editing experiments for phase discrimination, improves on *Daphnia* CMP-NMR metabolite assignments, and compares the biological differences in *Daphnia* at various life stages and across generations.

2. Materials and methods

2.1. Daphnia culturing

Sample preparation was followed using methods described by Simpson et al., [25]. *Daphnia magna* were obtained from Ward's Science Canada (St. Catherines, ON, Canada), but have been cultured in house for the past 8 years following guidelines set by Environment Canada. The *Daphnia* were cultured at 20 ± 1 °C in 4 L glass vessels filled with hard reconstituted water at a pH of 7.5–8.5 (tap-water dechlorinated using API tap-water conditioning agent for a period of 2 weeks) under a 16:8 h light to dark photoperiod. The *Daphnia* were fed a steady diet of only ^{13}C-enriched *Chlamydomonas reinhardtii* (Silantes, München, Germany) three times a week. The population density was 25 daphnids per liter of culture medium. Each week, ~50% of the culture medium was changed. *Daphnia magna* were separated by age and generation to elucidate any differences due to ^{13}C labeling. To ensure all signals observed in the NMR data were from the *Daphnia* biomass itself, rather than the algae in the guts of the organisms, the *Daphnia magna* were fed unlabelled *C. reinhardtii* for 24 h prior to the experiments to purge the gut contents of ^{13}C-labelled media. Organisms were freeze-dried and re-swollen with D_2O. ~20 mg of sample for each age and generation were collected for all NMR experiments.

2.2. NMR spectroscopy

The following NMR experiments were conducted using

protocols similar to McGill et al. and are standard for most multiphase NMR experiments [17]. All NMR experiments were performed using a Bruker Avance III 500 MHz ^{1}H spectrometer, equipped with a prototype CMP-MAS 4 mm ^{1}H-^{13}C-^{2}H probe (Bruker Biospin, Billerica, MA) with an actively shielded Z-gradient. All experiments were locked on D_2O (including solid state experiments) and maintained at 298 K. Samples were spun at a spinrate of 6666 Hz (which results in a rotor period of 150 μs) thus easily permitting rotor synchronization of pulses and delays. This is fast enough to position ^{1}H sidebands 500 MHz outside the spectral window. As this was an *ex vivo* multiphase study using deceased organisms, a faster spinning speed was used to obtain optimal NMR results. For perspective, living *Daphnia* can be spun at 2500 Hz at 5 °C, and will survive for ~4 h, however, to maintain them for 24 h they must be spun at 50 Hz [10].

To remove ^{1}H−^{13}C coupling from the labelled samples, decoupling was used in both one-dimensional (1D) and two-dimensional (2D) experiments. For the liquid/gel-state experiments, GARP-4 was used for the ^{1}H observed experiments, whereas a low-power WALTZ16 decoupling was used for ^{13}C observed experiments. The solid state cross-polarization magic angle spinning (CP-MAS) experiments used high-power SPINAL64 decoupling.

2.2.1. 1D NMR spectroscopy

All ^{1}H spectra were obtained using presaturation for water suppression and the 90° excitation pulse was calibrated for each sample. A standard inversion recovery approach was used to measure the T_1 time for each sample, and the recycle delay was set to 5 times the measured T_1 value. Due to the fast relaxation in the fully ^{13}C enriched sample, this equated to ~2.9 s between scans. Each experiment was recorded with a spectral width of 20 ppm, 16384 time-domain points and 1024 scans. All spectra were Fourier transformed and processed with a line broadening equivalent to 1 Hz (10 Hz for diffusion edited data).

All ^{13}C spectra (except for CP) were acquired using a spectral width of 400 ppm, 16384 time-domain points, 3072 scans and inverse gated ^{1}H decoupling. The recycle delay was set to 5 times the measured T_1 value (~3.1s between scans due to fast relaxation in the ^{13}C enriched samples). Spectra were processed using an exponential function corresponding to a line broadening of 1.5 Hz for ^{13}C inverse gated spectra and 25 Hz for diffusion edited spectra. CP-MAS was used to observe the more solid/rigid phase, using a recycle delay of 1 s, a spectral width of 300 ppm, 1024 time-domain points, 4096 scans, a contact time of 1 ms and a line broadening corresponding to 25 Hz.

2.2.2. Spectral editing and scaling

^{1}H and ^{13}C diffusion editing were acquired using a bipolar pulse pair longitudinal encode-decode (BPPLED) sequence. Scans were collected using encoding/decoding gradients of 1.8 ms at ~50 G cm^{-1} and a diffusion time of 180 ms. CPMG (Carr−Purcell−Meiboom−Gill) filtering was achieved using a total delay of 120 ms, with the exception of T_2-filtered CP-MAS which used 2 echoes separated by 15 μs prior to cross-polarization [15]. Inverse diffusion editing (IDE), Relaxation Recovery Arising from Diffusion Editing (RADE) and inverse T_2-filtered ^{13}C CP-MAS was done by weighted spectral subtraction as previously described [15] and will be discussed in more detail within this manuscript.

2.2.3. 2D NMR spectroscopy

^{1}H−^{13}C heteronuclear single-quantum coherence (HSQC) correlation experiments were collected in phase sensitive mode using Echo/Antiecho encoding and gradients for coherence selection. 128 scans were collected for each of the 128 increments in the F1 dimension. 2048 time-domain points were recorded in the F2 dimension with a ^{1}J ^{1}H-^{13}C of 145 Hz. The F2 dimension was processed using an exponential function corresponding to a line broadening of 8 Hz and F1 using a sine-squared function with a π/2 phase shift and a zero-filling factor of 2. 2D correlation spectroscopy (COSY) spectra were also acquired to confirm HSQC NMR assignments. The COSY NMR experiments were done in non phase-sensitive mode, using gradients for coherence selection. 2048 time-domain points and 128 scans were recorded for each of the 256 increments in the F1 dimension. Both dimensions were processed using an unshifted sine-squared function and zero-filling factor of 2. Magnitude mode was used for projection.

2.3. Compound identification

Assignments were performed using the Bioreference databases, versions 2-0-0 to 2-0-5 and AMIX (Analysis of MIXtures software package, version 3.9.15, Bruker BioSpin, Rheinstetten, Germany). The Bioreference Compound Databases in AMIX were used to calibrate 1D and 2D spectra using known metabolites, and identify key metabolites using a procedure previously developed for complex mixtures [26]. Only assignments that exhibited an R^2 (Fig. S6 in the supporting information) correlation >0.99 between observed and database shifts were retained as assignments. COSY was used to confirm assigned metabolites in the HSQC wherever possible. Further assignments were cross referenced against the literature (see Table S1 in the supporting information).

3. Results and discussion

3.1. Spectral editing

The principle behind CMP-NMR is to study multiphase samples in their intact state. As such, applying spectral editing approaches to identify which components are in the liquid, gel and solid state is desirable and provides important information as to the physical organization of components within complex samples [8]. Spectral editing was introduced with the first CMP-NMR probe [15] in 2012, and has been covered in a range of publications since [8−11,15−22]. However, due to its significance to CMP-NMR, it is important to revisit it here briefly in the context of *ex vivo* intact organisms. Spectral editing can be applied to both ^{1}H and ^{13}C. With samples at natural abundance, ^{1}H is most efficient for editing solutions, gels and semi-solids due to the increased sensitivity of the ^{1}H nucleus, while ^{13}C CP-MAS is used for the study of true solids due to the lack of resolution in ^{1}H detected solids [15,27,28]. However, in this study, as the organisms are ^{13}C enriched, it provides a rare opportunity to also apply the full suite (liquids, gels, semi-solids, solids) of ^{13}C detected editing experiments.

3.1.1. Spectral editing using ^{13}C

To demonstrate the spectral editing approaches, 3-week-old *D. magna* were used from the F_3 generation as they are a good representative of complete ^{13}C enriched *D. magna* in general (refer to section 3.3.4, Fig. 6). Fig. 1a shows the conventional carbon spectrum collected with low power ^{1}H decoupling. In general low power decoupling covers the standard ^{1}H range encountered for liquid samples [29] but decoupling efficiency typically falls off rapidly outside this bandwidth. As such, low power decoupling will be efficient for structures that have been narrowed to some extent via molecular motion induced by the water in the organisms (liquids, gels, and swollen solids) but will be inefficient for true solids where proton lineshape can be 100's of KHz wide due to strong ^{1}H-^{1}H dipolar interactions [30]. As such, Fig. 1a can be thought of as spectrum where all swollen components are well represented, but where the solids components are somewhat suppressed. As true

