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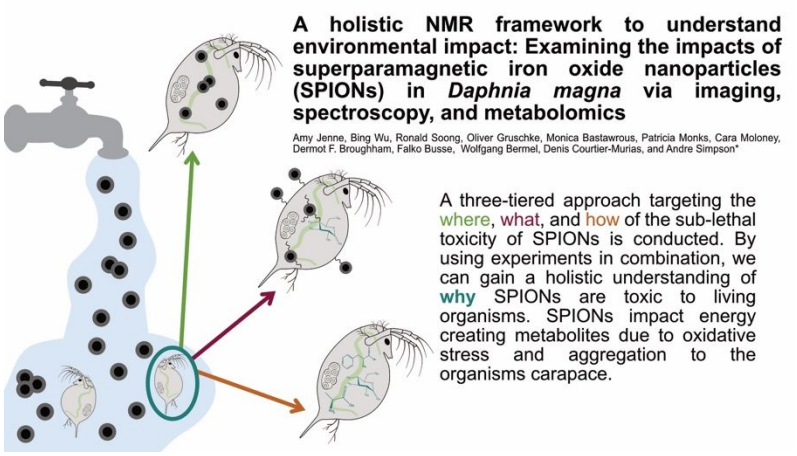

Abstract

Superparamagnetic Iron Oxide Nanoparticles (SPIONs) are a contaminant of emerging interest, often used in the medical field as an imaging contrast agent, with additional uses in wastewater treatment and as food additives. While the use of SPIONs is increasing, little research has been conducted on the toxic impacts to living organisms beyond traditional lethal concentration endpoints. *Daphnia magna* are model organisms for aquatic toxicity testing with a well understood metabolome and high sensitivity to SPIONs. Thus, as environmental concentrations continue to increase, it is becoming critical to understand their sub-lethal toxicity. Due to the paramagnetic nature of SPIONs, a range of potential Nuclear Magnetic Resonance Spectroscopy (NMR) experiments are possible, offering the potential to probe the physical location (via imaging), binding (via relaxation weighted spectroscopy), as well as the biochemical pathways impacted (via *in-vivo* metabolomics). Results indicate binding to carbohydrates, likely chitin in

the exoskeleton, along with a decrease in energy metabolites and specific biomarkers of oxidative stress. The holistic NMR framework used here helps provide a more comprehensive understanding of SPIONs impacts on *Daphnia magna,* and showcases NMR's versatility in providing physical, chemical and biochemical insights.

Keywords
NMR, MRI, ^{1}H, ^{13}C, *in-vivo*, metabolomics, sub-lethal toxicity, environmental, superparamagnetic, iron oxide nanoparticles

Introduction

Nanomaterials have become an increasingly prevalent substance used in numerous applications from food additives to preservatives, as well as in medicine and wastewater treatment, and production only continues to expand.[1–4] In the last decade there has been a 25-fold increase in products which used nanoparticles during their production.[3] However, despite their increased use, little research has been performed as to their environmental impact. Due to their small size, nanoparticles can move through the environment with ease and as many nanoparticles are metal-based, the potential ramifications to the biotic environment can be severe.[2] Superparamagnetic Iron Oxide Nanoparticles (SPIONs) are one such potential contaminant where impacts to the environment are not fully understood. The most common use of SPIONs is in the medical field as clinical Magnetic Resonance Imaging (MRI) contrast agents. The presence of SPIONs alters the surrounding water molecules spin-spin relaxation in particular, causing them to relax faster and resulting in local suppression of image intensity (darkening) under T_2-weighted imaging conditions.[5] This is particularly useful in disease monitoring as the suppression is proportional to the concentration of particles.[6] When surface functionalized with appropriate ligands, SPIONs can be selectively consumed by cancer cells, helping identify tumorous regions,[7] passive targeting is also well documented.

