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

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**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 13C 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 13C 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.

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**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...
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 (lipsids, 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 solution of study, 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 1H and 13C 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 13C-enriched Chlamydomonas reinhardtii (Sielantes, 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.

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The following NMR experiments were conducted using
protocols similar to McGill et al. and are standard for most multi-phase NMR experiments [17]. All NMR experiments were performed using a Bruker Avance III 500 MHz 1H spectrometer, equipped with a prototype CMP-MAS 4 mm 1H-13C-2H probe (Bruker Biospin, Billerica, MA) with an actively shielded 2-gradient. All experiments were locked on D2O (including solid state experiments) and maintained at 298 K. Samples were spun at a spindle frequency 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 1H 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 1H–13C 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 1H observed experiments, whereas a low-power WALTZ16 decoupling was used for 13C 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 1H 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 T1 time for each sample, and the recycle delay was set to 5 times the measured T1 value. Due to the fast relaxation in the fully 13C 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 13C spectra (except for CP) were acquired using a spectral width of 400 ppm, 16384 time-domain points, 3072 scans and inverse gated 1H decoupling. The recycle delay was set to 5 times the measured T1 value (~3.1s between scans due to fast relaxation in the 13C enriched samples). Spectra were processed using an exponential function corresponding to a line broadening of 1.5 Hz for 13C 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

1H and 13C diffusion editing were acquired using a bipolar pulse pair longitudinal encode-decode (BBPLED) 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 T2-filtered CP-MAS which used 2 echoes separated by 15 μs prior to cross-polarization [15]. Inverse diffusion editing (IDE), Relaxation Recovery Aising from Diffusion Editing (RADE) and inverse T2-filtered 13C 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

### 2H, 13C 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 1J 1H-13C 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 R² (Fig. S6 in the supporting information) correlation >0.99 between observed and calculated 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 briefly in the context of ex vivo intact organisms. Spectral editing can be applied to both 1H and 13C. With samples at natural abundance, 1H is most efficient for editing solutions, gels and semi-solids due to the increased sensitivity of the 1H nucleus, while 13CCP-MAS is used for the study of true solids due to the lack of resolution in 1H detected solids [15,27,28]. However, in this study, as the organisms are 13C enriched, it provides a rare opportunity to also apply the full suite (liquids, gels, semi-solids, solids) of 13C detected editing experiments.

#### 3.1.1. Spectral editing using 13C

To demonstrate the spectral editing approaches, 3-week-old *D. magna* were used from the F3 generation as they are a good representative of complete 13C enriched *D. magna* in general (refer to section 3.3.4, Fig. 6). Fig. 1a shows the conventional carbon spectrum collected with low power 1H decoupling. In general low power decoupling covers the standard 1H 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 1H-1H 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
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 BPPLIED (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 both rigid and dynamic solids.

Fig. 1. 1D $^{13}$C spectra of $^{13}$C-labelled, F3 - 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.
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 solid 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 10 $^{13}$C NMR is around 30,000 [35]. However, this increases significantly to around 2,000,000 for 2D $^1$H-$^1$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 $\text{^{1}H-^{1}H}$ 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 $\text{^{1}H-^{13}C}$ 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 $\text{^{13}C}$ and $\text{^{1}H}$ 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 $\text{^{1}H}$ spectrum is less resolved in comparison to $\text{^{13}C}$ NMR, only more general assignments can be provided for the $\text{^{1}H}$ 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
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 $^3$H-$^{13}$C NMR at such low 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 D2O which further reduces complications from a large H2O signal.

C) Reduction of Lipid Signals: In vivo lipid signals 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 (D2O). 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 12C algae prior to freeze-drying as previously described [25]. As the 12C algae is below NMR detection limits [25] using 13C and 1H-13C HSQC detection, only the 13C 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 13C spectra of Daphnia at different life stages. To simplify things, only 3 categories of spectra are shown: 1) 13C NMR due to the low power of the 13C spectrometer 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-T2 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 (CH2)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 (CH2)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 Daphnia’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
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 semisolid (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 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.
3.3.4. Generational (F0-F3) 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 F0 vs. F1 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 F0-F3 generations. For the

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.)
F₀-F₃ 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 F₀ and F₁ are quite different in that the F₀ 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 F₀ generation the neonate’s biomass starts as ¹³C (including initial lipid reserves inherited from their mothers), thus the selective uptake of ¹³C from their diet is amplified in the ¹³C NMR. Note the organisms’ guts were purged of ¹³C prior to lyophilization (see experimental, section 2.1) so the additional lipid signal is from the Daphni themselves and not just the algae in their guts. With the exception of the lipids, the remaining profile of the F₀ 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 ¹³C enriched biomass.

In summary, if costs are ignored, F₁ 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 F₁-F₃ generations is discussed in Fig. S8 and associated text. However, the only drawback of using the F₀ 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 F₀ generations are commonly used successfully in vivo response studies [36,65,66]. As such, the use of F₀ vs. F₁ 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

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**Fig. 6.** ¹³C Intergenerational spectra of freeze-dried Daphnia magna of F₀, F₁, F₂, and F₃ (top to bottom) generations. Columns: Dynamic components (Low power ¹³C NMR with semisolids removed; see Fig. 1f for reference) Semi-solids (¹³C RADE) and True solids (Inverse T₂-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

Rajeshree 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 Schmid: 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. Andre 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 influenced the work reported in this paper.

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Appendix A. Supplementary data

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Appendix A. Supplementary data

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