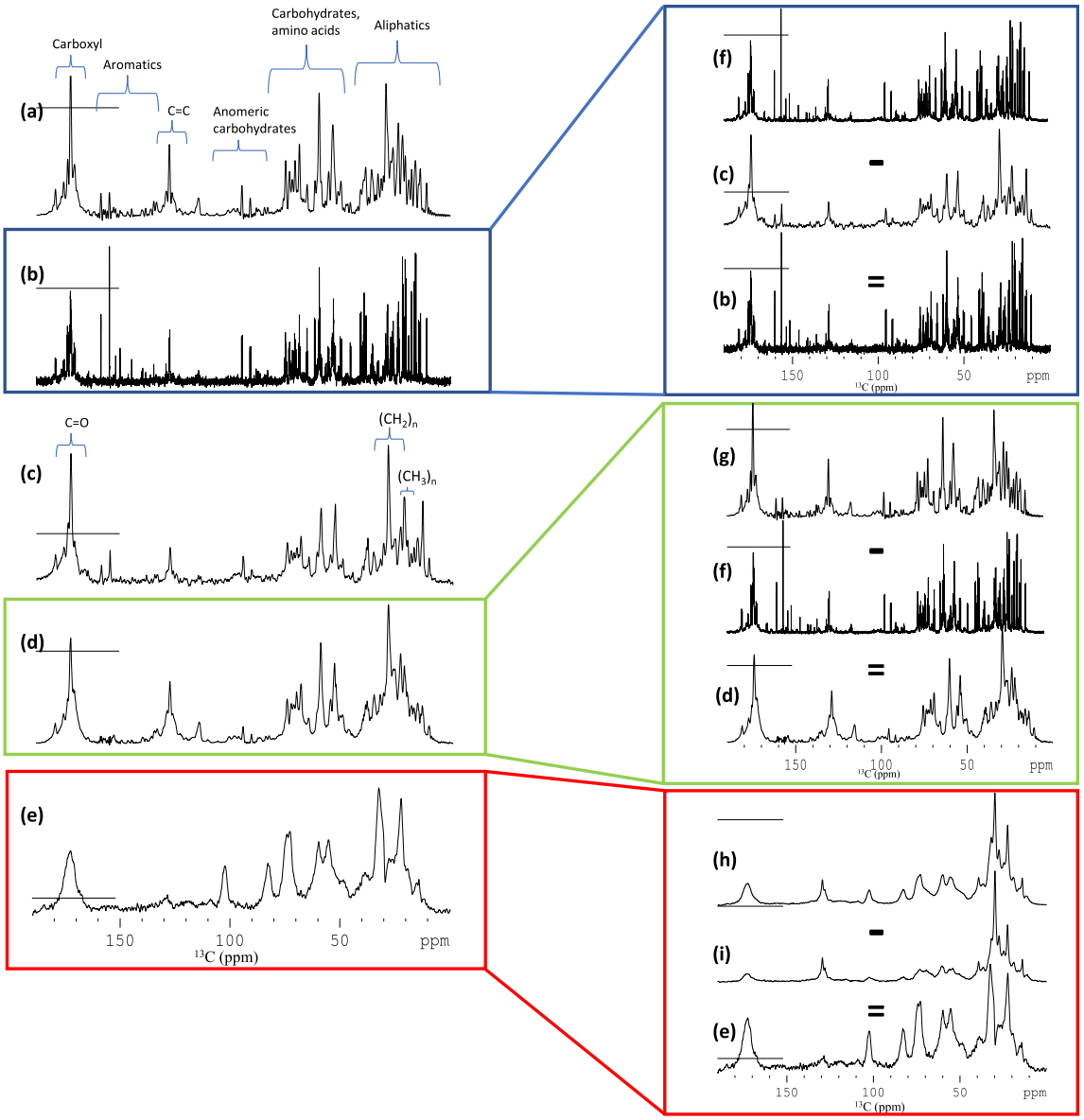


Fig. 1. 1D ^{13}C spectra of ^{13}C-labelled, F_3 - 3-week-old freeze-dried *Daphnia magna*. (a) ^{13}C NMR with low power decoupling, representing all components with the exception of solids that are partially suppressed (see section 3.1), (b) ^{13}C NMR of the truly dissolved component (mainly metabolites), termed inverse diffusion edited (IDE), created via subtraction of: (c) a conventional diffusion edited (DE) ^{13}C spectrum (i.e. molecules with restricted diffusion) from (f) DE sequence but with the diffusion gradient turned off. (d) ^{13}C semi-solid components, acquired using Relaxation Recovery Arising from Diffusion Editing (RADE), obtained by subtraction of (f) with no diffusion gradient but delays set, from (g) both gradient and delays set to zero. (e) ^{13}C NMR of the rigid solid components, obtained using "Inverse" ^{1}H T_2 – filtered ^{13}C CP-MAS, acquired from the subtraction of: (i) T_2-filtered CP-MAS, showcasing the dynamic solids, from (h) standard CP-MAS which shows both rigid and dynamic solids.

solids are specifically selected later (see section 3.1.5) using cross-polarization, and the goal is to provide clean separation of each of the phases, the partial suppression of true solids during the separation of the more mobile components is advantageous. Readers interested in absolute quantification of all components (liquids, gel, semi-solids and true solids) in a single spectrum should apply the stepped decoupling approach recently introduced by Ning et al. [28]. Spectral editing can be based on either relaxation or diffusion [28] however diffusion tends to give the cleanest, and most meaningful, separation of the phases [10] and will be employed here.

3.1.2. *Isolating solution/freely diffusing (dissolved) compounds via ^{13}C NMR*

Fig. 1b shows the inverse diffusion edited (IDE spectrum) which contains signals from only species freely moving in space (i.e. dissolved components). Diffusion editing (DE) encodes the position of molecules in a sample at the start of the experiment, and after a diffusion time, decodes them. Species that do not move during the diffusion time are recovered, whereas molecules that move positions are not fully decoded and their signals are attenuated [15,31]. The blue expansion from Fig. 1b shows how the IDE spectrum is created. Spectrum 1f is a spectrum using a BPPLED (reference spectrum) diffusion sequence with all the delays set for diffusion editing, but with the encoding/decoding gradient turned off. The result is that both freely diffusing components as well as those with restricted diffusion are detected, and this spectrum act as a reference. Fig. 1c is identical to Fig. 1f with the exception that the encoding/decoding gradient is turned on, and molecules that move are strongly attenuated. The IDE is created via difference by

subtracting Fig. 1c from 1f such that only molecules that have the ability to move within the sample (i.e. soluble components) are selected. Fig. 1b contains a range of sharp lines consistent with a wide range of metabolites. More detailed assignments will be performed later in this paper using 2D NMR (see section 3.2, Figs. 2 and 3).

3.1.3. Isolating components with restricted diffusion (gel-like) compounds via ^{13}C NMR

Selecting components with the restricted diffusion is simply achieved by applying diffusion editing as described above. Diffusion editing attenuates the signals from mobile molecules and retains signal from those components that do not move position in the sample. Restricted diffusion is defined here as molecules that move less than ~1 μm during the diffusion time of 180 ms [17]. Very generally, these components are termed gels, but could include very large macromolecules with very slow diffusion, or bound components as well as swollen gel-like materials. Fig. 1c shows the DE spectrum and has strong signals from lipids, likely in part from lipids in vesicles and micelles where their diffusion is suppressed via molecular associations and physical compartmentalization.

In a simple world, the combination of IDE and DE would differentiate all swollen components within a sample. However, this is not the case as relaxation can occur during the relatively long delays required for diffusion measurement. If these components are not accounted for, it is possible they could be missed. Luckily these components that are loosely termed "rigid gels/semi-solids" can be recovered using an experiment termed, Relaxation Recovery Arising from Diffusion Editing (RADE).

3.1.4. Isolating rigid gels/semi-solids compounds via ^{13}C NMR

RADE accounts for signals that are lost via relaxation when diffusion editing approaches are applied. In this case Fig. 1g is used as a reference spectrum (diffusion delays set to zero and diffusion gradients turned off) which is essentially identical to Fig. 1a (contains signals from all swollen components) but is based on a BPPLED sequence thus making it better suited for subtraction.

Fig. 1f is a diffusion sequence but collected with the delays set correctly for diffusion editing but with the encoding/decoding gradient turned off (i.e. no diffusion filter applied). The difference between 1g and 1f is the components that are lost via relaxation during the diffusion delays. Due to their fast relaxation, these components tend to be the broadest and can be detected by conventional ^{1}H NMR (i.e. without resorting to multi-pulse narrowing techniques such as CRAMPS [32]) and are best considered as rigid-gels/semi-solids (Fig. 1d). The spectra have strong contributions from lipids and carbohydrates sitting on a background from protein similar to the observations from CMP-NMR on living organisms [11]. Such components may include partially swollen materials, swollen cell walls, or swollen structural components.

3.1.5. Isolating solid components via ^{13}C NMR

While ^{1}H detection of solids is possible using techniques such as CRAMPS [32] the ^{1}H-^{1}H dipoles lead to broad lineshape and loss of the majority of chemical shift information in complex samples [15]. As such, ^{13}C detection via cross-polarization is much more informative and provides an abundance of chemical shift information. In cross-polarization, magnetization from ^{1}H can be passed via spatial dipolar interactions to ^{13}C. Cross-polarization is extremely inefficient in solution and dynamic gels [15] as molecular motion modulates the dipolar interactions, but is highly efficient for true solids. As such, cross-polarization acts as a filter that selects the most solid-like components in a mixture.

Fig. 1h shows a standard cross-polarization experiment. The result contains signals from the true solids as well as those from semi solids/rigid gels which also cross-polarize with reasonable efficiency [15]. The semi-solids can be selected by applying a very short (in this case 2 × 15 μs, CPMG echo on the ^{1}H channel prior to cross-polarization). The result is that ^{1}Hs in true solids are attenuated due their very fast relaxation, while signals from dynamic solids persist. Fig. 1i shows the dynamic solids that include signals from lipids (likely membrane or cell walls). The true solids (Fig. 1e) can be recovered via difference (Fig. 1h and i) and are dominated by carbohydrates and protein consistent with chitin which forms much of the structural framework of the organisms [11,33,34]. When combined, the editing approaches do not miss any components present in natural samples, but the CP-MAS and RADE approaches do tend to detect the rigid-gels and semi-solids components twice. In summary, moving down the left column of Fig. 1b-e, the components are edited in a "rigidity" gradient moving from true solutions (1b) at one extreme to true solids (1e) at the other.

3.1.6. ^{1}H NMR spectra of the isolated phases

^{1}H NMR is more commonly used for spectral editing as there is a high abundance of ^{1}H and is more sensitive relative to ^{13}C in samples of natural abundance. As a result, less sample is required, and experimental times are much shorter (few minutes). The main advantage of ^{13}C is the larger chemicals shift range that improves signals dispersion [35]. In the same manner as mentioned above with the ^{13}C NMR experiments, the various phases of *D. magna* may be isolated using ^{1}H NMR. For completeness, the spectral editing approach using ^{1}H NMR is shown in Fig. S1 of the supporting information.

3.2. Spectral assignments

Thus far, this paper has focused on the use of 1D NMR spectroscopy to help differentiate the phases within an *ex vivo* sample. This can be very useful for following, for example, how lipid changes with age or how the metabolite pool changes with environmental stress. However, due to the relatively low spectral dispersion in 1D NMR, directly identifying compounds in complex natural samples can be challenging. For example, Hertkorn reports the relative "peak capacity" or resolving power of 1D ^{1}H NMR to be around 3000 peaks whereas 1D ^{13}C NMR is around 30,000 [35]. However, this increases significantly to around 2,000,000 for 2D ^{1}H-^{13}C NMR [35]. In addition, a simple ^{1}H-^{13}C correlation (for example Heteronuclear Single Quantum Coherence, HSQC) provides additional connectivity information identifying which ^{1}H and ^{13}C nuclei are directly bonded. As such, when attempting to identify specific compounds *ex vivo*, 2D NMR is far superior to 1D NMR spectroscopy and allows a wider range of compounds, including ones at low concentration that may be masked by larger peaks in 1D, to be identified.