For human consumption, SPIONs are coated in a stable ligand, thus considered non-toxic. As a result, SPIONs move through the body based on their half-life (proportional to their size) and then are passed through the liver and are eventually eliminated as waste.[5,7,8] However, there has been limited research examining the toxicity of released particles to aquatic life, particularly from sub-lethal or mechanistic perspectives. Nanoparticles released by the body end up in wastewater, and as they enter our freshwater systems, they can be consumed by primary consumers such as *Daphnia magna. D. magna* are one of the most utilized model organisms in environmental toxicity testing and multiple studies have measured the impacts of other types of nanoparticles such as silver, titanium, and silicon oxide particles.[9–11] However, to date, metabolomic studies with iron oxide nanoparticles are lacking. The limited research that has been conducted on the toxic impacts of SPIONs have focused on apical endpoints with respect to lethal concentrations, rather than a mechanistic understanding. *Daphnia magna* have been shown to be incredibly susceptible to SPIONs, with an LC_{50} of 2.3 x 10^{-4} mg/mL.[12] However, information targeting the "where, what, how, and why" of SPION toxicity is still lacking.

Magnetic resonance imaging and spectroscopy when used in combination have a unique ability to provide comprehensive insights into the issue. As the contaminant is a contrast agent MRI has

potential to show within *Daphnia magna* physically "where" SPIONs accumulate, while also providing average or localized changes in relaxation. Hence relaxation measurements are useful when evaluating how effectively different size SPIONs are consumed. This is especially important as *Daphnia* are filter feeders, and little is known about the minimum size of particles they can consume or how specific ligand coatings may impact uptake. Another consideration is determining whether the nanoparticles are consumed at all, or if the toxic response is only as a result of the interactions with the outside of the organism.[9–11] If they are ingested, then "what" the nanoparticles bind with inside the organism at the molecular level must be examined. Here, relaxation filtered 2D NMR spectroscopy could be useful to study the categories of molecules or even structures that bind to SPIONs, aiding in understanding their excretion or bioaccumulation.[13,14] Finally, the question as to "how" the SPIONs are toxic can be tackled using *in-vivo* NMR metabolomics. This is the measurement of changes to an organism's metabolome as a result of an external stressor.[15] Sub-lethal toxicity in *Daphnia magna* has been assessed in this way, however utilizing *in-vivo* NMR spectroscopy is particularly desirable, as changes to the organism's metabolome can be measured in close to real time.[16–22] To the authors knowledge, to date there have been no studies of nanoparticles *in-vivo*. Perturbations to specific metabolic pathways could provide important information on the toxicity of SPIONs and help explain the toxic-mode-of-action inside *Daphnia magna,* as an exemplar aquatic organism, which are essential for setting updated environmental policies.

In this study, a-proof-of-concept study is presented that combines imaging, relaxation filtered 2D NMR, and *in-vivo* metabolomics to provide a more comprehensive overview addressing the "why" of SPION fate and toxicity. Given magnetic resonance's ability to provide detailed molecular information in a non-invasive fashion, such unified approaches drawing from different branches of NMR/MRI are shown to provide holistic understanding of the complex physiochemical processes in SPION toxicity, that may prove generally translatable.

Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma Aldrich (St. Louis, Missouri, U.S).

SPION Synthesis and Validation Measurements

Preparation of Primary Nanoparticles

4 nm (core size) primary SPIONs were prepared with slight modifications to the Pinna method.[23] In brief, 20 mL of 141.6 mM $Fe(acac)_3$ in benzyl alcohol was purged with N_2 for 20 minutes and refluxed at 205°C for 7 hours. The resulting black solution contained 10 mg/mL γ-Fe_2O_3 (90% yield) of 8.6 nm SPIONs as determined by transmission electron microscopy (TEM). To prepare the 12 nm SPIONs used in this study, 20 mL of 8.6 nm SPIONs were washed twice with benzyl alcohol and resuspended in 20 mL of 141.6 mM $Fe(acac)_3$ in benzyl alcohol. The solution was purged with N_2 for 20 minutes and refluxed at 205°C for 7 hours. The resulting black solution contained 20 mg/mL γ-Fe_2O_3 (90% yield) of 12 nm SPIONs as determined by TEM. SPIONs were stored under N_2 until use to prevent oxidation.