Fig. 2a shows an example HSQC with the major structural categories labelled. In HSQC, the X axis describes the ^{1}H chemical shift in an H-C fragment and the Y axis describes the corresponding ^{13}C chemical shift. A cross peak occurs only if the H and C are directly bonded to each other. In many ways the HSQC can be thought of as a high-resolution H-C fingerprint of a mixture. HSQC correlates the H to the C, and when a ^{1}J CH coupling constant in the 140–145 Hz range (represents an average value across all molecules and is by far the most common setting used) is used all compounds are well represented. As such HSQC is highly reproducible and thus ideal for database matching to identify compounds. ^{1}H-^{1}H COSY is highly complementary to HSQC and provides connectivity information between adjacent protons in a molecule. Unlike other ^{1}H-^{1}H correlations, such as TOCSY, which employs a user defined mixing time, which in turn impacts the number and type of cross peaks

seen, there are no adjustable parameters in COSY. This means the results from COSY (as with HSQC) are largely independent of experimental setup making them ideal for database matching.

Fig. 2b shows the ^{1}H-^{1}H COSY with matches against version 2-0-0 to 2-0-5 of the Bruker Bio-reference databases labelled. Similarly, Fig. 3 shows various expansions of the ^{1}H-^{13}C HSQC data with assignments of the major compounds present. The matching protocols are described in the experimental section (see section 2.3). The role of each compound is briefly described in Table S1 in the supporting information, as well as, references to other studies that identified the same compounds by NMR or other methods in freshwater crustaceans. In turn, where possible, assignments from the 2D NMR were transferred over to the corresponding 1D ^{13}C and ^{1}H spectra, which can be found in the supporting information (see Figs. S2 and S3). Due to the reduced resolution, it is not possible to offer complete assignments in 1D NMR, however, as 1D NMR is commonly acquired, adding assignments where possible could prove useful to future researchers studying *Daphnia* by NMR. As the ^{1}H spectrum is less resolved in comparison to ^{13}C NMR, only more general assignments can be provided for the ^{1}H data.

3.2.1. Advantages of ex vivo over in vivo

Before moving onto the biological differences detected by CMP-NMR, it is worth discussing the differences between *ex vivo* over *in vivo* CMP-NMR. While *in vivo* NMR is the obvious choice for studying real-time responses and the most biologically active state, when such information is not critical to the study, *ex vivo* NMR can offer some distinct advantages that can be summarized as:

A) **Increased Resolution and Signal Intensity:** The resolution from *ex vivo* under MAS is superior to that obtained *in vivo* (both under MAS [10,11,18] and static *in vivo* systems [5,6,12–14,36–38]) in large part due to the ability to spin faster. Further, *ex vivo* experiments can be run for much

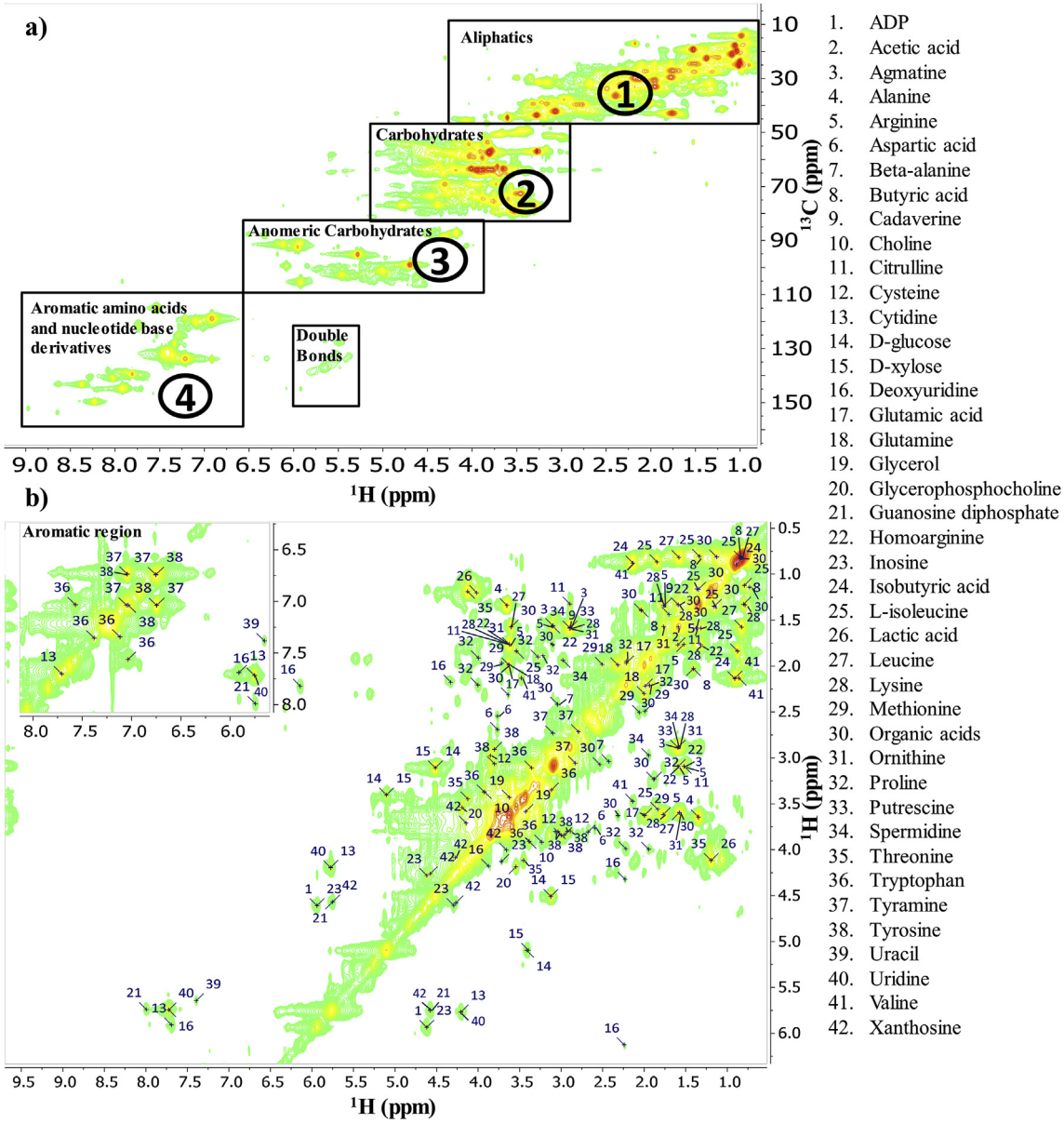
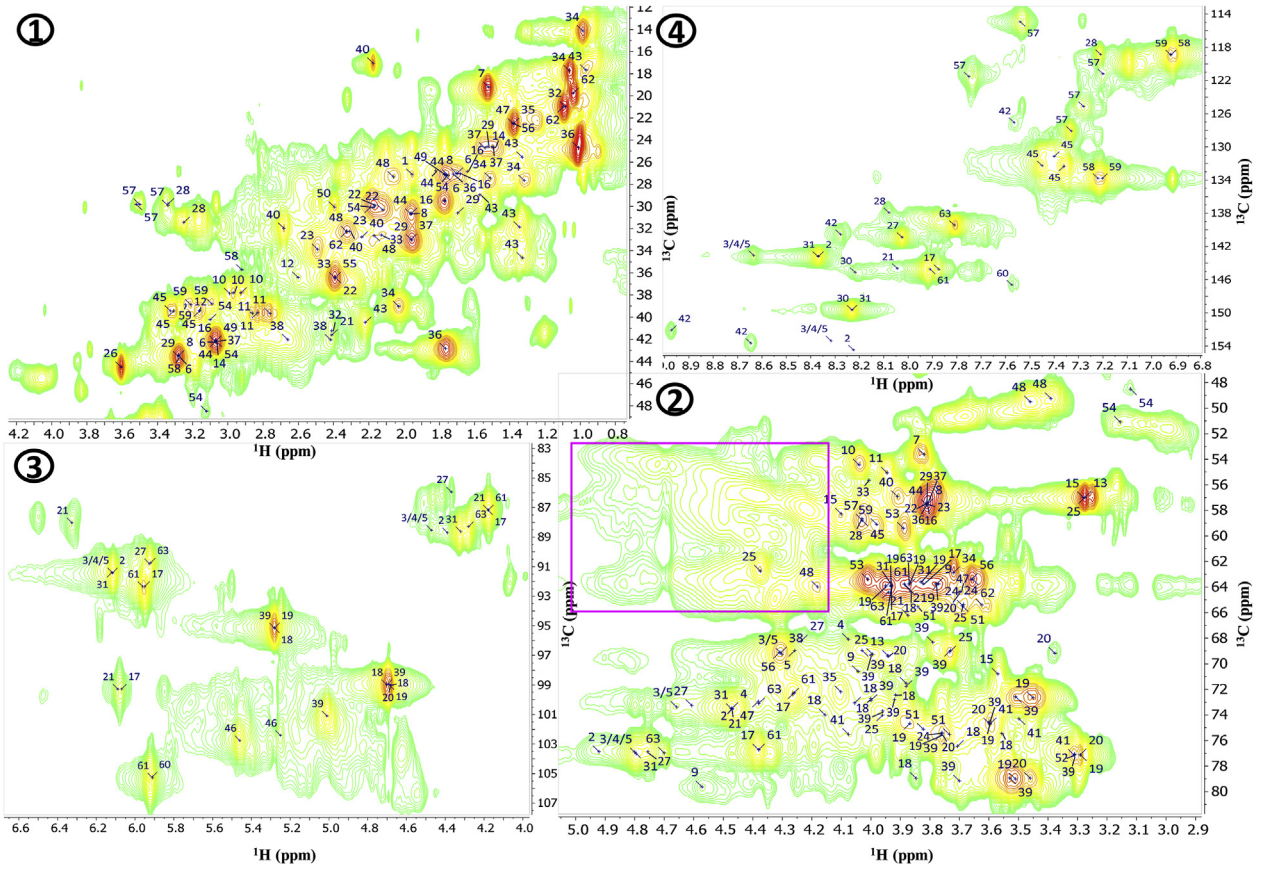


Fig. 2. 2D NMR spectra of ^{13}C labelled F_3-3-week-old *Daphnia magna*. (a): 2D (^{1}H-^{13}C) HSQC with major structural categories labelled, and (b): (^{1}H-^{1}H) COSY with metabolite assignments using Bruker's Bioreference Databases. Lipids are present mainly in the form of triacylglycerides see Fig. S7.