Stabilization of Primary Nanoparticles

5 mL of nanoparticles were washed twice with acetone using a magnetic separator. 37 µL of GLYMO in 2 mL THF was added to the wet nanoparticles and tumbled for 24 h. 10 µL of hydrochloric acid (1 M) and ethanol were also added to catalyze the hydrolysis and condensation of the alkoxysilane groups. This process yields a stable dispersion of GLYMO-functionalized Fe_3O_4 nanoparticles. PEG-NH_2 (of different M_n) in THF was added to the SPIONs in a 10:1 polymer:GLYMO ratio. Following the addition of 50 µL KOH (1 M), the PEG-grafted SPIONs precipitated out, were washed with THF and redispersed in water. The procedure has previously been reported.[24]

TEM

Transmission electron microscopy was performed on a FEI Tecai G2 TWIN 200 kV microscope. Aqueous suspensions of SPIONs were prepared (concentrations of 5 mM Fe) and 5 µL was pipetted onto a carbon TEM grid (Formvar and carbon films on 400 µm copper grid, Agra Scientific). Size analysis was performed using ImageJ software.

DLS

Dynamic light scattering (DLS) measurements were performed using a Malvern Nano ZetaSizer (Malvern Instruments, Malvern UK). A 3 mW He-Ne laser operating at a wavelength of 633 nm was used and back scattered light is detected at an angle of 173° to the incident beam. Data was processed using Dispersion technology software (v 7.13, Malvern instruments, Malvern UK) using the multiple narrow modes algorithm based upon a non-negative least square fit to calculate the hydrodynamic diameter (d_{hyd}) and the polydispersity Index (PDI). Samples were diluted to 5 mM Fe and analysis was performed at 25 °C, with a thermal equilibration time of 30 seconds prior to measurement.

<u>*Daphnia magna* Culturing</u>

D. magna were cultured from a colony originally purchased from Ward's Scientific but have been maintained in the lab since 2017. Organisms were cultured at 20°C, a water hardness of 124 mg/L $CaCO_3$, and pH 7.5-8.5, consistent with local freshwater conditions. Cultures were kept under a 16:8 light/dark cycle and a 40% water media exchange occurred three times per week. For *in/ex-vivo* experiments, species were cultured on a diet of 99% ^{13}C enriched *Chlamydomonas reinhardtii* (purchased from Silantes, GmbH)[25] as their sole food source from birth. Prior to experiments, organisms were placed in fresh dechlorinated water to clear off residual algae and fed ^{12}C algae to clear their gut, meaning only signals from the *Daphnia* would be measured. During *in-vivo* experiments, organisms were continuously fed unenriched *Chlamydomonas reinhardtii* cultured in the lab. Adult *Daphnia magna* (~two weeks old) born from ^{13}C enriched parents, are used in the study which have been shown to be ~99% ^{13}C enriched.[26,27] For imaging experiments, organisms were not isotopically enriched, but other culturing parameters remained the same.

<u>Nanoparticle Exposure</u>

In *Daphnia* samples used for *ex-vivo* exposure, 250 mL beakers were filled with 100 mL of culturing media (as above), 10 adult *Daphnia magna* were added to each beaker and nanoparticles were dosed in at a volume creating a total concentration of 10% of the LC_{50} (2.3 x

10^{-5} mg/mL).[12] Organisms were exposed for 48 hours and fed halfway through exposure to ensure a lack of stress response from starvation. Once completed, organisms were removed from exposure vessels and placed in beakers with clean media to allow any aggregated nanoparticles on the outside of their bodies to be washed. These were then prepared for the imaging and T_1 weighted spectroscopy. Note for the *in-vivo* metabolomics, the organisms were exposed inside the NMR as described below.