1. Acetic acid
2. Adenosine
3. Adenosine-5-diphosphate (ADP)
4. Adenosine-5-monophosphate (AMP)
5. Adenosine-5-triphosphate (ATP)
6. Agmatine
7. Alanine
8. Arginine
9. Ascorbic acid
10. Asparagine
11. Aspartic acid
12. Beta-alanine
13. Betaine
14. Cadaverine
15. Choline
16. Citrulline
17. Cytidine
18. D-galactose
19. D-glucose
20. D-xylose
21. Deoxycytidine monophosphate (dCMP)
22. Glutamic acid
23. Glutamine
24. Glycerol
25. Glycerophosphocholine
26. Glycine
27. Guanosine diphosphate (GDP)
28. Histidine
29. Homoarginine
30. Hypoxanthine
31. Inosine
32. Isobutyric acid
33. Isoglutamine
34. L-isoleucine
35. Lactic acid
36. Leucine
37. Lysine
38. Malic acid
39. Melibiose
40. Methionine
41. Myo-inositol
42. Niacin
43. Organic acids
44. Ornithine
45. Phenylalanine
46. Phosphoenolpyruvate
47. Phosphothreonine
48. Proline
49. Putrescine
50. Pyruvic acid
51. Ribitol
52. Scyllo-inositol
53. Serine
54. Spermidine
55. Succinate
56. Threonine
57. Tryptophan
58. Tyramine
59. Tyrosine
60. Uracil
61. Uridine
62. Valine
63. Xanthosine

Fig. 3. 2D (^{1}H-^{13}C) HSQC ^{13}C-labelled F_3-3-week *Daphnia magna*. Regions of the HSQC are enlarged to visualize the assigned metabolites, where (1) are the aliphatics, (2) are the carbohydrates, (3) are the anomeric carbohydrates and (4) are the aromatics. The magenta box highlights where α-H-C units in proteins resonate. The metabolites were assigned using Bruker's Bioreference Databases.

longer (i.e. the time available to collect data is not limited by a relatively short window over which the organisms can survive) leading to increased resolution in the indirect dimension (i.e. more slices increases resolution) as well as improved detection limits (more scans can be used), in addition to a wider range of NMR experiments that can be employed. Figs. S4a and S4b in the supporting information compares ^{13}C and ^{1}H spectrum collected *ex vivo* under MAS to that of collected in a flow system *in vivo*. Note flow systems are commonly used for toxicity based in-vivo studies [12,39–41]. The increase in resolution, and thus, the information content of the *ex vivo* spectrum, is impressive and clearly shows how *ex vivo* NMR is complementary to *in vivo*. However, it is worth noting, that if only the metabolic profile is of interest, this can be obtained by simply crushing the dead organisms and collecting solution state NMR (see Fig. S5 and associated text) although information from the gel and solid phase cannot be accessed using this approach.

B) **Complications from an intense water signals are reduced:** For *in vivo* studies, organisms have to be maintained in pure water, this leads to an intense and broad signal. While it can be suppressed at faster spinning rates, suppression at slow spinning speeds required for *in vivo* becomes very challenging [5]. For example, 2500 Hz is the fastest spin rate that can be performed while keeping freshwater shrimp alive [11], and *Daphnia* can only survive spinning at 50 Hz for 24 h [10] leading to significant artifacts in 2D ^{1}H-^{13}C NMR at such slow spin rates. However, here the *ex vivo* samples are spun

at 6666 Hz improving lineshape and moving spectral sidebands (artifacts) outside of the spectral window of interest. Furthermore, if freeze-dried organisms are used (as is the case here) they can be re-swollen using D_2O which further reduces complications from a large H_2O signal.

C) **Reduction of Lipid Signals:** *In vivo* lipids signal dominate the spectral profile in both the 1D and 2D NMR, masking many other key signals such as amino acids, carbohydrates, etc. Due to the faster spinning *ex vivo,* the *Daphnia* do not remain intact and rupture from the faster spinning speed. While on one hand this could be argued as detrimental, the advantage is that the lipids float to top of the rotor above the coil region, and the lipids signals are greatly reduced. As such, *ex vivo* NMR is complementary as signals hidden below the lipids are much easier to discern. Supporting Fig. S4b shows this clearly by comparing an *in vivo* solution state NMR spectrum of *Daphnia*, dominated largely by lipids, with an *ex vivo* MAS NMR that shows a wider range of components, such as amino and carbohydrates, that were previously hidden under the lipids.

D) **Increased Biomass:** In a recent flow-based study, it was only possible to maintain ~10-15 medium size *Daphnia in vivo* [18] in a 5 mm flow cell for 2D NMR. If we consider an adult *Daphnia magna* contains ~100 μg of dry biomass only ~1–1.5 mg of biomass is present. However, in this study (*ex vivo*) it was possible to fit up to 20 mg of biomass and still provide ample room for the swelling solvent (D_2O). As such, up to 20 times the biomass can be used *ex vivo,* which converts into saving 400X the time in the NMR (i.e. an experiment which took 400 min *in vivo* would provide the same SNR in 1 min with 20 times the biomass *ex vivo*). In summary, *ex vivo* NMR should prove complementary to *in vivo* NMR by providing more detailed assignments in a more accessible manner. In turn, this should help provide a better understanding of the signals *in vivo*, which while broader and more challenging to discern, provide access to the most biologically relevant state. However, while *in vivo* NMR is arguably unrivaled in providing information on real-time stress responses, *ex vivo* NMR is still useful for studying compositional and structural changes in organisms, for example, with age, over different generations or with pregnancy.

E) **Other considerations:** Organisms survive far longer at lower temperatures ~5 °C as their respiration is reduced [10,11]. However, this requires strict temperature control (not available in all labs) and must be converted from nitrogen (used in many NMR labs) to very low dew point air (to protect the equipment), so that organisms can breathe. Moreover, deceased organisms are much easier to transport between labs especially when this involves international borders. *In vivo* NMR experiments are harder to schedule, especially if organisms are being brought in from the environment. In such studies, the goal is often to capture the natural state of the organisms as they would have been in the environment. The only way to achieve this is to run the organisms on arrival before they become climatized to lab conditions. Conversely, *ex vivo* organisms can be frozen and/or freeze-dried and then stored until analysis.

3.3. Biological significance

Sections 3.1 and 3.2 of this manuscript focused on the spectral editing approaches and detailed assignments that complement *in vivo* studies. This section demonstrates how the approaches can be applied to understand biological changes in *Daphnia* over, for example, different life stages and across generations. It should also be noted that the guts of the *Daphnia* were purged with ^{12}C algae prior to freeze-drying as previously described [25]. As the ^{12}C algae is below NMR detection limits [25] using ^{13}C and ^{1}H-^{13}C HSQC detection, only the ^{13}C enriched biomass of the organisms themselves, and not the residual food in their stomachs is detected. Each subsection will deal with different aspects of the growth and development of *D. magna* to demonstrate the application of *ex vivo* CMP-NMR for whole organism analysis.

3.3.1. Studies of Daphnia magna at different life stages

Daphnia magna become sexually mature after 5–10 days following birth, and can reproduce asexually (parthenogenesis) in ideal environmental conditions [22]. Pregnant Daphnids carry their eggs on their backs within their brood chamber, where a single female may have as many as 100 neonates within one clutch [22]. The neonates are considered to be juvenile after they have undergone two molting periods (1-week-old). Following two more molting periods, the juvenile *Daphnia* become sexually mature [42]. *Daphnia* have an average lifespan of 40–56 days (in laboratory settings) [23] and can reproduce until death.

Fig. 4 shows an overview of the spectral editing for ^{13}C spectra of *Daphnia* at different life stages. To simplify things, only 3 categories of spectra are shown; 1) the low power DE ^{13}C spectrum with the delays set but diffusion gradient turned off (refer to Fig. 1f earlier for details) which provides an overview of the dynamic components (liquids and gels), 2) RADE emphasizing the semi-solids and 3) inverse-T_2 CP-MAS highlighting the true solid components. Specific changes seen in the aromatic region (red boxes) will be discussed later in combination with the 2D spectra. In the dynamic components (Fig. 4) the most interesting observation is the highlighted $(CH_2)_n$ peak corresponding to the lipid signals.

Lipids are key moieties required for cell structure, growth, reproduction and energy [43,44]. Like many crustaceans, *D. magna* do not have the ability to synthesize many of their own lipids and must obtain them through dietary means [45,46]. In the dynamic components, the lipids are highest in the pregnant *Daphnia* and lowest in the juveniles. The same trend is true for the semi-solids. However, the lipids are significantly depleted in the true solids in pregnant organisms. When considered together, this is consistent with the mothers depleting their own energy reserves (true solids) and transferring them into the lipid sacs that make up a large proportion of *Daphnia* eggs [47]. These energy reserves are used to help the neonates grow during their early growth when they are not yet large enough to efficiently uptake external food from algae [45,47]. As the organisms age, it can be seen that the *Daphnia* store additional lipids in a more solid form, supported by the increase in the peak at ~33 ppm corresponding to rigid $(CH_2)_n$ groups [48], in the 3-week-old Daphnids (Fig. 4k). A decrease in this peak in the 5-week-old organisms (Fig. 4l (note: 5 weeks is the end of a Daphnid's life cycle)) suggests that the lipid stores are used up in the ageing process alongside reproduction and is consistent with studies in aging adult *D. magna* that showed lower contents of monounsaturated fatty acids [49].

Unsaturated lipids are the most challenging for *Daphnia* to synthesize, as such it is logical that the lipids enriched by the mothers are highly unsaturated. For example, see the ratio between the C=O and C=C in Fig. 4e and f (see dashed blue line) showcasing the high C=C content, consistent with the abundance of unsaturated lipids within the pregnant mothers, which are essential to the early growth of her offspring [45,46]. In turn, after one week of growth (see juvenile Fig. 4b and f), the lipids in both the dynamic and semi-solids fractions are largely depleted in the juveniles, consistent with the organisms using up these more accessible unsaturated-rich lipid reserves during their first week. To a lesser extent carbohydrates, which are also primary energy stores and are

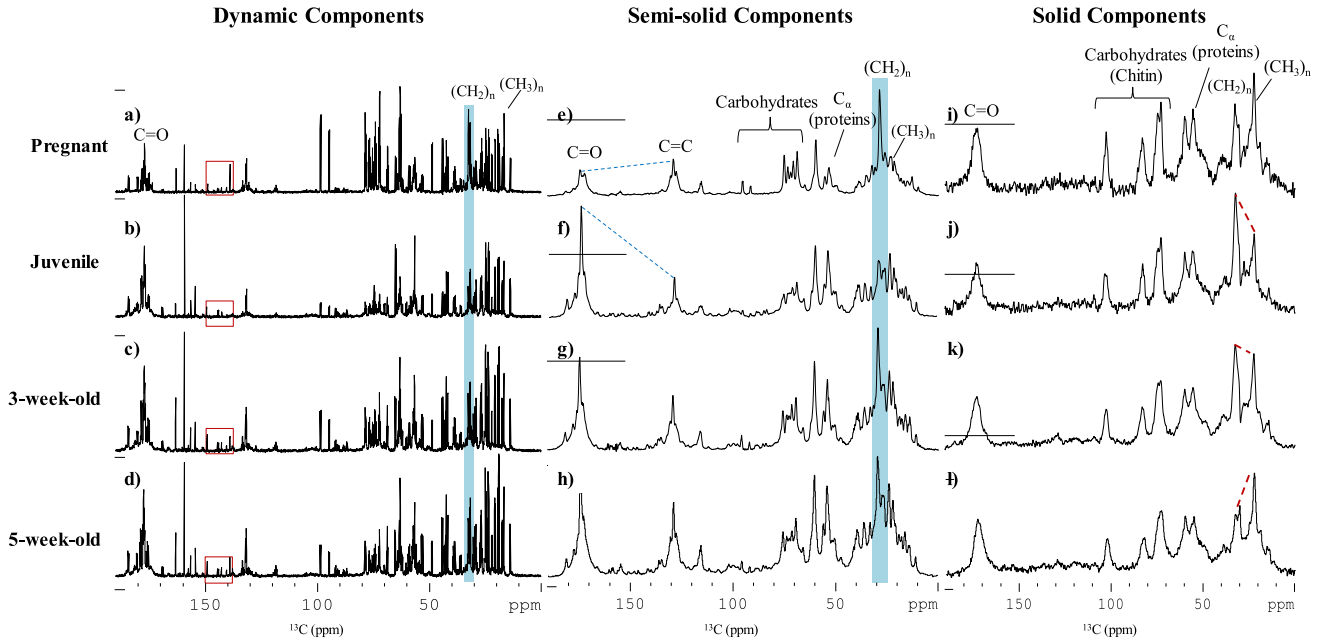


Fig. 4. ^{13}C spectra of ^{13}C labelled *Daphnia magna* that are Pregnant, juvenile (1-week-old), 3-week-old adult and 5-week-old adult *D. magna* (rows). (a-d): Dynamic components (Low power ^{13}C NMR with semisolids removed; see Fig. 1f for reference); (e-h): Semi-solids (^{13}C RADE); and (i-l): True solids (Inverse T_2-filtered CP-MAS). The red box in the dynamic components highlights changes in the aromatic region (refer to text, section 3.3.3). The blue dashed lines (e–f) showcases the ratio between the C=C and C=O bonds in the semi-solid components, where C=C arises from unsaturated lipids within the *Daphnia*. The red dashed lines (j–l) highlights the CH_3:CH_2 ratio in the true solids, whereby, the CH_3 signals are dominated by protein structures (in the exoskeleton) and CH_2 lipid chains. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

metabolized often before lipids [50,51], are also slightly depleted in the juveniles.

Interestingly, the contribution of lipids to the true solids component in the juveniles is considerable. There are two possible explanations for this: 1) The juveniles store some lipids in a solid form likely as a future energy reserve. The fact that the mothers deplete their solid lipid stores during pregnancy (see above) supports the Daphnids' ability to transfer lipids from a stored solid form into a more readily accessible mobile form. 2) The lipids appear prominent in comparison to other solid components (mainly chitin in exoskeleton [33,34,42] (protein/carbohydrate complex)) as the exoskeleton in the juveniles is still developing, making the lipids stand out on a relative basis.

3.3.2. Studies of aging in D. magna

Morphologically, young *Daphnia* are similar to adults [22], and for the most part, there are few significant changes with aging in adult *Daphnia* (except for the metabolism of lipids as noted above) making them ideal model organisms for laboratory studies. Fig. 4 depicts changes seen in *Daphnia* during the aging process, comparing Daphnids at 1-, 3- and 5- weeks of age. The spectra of the 3-week and 5-week old adults (dynamic (4c and 4d) and semi-solid (4g and 4h) components) are extremely similar, demonstrating that the biochemistry in adults, as well as, the acquisition of the CMP-NMR data are both highly reproducible. The largest difference can be seen in the true solids. While a small fraction of the rigid CH_3 can come from lipids, a large portion arises from protein in the chitin complex [11,52] and that comprises the majority of the exoskeleton. The increase in rigid protein in the 3-week and 5-week-old adults over the juveniles (see red dashed lines in Fig. 4j, k, l, highlighting the CH_3:CH_2 ratio) is consistent with the exoskeleton and shell thickening with age [53].

3.3.3. 2D NMR

2D NMR provides additional information to support the findings from spectral editing. Fig. 5a shows the carbon spectra (dynamic components) along with a corresponding expansion of the aromatic region of in both ^{13}C and 2D ^{1}H-^{13}C 2D NMR. The aromatic region is of particular interest as it contains key nucleic acids that are linked to reproduction. Xanthosine levels are seen to vary across the life stages, where the highest levels are observed in the pregnant *Daphnia*, and are almost depleted in the juveniles. Xanthosine, alongside hypoxanthine and inosine, is a precursor to purine DNA nucleotides [54]. Inosine, a nucleoside commonly found in tRNA, is also a precursor for hypoxanthine [55]. Furthermore, these metabolites are constituents of the purine salvage pathway in which purine nucleotides are catabolized into smaller precursors, and in time of need, are then cycled back to generate more nucleotides. There has been very little study of the purine salvage pathway outside of animals [56], plants [57,58], and bacteria [54,56], however this pathway is thought to exist in *Daphnia pulex* [59] (according to the KEGG: Kyoto Encyclopedia of Genes and Genomes Pathway Database), a close relative of *Daphnia magna,* implying its likely occurrence in *D. magna*. Pregnant *Daphnia* transfer RNA, amongst other nutrients, to the eggs such that the offspring have a higher chance of survival and development [42]. It seems likely that the mothers are concentrating xanthosine within the eggs, which later the juveniles will convert into the required nucleosides. This is consistent with the xanthosine levels decreasing in the juveniles as other nucleotides, in the 144–146 ppm window (mainly cytidine and uridine) in the ^{13}C NMR spectrum, increase. This is further exemplified by higher levels of uracil in pregnant organisms (most clearly seen in the HSQC, showcased by the red boxes in Fig. 5b) which become depleted in the juveniles, consistent with their use for the formation of DNA/RNA [60,61] during their early growth.

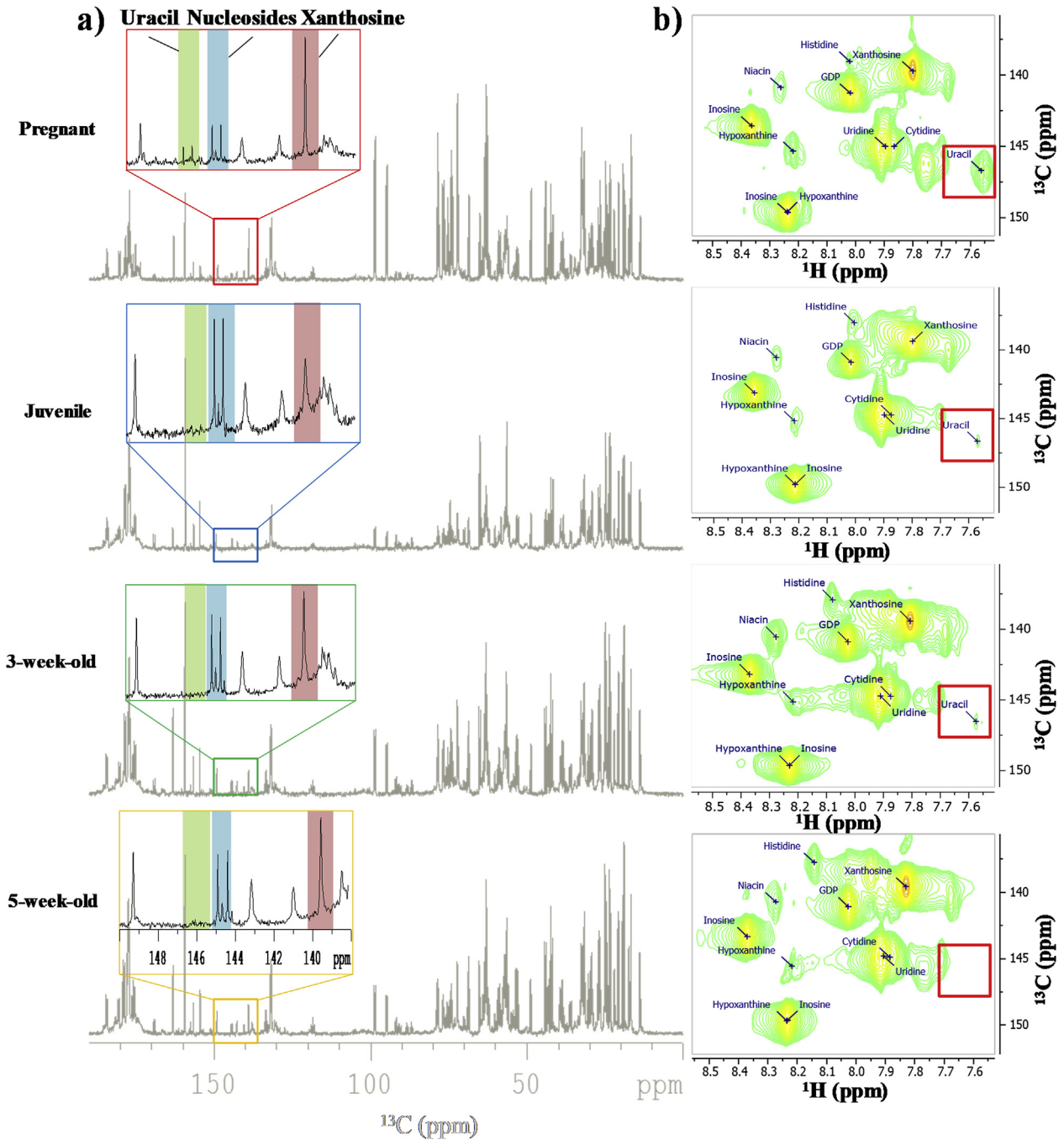


Fig. 5. ^{13}C spectra of pregnant, juvenile, and 3-week-old, and 5-week-old adult (top to bottom) ^{13}C labelled *Daphnia magna*. (a): The aromatic region is enlarged to show changes in nucleosides in the different life stages and ages. b) Corresponding assigned (^{1}H-^{13}C) HSQC of the aromatic region. The red box highlights the aromatic signal from uracil. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3.4. *Generational (F_0-F_3) study of Daphnia magna*

In addition to the developmental stages and ages, discussed above, it is important to consider if there are any intergenerational changes with ^{13}C carbon enrichment. This is driven by two main reasons: 1) To the authors' knowledge, intergenerational studies of carbon enrichment have not been assessed by NMR in any organisms, 2) the F_0 vs. F_1 comparison is very interesting as it has considerable impact on the culturing time and cost of raising organisms for ^{13}C enriched *in vivo* NMR studies [5,6,12–14,36,37]. For example, for *in vivo* studies, two enrichment approaches are feasible; 1) maintain fully ^{13}C enriched cultures all the time in the lab and 2) maintain ^{12}C organisms in the lab and only enrich the neonates from birth for "X" number of days on ^{13}C food prior to NMR analysis. While option 1 is ideal, in that all the carbon will be enriched and NMR studies can be performed with less lead-time, this approach could be cost prohibitive over the long term. Conversely, feeding ^{13}C only when needed, utilizes the expensive ^{13}C enriched food source efficiently, but the question becomes "does the ^{12}C present in the organisms at birth impact ^{13}C metabolic profile at 3-weeks (a common time point for analysis of enriched ^{13}C *D. magna* by NMR [36])?"