Imaging *Daphnia magna* Experiments

Immediately prior to experiments, *Daphnia magna*, one from control cultures and one from exposed, were placed in a 2.5 mm glass NMR tube, cut to the size of the imaging probe. Organisms were alive when they were placed in the tube and suspended in Fomblin (Solvay Fomblin® Y, LVAC 06/6, average mol wt. 1800), but died once immersed in the oil. Note, Fomblin is a perfluorinated oil and contributes no ^{1}H signal. In each tube there was a single control and exposed *Daphnia* to measure the differences in the contrast through proton imaging. Experiments were run in triplicate for each ligand size (3 SPION samples, 9 samples total).

Ex-vivo Daphnia magna – T_1 Weighted Spectroscopy

360 fully grown *Daphnia magna* were removed from the culture (180 from the control culture, 180 from the nanoparticles exposure), immediately frozen with liquid nitrogen to halt metabolite response, and lyophilized. 30 minutes prior to experimentation 60 freeze-dried organisms (from either control or exposed samples) were placed in a 5 mm NMR tube and re-swollen with 600 µL of 90% H_20/10% D_2O with 0.5% W/W sodium azide to prevent bacterial growth. Organisms were kept intact and analyzed at 5°C inside the NMR to prevent degradation. Experiments were conducted in triplicate.

In-vivo Daphnia magna – Metabolomics

Immediately prior to experiments, 30 *Daphnia magna* from standard cultures (not yet exposed to nanoparticles) were transferred into a specially designed low-flow *in-vivo* flow system (including an external lock capillary isolated from the organisms), as previously described.[22] The water reservoir contained the same media as the culture, and algae concentrations were maintained for the duration of the exposure to ensure no starvation responses. The sample was kept at 5°C for the experiment.[19] After 6 hours, SPIONS were added to the flow system to create a total concentration the same as that used above (10% of the LC_{50}). Organisms were monitored for a total of 24 hours. Control experiments were also conducted for a full 24 hours maintaining the flow to show differences between exposed and non-exposed organisms.

Experimental Parameters

Unless otherwise stated, all experiments were performed at 278 K using a Bruker Avance III HD NMR 500 MHz spectrometer, fitted with a 5 mm ^{13}C-^{15}N TCI Prodigy cryoprobe fitted with an actively shielded Z-gradient.

Imaging

^{1}H 2D/3D NMR imaging measurements were performed on a Bruker Avance III HD 500 MHz spectrometer equipped with Bruker micro-imaging accessory including a GREAT-60 amplifier and

a Bruker DIFF50 probe with a Z-gradient of 28.5 T m^{-1} maximum (at Bruker, Rheinstetten/Germany). For 3D microimaging acquisition with T_2-weighting, a multi-slice spin-echo sequence (MSME) was used with a repetition time (TR) of 0.5 s, an echo time (TE) of 4 ms and 16 average (NA). For T_2 analyses, a MSME-based protocol (Bruker MSME-T_2-map) was used with a resolution of 0.1 mm × 0.1 mm. All images were collected with Bruker ParaVision 6.0.1.

T_1 Weighted Spectroscopy

A pseudo 3D ^{1}H-^{13}C HSQC correlation via double inept transfer using an Echo/Antiecho-TPPI gradient, for selection and sensitivity improvement. Data were collected with 96 increments, 24 scans, at 12 different T_1's (from 0 – 2.0 s). The T_1 delay was applied with ^{13}C magnetization along Z, while a train of 180° ^{1}H pulses was applied to refocus H-C couplings. Experiments were collected with 2048-time domain points, a recycle delay of 5.0 s, $^{1}J_{CH}$ of 145 Hz, an adiabatic shape pulse for inversion (500 μs, Crp60,0.5,20.1), and an adiabatic shape pulse for refocusing (2 ms, Crp60comp.4). The 90° pulses were determined for each sample. Data were processed with a sine-squared function phase shifted by 90° in both dimensions and a zero-filling factor of 2. Fitting was performed in Bruker Protein Dynamic Center to extract the T_1 relaxation times. The 3D T_1 heat map (Figure 5) was generated using Python 3.0 via Thonny based on the percent changes of the T_1 relative to the controls. The code can be found in the supplementary materials. Briefly, a 3D gaussian shape was used to generate the peak shape for each T_1 value where the width of the gaussian was adjusted to match that of the ^{1}H-^{13}C HSQC.

In-vivo NMR Experiments

A phase sensitive ^{1}H-^{13}C HSQC using Echo/Antiecho-TPPI gradient selection was run using a total of 128 increments, each with 52 scans, 2048-time domain points, a recycle delay of 1.0 s, $^{1}J_{CH}$ of 145 Hz, and an adiabatic shape pulse for inversion (500 μs, Crp60,0.5,20.1). 90° pulses were determined for each sample. Data were processed with a sine-squared function phase shifted by 90° in both dimensions and a zero-filling factor of 2.

Spectral Assignments

Compound identification and assignment was completed using AMIX (Analysis of MIXtures software package, version 3.9.15, Bruker BioSpin, in combination with the Bruker Bio-reference NMR databases version 2-0-0 through 2-0-5). Spectra were calibrated against the Bruker Bio-reference NMR databases using tyrosine and D-glucose resonances as reference peaks. Assignments were performed using a procedure previously described.[28,29] Relative changes in metabolites during *in-vivo* exposure were found using MestReNova 14.1.0 using spectral stacks and the peaks graphs resulting from global spectral deconvolution.

Results and Discussion

Nanoparticle Size

Daphnia magna are filter feeding organisms. As they move through water, food (or particulate matter) is taken in and must pass through a filter before being ingested.[30,31] Only if the particles are the correct size, will they proceed into the gut.[31] If a particle is too large they will be removed by the *Daphnia* furcal claws, while particles that are too small pass through without interacting with the organism.[30] Previous studies have shown 0.5 μm particles result in the highest uptake

by *Daphnia magna,* but particles down to 0.1 μm have been measured.[30] To our knowledge, no studies have examined uptake and accumulation in the lower nanometer range. However, the increased prevalence of nano-sized contaminants (micro/nano plastics and nanoparticles) support the need for more research in this area. In addition, acute toxicity tests have shown that SPIONs are toxic to *Daphnia*,[12] yet the wide-spread implications and toxic mechanisms are not well understood.[32,33]

To address this gap, nanoparticles of *c.*12 nm core size were synthesized with three different ligand shell sizes of polyethylene glycol (PEG): 1, 5, and 10 kDa. PEG has low cytotoxicity, is clinically approved for some applications, and it is one of the most used ligands with SPIONs. The highly hydrated and dynamic grafted ethyleneglycol chains can provide colloidal stabilization and dispersion of the particles in isotonic media at different pH.[32] The chains also reduce protein binding *in-vivo*, which reduces uptake by macrophages in the human body.[5,32] TEM and DLS measurements were undertaken, see Figure 1. The average core size, from TEM, is 12.4 nm and the average hydrodynamic size, from DLS, progressively increasing from ~16.5, to ~18.0 to ~19.1 nm, for 1, 5 and 10 kDa grafts respectively, and the polydispersity indices were low. Hence the difference in d_{hyd} arise from the chain length, which confirms full particle dispersion (absence of aggregates in suspension). This is important as we have shown that for fully dispersed particles the MRI contrast effect is unchanged for different graft types and lengths.[34] *Daphnia magna* were then exposed to determine which size was most impactful in terms of consumption.