Fig. 6 compares the NMR data for the F_0-F_3 generations. For the

F_1-F_3 generations all the data from the most dynamic to the most solid are very similar. Indeed this is expected, given that under low-stress conditions (as is the case of the lab environment here) *Daphnia* reproduce asexually (parthenogenetic reproduction), in which the parent, female *Daphnia* gives rise to genetically identical daughters [62–64]. As a result, no significant differences are expected in their metabolites or structural components amongst the generations. This is a key reason as to why *Daphnia* are often the ideal choice of organisms in multigenerational studies [63,64].

However, the F_0 and F_1 are quite different in that the F_0 generation has a stronger contribution from lipid signals in the dynamic (to a small extent) and semi-solids fractions (to a great extent). This is in-line with the organisms "stock piling lipids" during early growth. This is consistent with the fact that lipids need to be assimilated from their diet and are used as the primary energy source for growth [45,46]. While this will also happen in later generations, in the F_0 generation the neonate's biomass starts as ^{12}C (including initial lipid reserves inherited from their mothers), thus the selective uptake of ^{13}C from their diet is amplified in the ^{13}C NMR. Note the organisms' guts were purged of ^{13}C prior to lyophilization (see experimental, section 2.1) so the additional lipid signal is from the *Daphnia* themselves and not just the algae in their guts. With the exception of the lipids, the remaining profile of the F_0 *Daphnia* is similar to the other generations, and strong signals from amino acids and carbohydrates are seen, consistent with the lipids overtime being converted in ^{13}C enriched biomass.

In summary, if costs are ignored, F_1 generation organisms are ideal for most *in vivo* NMR as the contributions from key metabolites, such as amino acids and sugars, are on a relative basis more pronounced, and it is often these categories of metabolites that flux during important processes or stress responses [36,65,66]. The reproducibility of CMP-NMR across the F_1-F_3 generations is discussed in Fig. S8 and associated text. However, the only drawback of using the F_0 generation is the over emphasized lipid signal. While this is not ideal, as lipids often mask other interesting signals [67], all components are labelled during 3 weeks of growth [14] and F_0 generations are commonly used successfully *in vivo* response studies [36,65,66]. As such, the use of F_0 vs. F_1 will likely come down to the cost of raising multiple generations of organisms, which will be driven by the reproduction rate, and food consumption rate of the organisms.

4. Conclusions

CMP-NMR is still a relatively new technique that provides an overview of organic components in all phases in complex heterogeneous samples. Studies thus far have mainly focused on either non-living materials such as rubber [68], soil [8,9,69] or on living systems [10,11]. However, no studies have applied CMP-NMR to non-living (*ex vivo*) organisms. This research presents the application of CMP-NMR to *ex vivo* samples, for the first time, and shows that the use of deceased, intact organisms can provide a wealth of information complementary to *in vivo* studies. Here CMP-NMR is applied to deceased, intact organisms and a wealth of information complementary to *in vivo* studies can be obtained. Advantages of *ex vivo* (vs. *in vivo*) CMP-NMR include; easier preservation of samples; samples can be spun faster thus increasing spectral resolution; more biomass can be analyzed; and as organisms do not need to be kept alive (i.e. a restricted time window for experiments) experiments can be run longer or more experiments can be performed. The higher quality data obtained from *ex vivo* NMR can be used to better interpret the less resolved *in vivo* NMR signals, thus providing a more detailed and extensive understanding of the biological processes in organisms. In summary, the studies on the various *Daphnia* life stages demonstrate that *ex vivo* CMP-NMR can provide important information on biological processes and could be of interest to researchers monitoring and understanding molecular changes inside small organisms.

Spectral editing provides a unique insight into not just the chemical components, but the physical state of the components as well, while 2D NMR can provide details regarding exact metabolites, thus helping to produce a holistic overview of growth and reproduction processes within an organism. Although the ultimate goal of environmental toxicology may be to understand the fate and

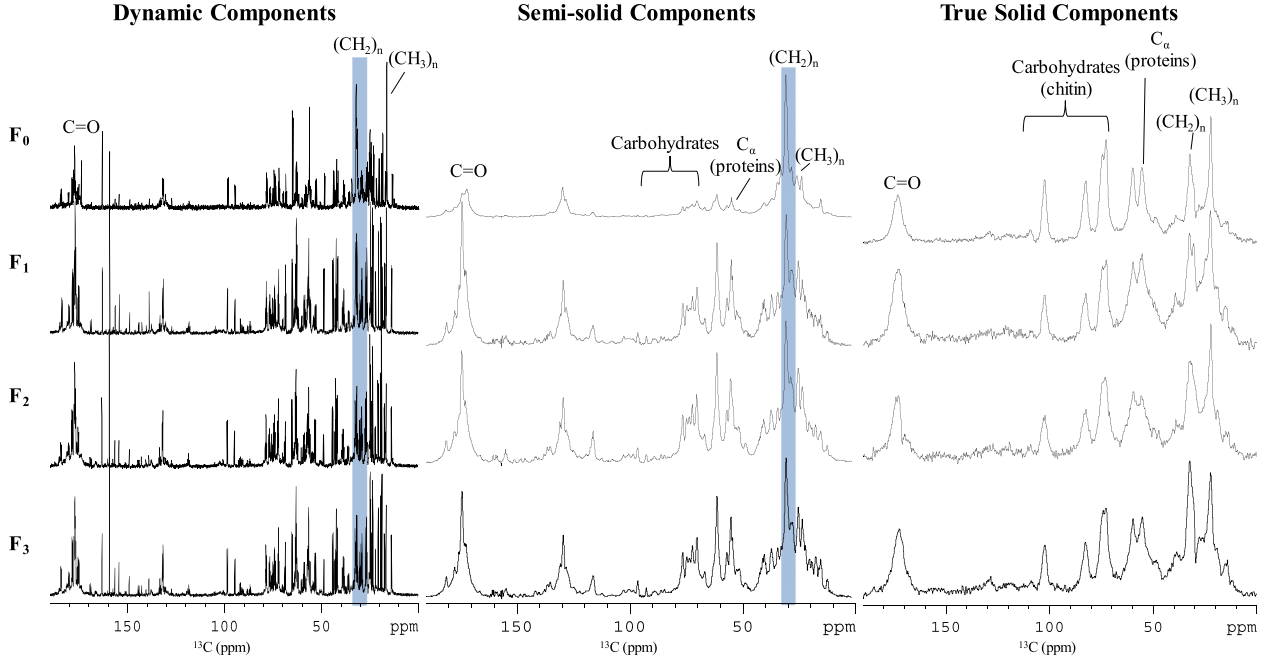


Fig. 6. ^{13}C Intergenerational spectra of freeze-dried *Daphnia magna* of F_0, F_1, F_2, and F_3 (top to bottom) generations. Columns: Dynamic components (Low power ^{13}C NMR with semisolids removed; see Fig. 1f for reference) Semi-solids (^{13}C RADE) and True solids (Inverse T_2-filtered CP-MAS).

effect of contaminants in living systems and their environment, *ex vivo* studies can be used to overcome some of the limitations *in vivo*. In conclusion, the authors suggest both approaches are useful and highly complementary and suggest *ex vivo* NMR to be very helpful for detailed characterization and assignments of organisms, which can then be extrapolated to better understand the broad resonances that are observed *in vivo*.

CRediT authorship contribution statement

Rajshree Ghosh Biswas: Conceptualization, Formal analysis. **Blythe Fortier-McGill:** Investigation. **Mohammad Akhter:** Investigation, Resources. **Ronald Soong:** Investigation, Validation, Software. **Paris Ning:** Investigation. **Monica Bastawrous:** Investigation. **Amy Jenne:** Investigation. **Daniel Schmidig:** Resources, Methodology. **Peter De Castro:** Resources, Methodology. **Stephan Graf:** Resources, Methodology. **Till Kuehn:** Resources, Methodology. **Falko Busse:** Resources, Project administration. **Jochem Struppe:** Resources, Resources. **Michael Fey:** Resources, Methodology. **Hermann Heumann:** Resources, Investigation. **Holger Boenisch:** Resources, Investigation. **Marcel Gundy:** Resources, Investigation. **Myrna J. Simpson:** Writing - review & editing, Funding acquisition. **André J. Simpson:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

We would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) (Strategic (STPGP 494273-16) and Discovery Programs (RGPIN-2019-04165)), the Canada Foundation for Innovation (CFI), the Ontario Ministry of Research and Innovation (MRI), and the Krembil Foundation for providing funding. A. J. S. would like to thank the Government of Ontario for an Early Researcher Award.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.acax.2020.100051.