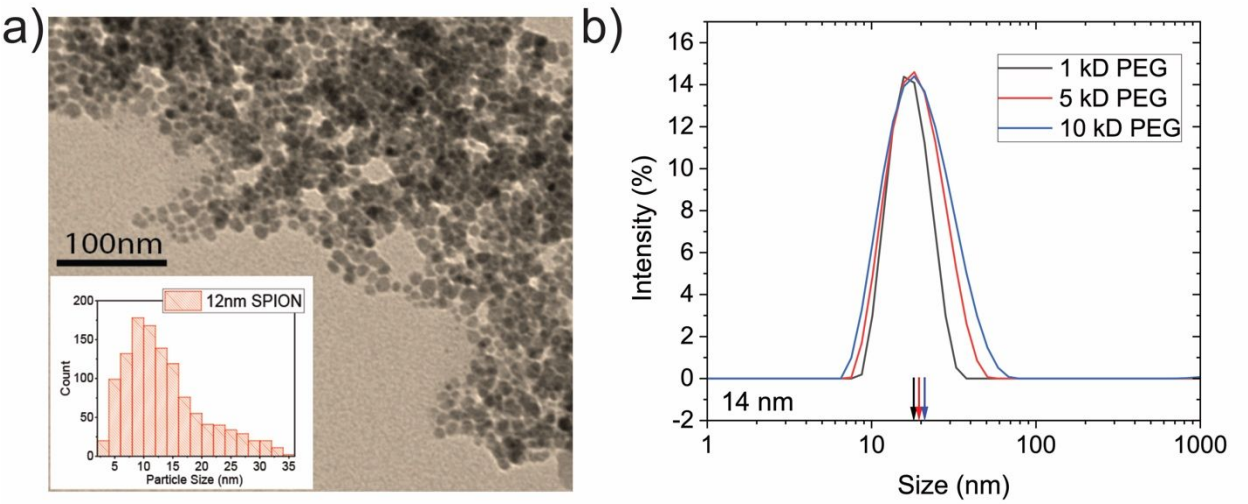

Figure 1. Confirmation of nanoparticle core and ligand size. a) Transmission electron microscopy (TEM) of the SPION cores to measure average sizing, determined to be 12.4 nm. b) Dynamic light scattering analyses of ligand (PEG)-stabilized SPION particle suspensions in H_2O. The average d_{hyd} size (core plus PEG ligands) progressively increases from 16.5 to 19.1 nm. The three suspensions were used to image *Daphnia magna* to determine the most impactful uptake based on the change in T_2 relaxation within the organism.

Imaging

In ^{1}H MRI, water inside the subject is imaged allowing for the visualization of different internal structures. One of the primary uses of SPIONs is in the medical field as an MRI contrast agent, resulting in localized darkening. To our knowledge, MR imaging of *Daphnia magna* has not yet

been reported. A 3D video with all the internal structures of a *Daphnia magna* is linked in the supporting information file, while representative still images are shown in Figure 2 to provide an overview of *Daphnia* physiology.