References

[1] P.S. Cranston, Biomonitoring and invertebrate taxonomy, Environ. Monit. Assess. 14 (1990) 265–273, https://doi.org/10.1007/BF00677921.
[2] M. Zuykov, E. Pelletier, D.A.T. Harper, Bivalve mollusks in metal pollution studies: from bioaccumulation to biomonitoring, Chemosphere 93 (2013) 201–208, https://doi.org/10.1016/j.chemosphere.2013.05.001.
[3] C. Wang, G. Lu, W. Peifang, H. Wu, P. Qi, Y. Liang, Assessment of environmental pollution of Taihu Lake by combining active biomonitoring and integrated biomarker response, Environ. Sci. Technol. 45 (2011) 3746–3752, https://doi.org/10.1021/es1037047.
[4] R. Van Der Oost, A. Goksøyr, M. Celander, H. Heida, N.P.E. Vermeulen, Biomonitoring of aquatic pollution with feral eel (Anguilla anguilla). II. Biomarkers: pollution-induced responses, Aquat. Toxicol. 36 (1996) 189–222, https://doi.org/10.1016/S0166-445X(96)00802-8.
[5] A.J. Simpson, Y. Liaghati, B. Fortier-McGill, R. Soong, M. Akhter, Perspective: *in vivo* NMR - a potentially powerful tool for environmental research, Magn. Reson. Chem. 53 (2015) 686–690, https://doi.org/10.1002/mrc.4142.
[6] M. Bastawrous, A. Jenne, M. Tabatabaei Anaraki, A. Simpson, In-vivo NMR spectroscopy: a powerful and complimentary tool for understanding environmental toxicity, Metabolites 8 (2018) 35, https://doi.org/10.3390/metabo8020035.
[7] A.J. Simpson, M.J. Simpson, R. Soong, Environmental nuclear magnetic resonance spectroscopy: an overview and a primer, Anal. Chem. 90 (2018) 628–639, https://doi.org/10.1021/acs.analchem.7b03241.
[8] H. Masoom, D. Courtier-Murias, H. Farooq, R. Soong, B.P. Kelleher, C. Zhang, W.E. Maas, M. Fey, R. Kumar, M. Monette, H.J. Stronks, M.J. Simpson, A.J. Simpson, Soil organic matter in its native state: unravelling the most complex biomaterial on earth, Environ. Sci. Technol. 50 (2016) 1670–1680, https://doi.org/10.1021/acs.est.5b03410.
[9] H. Masoom, D. Courtier-Murias, R. Soong, W.E. Maas, M. Fey, R. Kumar, M. Monette, H.J. Stronks, M.J. Simpson, A.J. Simpson, From spill to sequestration: the molecular journey of contamination via comprehensive multiphase NMR, Environ. Sci. Technol. 49 (2015) 13983–13991, https://doi.org/10.1021/acs.est.5b03251.
[10] Y.L. Mobarhan, R. Soong, D. Lane, A.J. Simpson, In vivo comprehensive multiphase NMR, Magn. Reson. Chem. (2019), https://doi.org/10.1002/mrc.4832.
[11] Y.L. Mobarhan, B. Fortier-McGill, R. Soong, W.E. Maas, M. Fey, M. Monette, H.J. Stronks, S. Schmidt, H. Heumann, W. Norwood, A.J. Simpson, Comprehensive multiphase NMR applied to a living organism, Chem. Sci. 7 (2016) 4856–4866, https://doi.org/10.1039/c6sc00329j.
[12] R. Soong, Y. Liaghati Mobarhan, M. Tabatabaei, M. Bastawrous, R.G. Biswas, M. Simpson, A. Simpson, Flow-based *in vivo* NMR spectroscopy of small aquatic organisms, Magn. Reson. Chem. (2019), https://doi.org/10.1002/mrc.4886.
[13] R. Soong, E. Nagato, A. Sutrisno, B. Fortier-McGill, M. Akhter, S. Schmidt, H. Heumann, A.J. Simpson, *In vivo* NMR spectroscopy: toward real time monitoring of environmental stress, Magn. Reson. Chem. 53 (2015) 774–779, https://doi.org/10.1002/mrc.4154.
[14] R.D. Majumdar, M. Akhter, B. Fortier-McGill, R. Soong, Y. Liaghati-Mobarhan, A.J. Simpson, M. Spraul, S. Schmidt, H. Heumann, Vivo solution-state NMR-based environmental metabolomics, EMagRes. 6 (2017) 133–148, https://doi.org/10.1002/9780470034590.emrstm1533.
[15] D. Courtier-Murias, H. Farooq, H. Masoom, A. Botana, R. Soong, J.G. Longstaffe, M.J. Simpson, W.E. Maas, M. Fey, B. Andrew, J. Struppe, H. Hutchins, S. Krishnamurthy, R. Kumar, M. Monette, H.J. Stronks, A. Hume, A.J. Simpson, Comprehensive multiphase NMR spectroscopy: basic experimental approaches to differentiate phases in heterogeneous samples, J. Magn. Reson. 217 (2012) 61–76, https://doi.org/10.1016/J.JMR.2012.02.009.
[16] L. Lam, R. Soong, A. Sutrisno, R. de Visser, M.J. Simpson, H.L. Wheeler, M. Campbell, W.E. Maas, M. Fey, A. Gorissen, H. Hutchins, B. Andrew, J. Struppe, S. Krishnamurthy, R. Kumar, M. Monette, H.J. Stronks, A. Hume, A.J. Simpson, Comprehensive multiphase NMR spectroscopy of intact 13C-labeled seeds, J. Agric. Food Chem. 62 (2014) 107–115, https://doi.org/10.1021/jf4045638.
[17] B.E. Fortier-McGill, R. Dutta Majumdar, L. Lam, R. Soong, Y. Liaghati-Mobarhan, A. Sutrisno, R. de Visser, M.J. Simpson, H.L. Wheeler, M. Campbell, A. Gorissen, A.J. Simpson, Comprehensive multiphase (CMP) NMR monitoring of the structural changes and molecular flux within a growing seed, J. Agric. Food Chem. 65 (2017) 6779–6788, https://doi.org/10.1021/acs.jafc.7b02421.
[18] Y. Liaghati Mobarhan, R. Soong, W. Bermel, M.J. Simpson, J. Struppe, H. Heumann, S. Schmidt, H. Boenisch, D. Lane, A.J. Simpson, Vivo ultraslow MAS ^{2} H/^{13} C NMR emphasizes metabolites in dynamic flux, ACS Omega 3 (2018) 17023–17035, https://doi.org/10.1021/acsomega.8b02882.
[19] Y.L. Mobarhan, J. Struppe, B. Fortier-McGill, A.J. Simpson, Effective combined water and sideband suppression for low-speed tissue and in vivo MAS NMR, Anal. Bioanal. Chem. 409 (2017) 5043–5055, https://doi.org/10.1007/s00216-017-0450-3.
[20] R. Guan, W.-X. Wang, Dietary assimilation and elimination of Cd, Se, and Zn by Daphnia magna at different metal concentrations, Environ. Toxicol. Chem. 23 (2004) 2689, https://doi.org/10.1897/03-503.
[21] X.-J. Liu, I.-H. Ni, W.-X. Wang, Trophic transfer of heavy metals from freshwater zooplankton Daphnia magna to zebrafish Danio reiro, Water Res. 36 (2002) 4563–4569, https://doi.org/10.1016/S0043-1354(02)00180-X.
[22] D. Ebert, Ecology, Epidemiology, and Evolution of Parasitism in Daphnia, National Library of Medicine US National Center for Biotechnology Information, Bethesda, Maryland, 2005. https://www.ncbi.nlm.nih.gov/books/NBK2042/. accessed March 22, 2019.
[23] Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms fifth edition. https://www.epa.gov/sites/production/files/2015-08/documents/acute-freshwater-and-marine-wet-manual_2002.pdf, 2002 accessed July 25, 2019.
[24] G. Persoone, R. Baudo, G.P. Be, Review on the acute Daphnia magna toxicity test-Evaluation of the sensitivity and the precision of assays performed with organisms from laboratory cultures or hatched from dormant, eggs 393 (2009) 1, https://doi.org/10.1051/kmae/2009012.
[25] A.J. Simpson, D. Courtier-Murias, J.G. Longstaffe, H. Masoom, R. Soong, L. Lam, A. Sutrisno, H. Farooq, M.J. Simpson, W.E. Maas, M. Fey, B. Andrew, J. Struppe, H. Hutchins, S. Krishnamurthy, R. Kumar, M. Monette, H.J. Stronks, Environmental comprehensive multiphase NMR, EMagRes. 2 (2013) 399–414, https://doi.org/10.1002/9780470034590.emrstm1337.
[26] G.C. Woods, M.J. Simpson, P.J. Koerner, A. Napoli, A.J. Simpson, HILIC-NMR: toward the identification of individual molecular components in dissolved organic matter, Environ. Sci. Technol. 45 (2011) 3880–3886, https://doi.org/10.1021/es103425s.
[27] H.L. Wheeler, R. Soong, D. Courtier-Murias, A. Botana, B. Fortier-Mcgill, W.E. Maas, M. Fey, H. Hutchins, S. Krishnamurthy, R. Kumar, M. Monette,