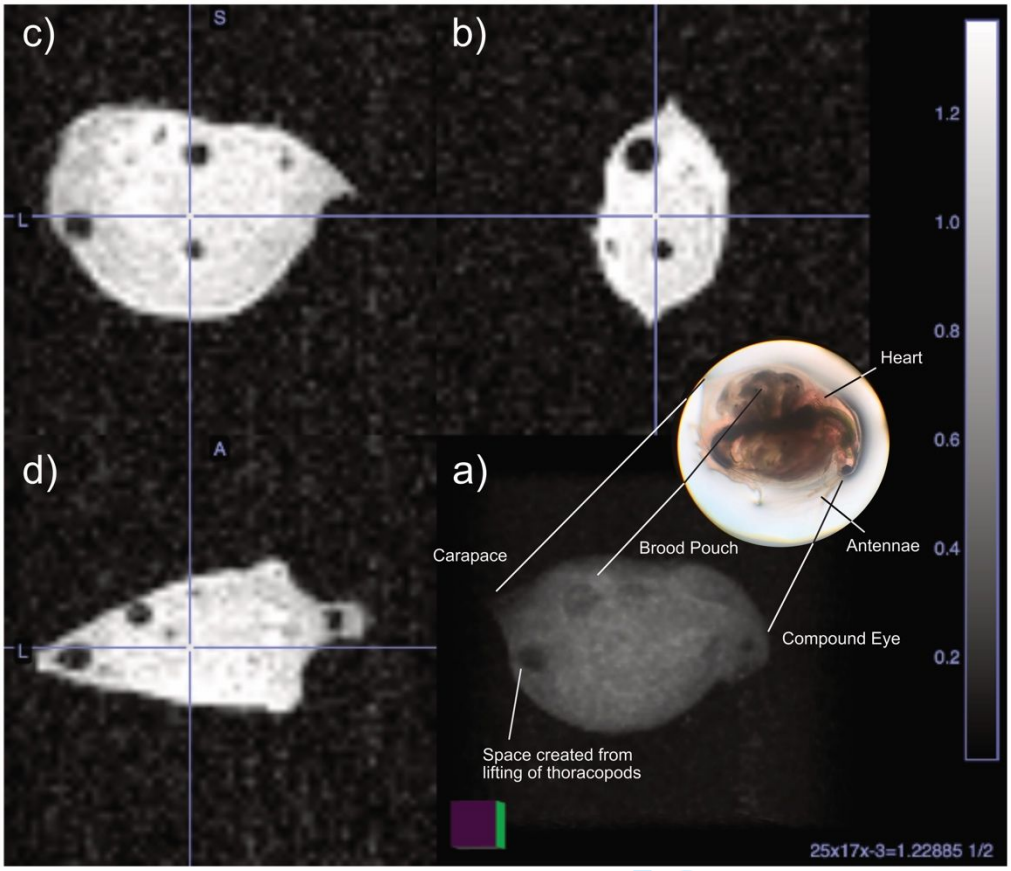

Figure 2. MRI of dead *Daphnia magna*. a) Standard spin echo image of a *Daphnia magna* with internal components labelled including compound eye, external carapace (uncalcified shell surrounding the *Daphnia*), and the spaces between the embryos inside the brood pouch. The dark space at the bottom of the *Daphnia magna* is a vacant spot due to the lifting of the thoracopods ('feet'). Note: the antennae labelled in the reference light microscope image would also be present in the MRI, but in this organism, it is tucked along the carapace rather than facing outwards. b-d) 2D sagittal, coronal, and axial planes extrapolated from the 3D image organism allowing for additional visualization of the labelled components in the 3D image.

Of more interest to this specific study is the impact of SPIONs on *Daphnia magna* images, which provide may insight as to "where" the SPIONs accumulate. Figure 3 shows an example of SPIONs inside *Daphnia magna*. On the right side is a control organism, while the left is one that has been

exposed to 10% of the LC_{50} of SPIONs for 48 hours prior to imaging.[12] Note, the organisms were washed to remove any nanoparticles interacting with the outside of the organism, allowing internal uptake by the *Daphnia* to be better evaluated. SPIONs here are T_2 contrast agents, meaning they reduce the relaxation time of the surrounding water, darkening the image where they are localized. Thus, imaging can theoretically show where SPIONs accumulate internally in the organism. Firstly, it can be confirmed that the washing was successful, and nanoparticles are not simply stuck to the outside of the organism, as the image would have been completely dark around all the edges (Figure 3). Additionally, considerable darkening is seen within the organism interior, confirming nanoparticle uptake. Nanoparticles are clearly present in the stomach and other internal compartments of the organism, but not the carapace (the outer exoskeleton of the organism). There is also a lack of darkening in the brood pouch on top of the organism, indicating the SPIONs had not yet accumulated in the eggs. Previous work examined the results of feeding 10 μm microbeads to *Daphnia,* in which they were contained strictly in the digestive track.[35] However, the widespread darkening here, especially the region around the heart, suggests the SPIONs can access many of the organs following ingestion. In particular, the heart is not visible at all. This suggests the SPIONs may be able to enter the hemolymph and thus may be transported within the organism's circulatory system.