H.J. Stronks, M.M. Campbell, A. Simpson, Comprehensive multiphase NMR: a promising technology to study plants in their native state, Magn. Reson. Chem. 53 (2015) 735–744, https://doi.org/10.1002/mrc.4230.
[28] P. Ning, R. Soong, W. Bermel, D. Lane, M.J. Simpson, A.J. Simpson, 13C quantification in heterogeneous multiphase natural samples by CMP-NMR using stepped decoupling, Anal. Bioanal. Chem. 410 (2018) 7055–7065, https://doi.org/10.1007/s00216-018-1306-1.
[29] A. Shaka, J. Keeler, R. Freeman, Evaluation of a new broadband decoupling sequence: WALTZ-16, J. Magn. Reson. 53 (1983) 313–340, https://doi.org/10.1016/0022-2364(83)90035-5.
[30] U. Sternberg, R. Witter, I. Kuprov, J.M. Lamley, A. Oss, J.R. Lewandowski, A. Samoson, 1H line width dependence on MAS speed in solid state NMR – comparison of experiment and simulation, J. Magn. Reson. 291 (2018) 32–39, https://doi.org/10.1016/J.JMR.2018.04.003.
[31] D.H. Wu, A. Chen, C.S. Johnson, An improved diffusion-ordered spectroscopy experiment incorporating bipolar-gradient pulses, J. Magn. Reson., Ser. A (1995), https://doi.org/10.1006/jmra.1995.1176.
[32] D.P. Burum, Combined rotation and multiple pulse spectroscopy (CRAMPS), concepts magn, Reson 2 (1990) 213–227, https://doi.org/10.1002/cmr.1820020404.
[33] B.G. Anderson, L.A. Brown, A study of chitin secretion in Daphnia magna, Physiol. Zool. 3 (1930) 485–493, https://doi.org/10.1086/physzool.3.4.30151118.
[34] M. Giraudo, M. Douville, G. Cottin, M. Houde, Transcriptomic, cellular and life-history responses of Daphnia magna chronically exposed to benzotriazoles: endocrine-disrupting potential and molting effects, PLoS One 12 (2017), e0171763, https://doi.org/10.1371/journal.pone.0171763.
[35] N. Hertkorn, C. Ruecker, M. Meringer, R. Gugisch, M. Frommberger, E.M. Perdue, M. Witt, P. Schmitt-Kopplin, High-precision frequency measurements: indispensable tools at the core of the molecular-level analysis of complex systems, Anal. Bioanal. Chem. 389 (2007) 1311–1327, https://doi.org/10.1007/s00216-007-1577-4.
[36] M. Tabatabaei Anaraki, R. Dutta Majumdar, N. Wagner, R. Soong, V. Kovacevic, E.J. Reiner, S.P. Bhavsar, X. Ortiz Almirall, D. Lane, M.J. Simpson, H. Heumann, S. Schmidt, A.J. Simpson, Development and application of a low-volume flow system for solution-state *in vivo* NMR, Anal. Chem. 90 (2018) 7912–7921, https://doi.org/10.1021/acs.analchem.8b00370.
[37] M. Tabatabaei Anaraki, M.J. Simpson, A.J. Simpson, Reducing impacts of organism variability in metabolomics via time trajectory in vivo NMR, Magn. Reson. Chem. 56 (2018) 1117–1123, https://doi.org/10.1002/mrc.4759.
[38] I. Fugariu, W. Bermel, D. Lane, R. Soong, A.J. Simpson, In-phase ultra high-resolution in vivo NMR, Angew. Chem. Int. Ed. 56 (2017) 6324–6328, https://doi.org/10.1002/anie.201701097.
[39] C.A. Pincetich, M.R. Viant, D.E. Hinton, R.S. Tjeerdema, Metabolic changes in Japanese medaka (Oryzias latipes) during embryogenesis and hypoxia as determined by in vivo 31P NMR, Comp, Biochem. Physiol. -C Toxicol. Pharmacol. 140 (2005) 103–113, https://doi.org/10.1016/j.cca.2005.01.010.
[40] M.R. Viant, C.A. Pincetich, D.E. Hinton, R.S. Tjeerdema, Toxic actions of dinoseb in medaka (Oryzias latipes) embryos as determined by in vivo 31P NMR, HPLC-UV and 1H NMR metabolomics, Aquat. Toxicol. 76 (2006) 329–342, https://doi.org/10.1016/j.aquatox.2005.10.007.
[41] W.T. Waller, A.D. Sherry, Whole organism31P nuclear magnetic resonance spectroscopy: a potential application in aquatic toxicity evaluations, Bull. Environ. Contam. Toxicol. 26 (1981) 73–76, https://doi.org/10.1007/BF01622057.
[42] B. Campos, D. Fletcher, B. Piña, R. Tauler, C. Barata, Differential gene transcription across the life cycle in Daphnia magna using a new all genome custom-made microarray, BMC Genom. 19 (2018) 370, https://doi.org/10.1186/s12864-018-4725-7.
[43] I. Fuertes, R. Jordão, J. Casas, C. Barata, Allocation of glycerolipids and glycerophospholipids from adults to eggs in Daphnia magna: perturbations by compounds that enhance lipid droplet accumulation, Environ. Pollut. 242 (2018) 1702–1710, https://doi.org/10.1016/J.ENVPOL.2018.07.102.
[44] R. Jordão, E. Garreta, B. Campos, M.F.L. Lemos, A.M.V.M. Soares, R. Tauler, C. Barata, Compounds altering fat storage in Daphnia magna, Sci. Total Environ. (2016), https://doi.org/10.1016/j.scitotenv.2015.12.097.
[45] A. Wacker, D. Martin-Creuzburg, Allocation of essential lipids in Daphnia magna during exposure to poor food quality, Funct. Ecol. 21 (2007) 738–747, https://doi.org/10.1111/j.1365-2435.2007.01274.x.
[46] C. Becker, M. Boersma, Differential effects of phosphorus and fatty acids on Daphnia magna growth and reproduction, Limnol. Oceanogr. 50 (2005) 388–397, https://doi.org/10.4319/LO.2005.50.1.0388.
[47] A. Putman, D. Martin-Creuzburg, B. Panis, L. De Meester, A comparative analysis of the fatty acid composition of sexual and asexual eggs of *Daphnia magna* and its plasticity as a function of food quality, J. Plankton Res. 37 (2015) 752–763, https://doi.org/10.1093/plankt/fbv043.
[48] Wei-Guo Hu, Jingdong Mao, Baoshan Xing, Klaus Schmidt-Rohr, Poly(-methylene) crystallites in humic substances detected by, Nucl. Magn. Reson. 34 (1999) 530–534, https://doi.org/10.1021/ES990506L.
[49] C. Barata, J. Carlos Navarro, I. Varo, M. Carmen Riva, S. Arun, C. Porte, Changes in antioxidant enzyme activities, fatty acid composition and lipid peroxidation in Daphnia magna during the aging process, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 140 (2005) 81–90, https://doi.org/10.1016/J.CBPC.2004.09.025.
[50] B.N. Fidder, E.G. Reátegui-Zirena, C.J. Salice, Diet quality affects chemical tolerance in the freshwater snail *Lymnaea stagnalis*, Environ. Toxicol. Chem. 37 (2018) 1158–1167, https://doi.org/10.1002/etc.4064.
[51] S.J. Taipale, A.W.E. Galloway, S.L. Aalto, K.K. Kahilainen, U. Strandberg, P. Kankaala, Terrestrial carbohydrates support freshwater zooplankton during phytoplankton deficiency, Sci. Rep. 6 (2016), https://doi.org/10.1038/srep30897, 30897.
[52] B. Artur Stankiewicz, M. Mastalerz, C.H.J. Hof, A. Bierstedt, M.B. Flannery, D.E.G. Briggs, R.P. Evershed, Biodegradation of the chitin-protein complex in crustacean cuticle, Org. Geochem. 28 (1998) 67–76, https://doi.org/10.1016/S0146-6380(97)00113-7.
[53] D.M. Skinner, S.S. Kumari, J.J. O'Brien, Proteins of the Crustacean exoskeleton, Am. Zool. 32 (1992) 470–484, https://doi.org/10.1093/icb/32.3.470.
[54] G. Liechti, J.B. Goldberg, Helicobacter pylori relies primarily on the purine salvage pathway for purine nucleotide biosynthesis, J. Bacteriol. 194 (2012) 839–854, https://doi.org/10.1128/JB.05757-11.
[55] L. McNaughton, B. Dalton, J. Tarr, Inosine supplementation has No effect on aerobic or anaerobic cycling performance, Int. J. Sport Nutr. 9 (1999) 333–344, https://doi.org/10.1123/ijsn.9.4.333.
[56] W.L. Nyhan, Nucleotide Synthesis via Salvage Pathway, 2014, https://doi.org/10.1002/9780470015902.a0001399.pub3.
[57] H. Ashihara, C. Stasolla, T. Fujimura, A. Crozier, Purine salvage in plants, Phytochemistry 147 (2018) 89–124, https://doi.org/10.1016/J.PHYTOCHEM.2017.12.008.
[58] A. Guranowski, Purine catabolism in plants. www.plantphysiol.org, 1982 accessed March 25, 2019.
[59] Purine metabolism - Daphnia pulex (common water flea), (n.d.), https://www.genome.jp/kegg-bin/show_pathway?dpx00230. accessed March 25, 2019.
[60] H.E. Krokan, F. Drabløs, G. Slupphaug, Uracil in DNA – occurrence, consequences and repair, Oncogene 21 (2002) 8935–8948, https://doi.org/10.1038/sj.onc.1205996.
[61] L.H. Pearl, Structure and function in the uracil-DNA glycosylase superfamily, Mutat. Res. Repair. 460 (2000) 165–181, https://doi.org/10.1016/S0921-8777(00)00025-2.
[62] H. Stibor, D.M. Navarra, Constraints on the plasticity of Daphnia magna influenced by fish-kairomones, Funct. Ecol. 14 (2000) 455–459, https://doi.org/10.1046/j.1365-2435.2000.00441.x.
[63] J. Routtu, M.D. Hall, B. Albere, C. Beisel, R. Bergeron, A. Chaturvedi, J.-H. Choi, J. Colbourne, L. De Meester, M.T. Stephens, C.-P. Stelzer, E. Solorzano, W. Thomas, M.E. Pfrender, D. Ebert, An SNP-based second-generation genetic map of Daphnia magna and its application to QTL analysis of phenotypic traits, BMC Genom. 15 (2014) 1033, https://doi.org/10.1186/1471-2164-15-1033.
[64] D.A. Arndt, J. Chen, M. Moua, R.D. Klaper, Multigeneration impacts on Daphnia magna of carbon nanomaterials with differing core structures and functionalizations, Environ. Toxicol. Chem. 33 (2014) 541–547, https://doi.org/10.1002/etc.2439.
[65] D. Lane, R. Soong, W. Bermel, P. Ning, R. Dutta Majumdar, M. Tabatabaei-Anaraki, H. Heumann, M. Gundy, H. Bönisch, Y. Liaghati Mobarhan, M.J. Simpson, A.J. Simpson, Selective amino acid-only in vivo NMR: a powerful tool to follow stress processes, ACS Omega 4 (2019) 9017–9028, https://doi.org/10.1021/acsomega.9b00931.
[66] D. Lane, T.E. Skinner, N.I. Gershenzon, W. Bermel, R. Soong, R. Dutta Majumdar, Y. Liaghati Mobarhan, S. Schmidt, H. Heumann, M. Monette, M.J. Simpson, A.J. Simpson, Assessing the potential of quantitative 2D HSQC NMR in 13C enriched living organisms, J. Biomol. NMR 73 (2019) 31–42, https://doi.org/10.1007/s10858-018-0221-2.
[67] Q. Hassan, R. Dutta Majumdar, B. Wu, D. Lane, M. Tabatabaei-Anraki, R. Soong, M.J. Simpson, A.J. Simpson, Improvements in lipid suppression for ^{1}H NMR-based metabolomics: applications to solution-state and HR-MAS NMR in natural and in vivo samples, Magn. Reson. Chem. 57 (2019) 69–81, https://doi.org/10.1002/mrc.4814.
[68] L.M.A. Silva, E.G.A. Filho, A.J. Simpson, M.R. Monteiro, T. Venâncio, Comprehensive multiphase NMR spectroscopy: a new analytical method to study the effect of biodiesel blends on the structure of commercial rubbers, Fuel 166 (2016) 436–445, https://doi.org/10.1016/J.FUEL.2015.10.105.
[69] H. Farooq, D. Courtier-Murias, M.J. Simspon, W.E. Maas, M. Fey, B. Andrew, J. Struppe, H. Hutchins, S. Krishnamurthy, R. Kumar, M. Monette, H.J. Stronks, A.J. Simpson, Characterisation of oil contaminated soils by comprehensive multiphase NMR spectroscopy, Environ. Chem. 12 (2015) 227, https://doi.org/10.1071/EN14129.