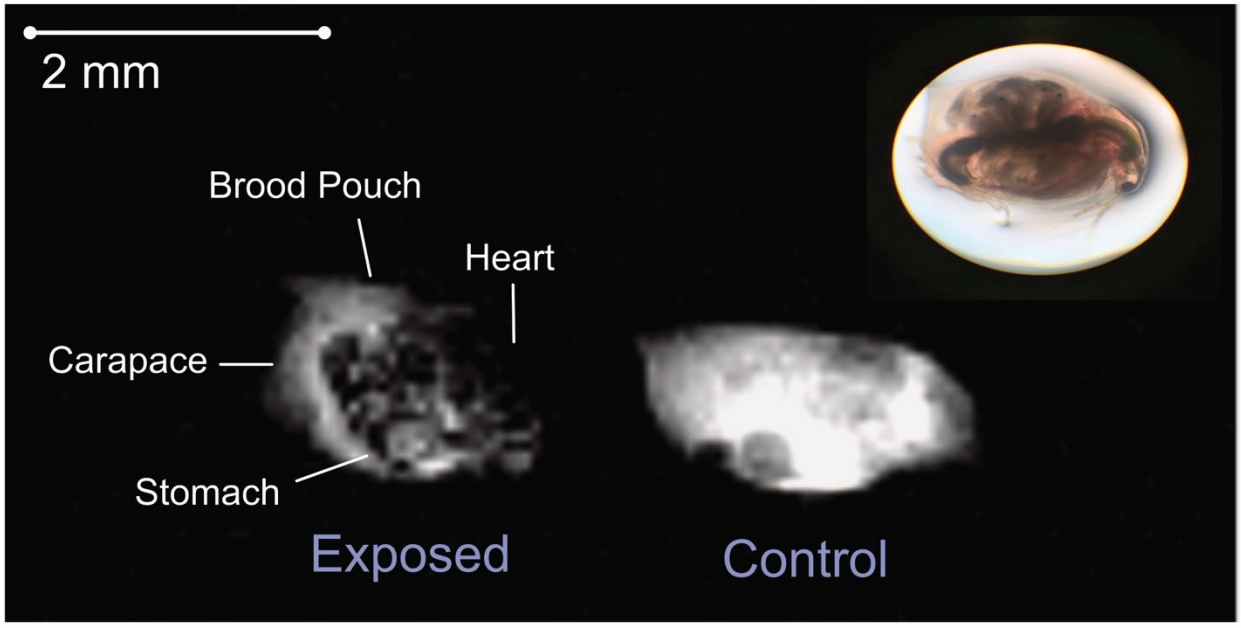

Figure 3. MRI of dead *Daphnia magna* using SPIONs as a T_2 contrast agent. The right represents an organism that was not exposed to SPIONs. The left represents a *Daphnia* which had been exposed to 10% of the LC_{50} for 48 hours prior to imaging. In the exposed organism the areas in proximity to the SPIONs are darkened. Darkening can be seen in the internal organs, stomach, and especially the heart. The regions least darkened are the carapace (the outer exoskeleton) and the brood pouch. In past studies nanoparticles have been seen to bind to the outside of the carapace, this has been reduced here via washing so that internal uptake is easier to discern.

While localization of the nanoparticles within the organism is difficult to assess given the size of the animal, the average change in the T_2 relaxation caused by the SPIONs provide useful insights

into how the different ligand sizes impact *Daphnia* consumption. The changes in the organism averaged T_2 relaxation times are shown in Figure 4. The smallest size (1 kDa ligand) appears to have little impact on the T_2 relaxation indicating it was either too small for *Daphnia* to ingest, quickly passed through the digestive system, or sedimented from the suspension (due to the shorter chain length) and was not available for uptake. All suspensions were sonicated prior to of dosing the *Daphnia* and the DLS analysis suggests no difference in the colloidal stability, so it is not clear what the cause is. In any case, of the two larger sizes, the 5 kDa ligand reduced the average relaxation time the most, consistent with it accumulating to the highest extent within the organisms. Ultimately, imaging provides an excellent way to quickly gauge nanoparticle uptake following exposure and confirms consumption. Based on the changes in average T_2 relaxation time, the 12 nm:5 kDa nanoparticles were the most impactful and were therefore selected for use in the remainder of this study.

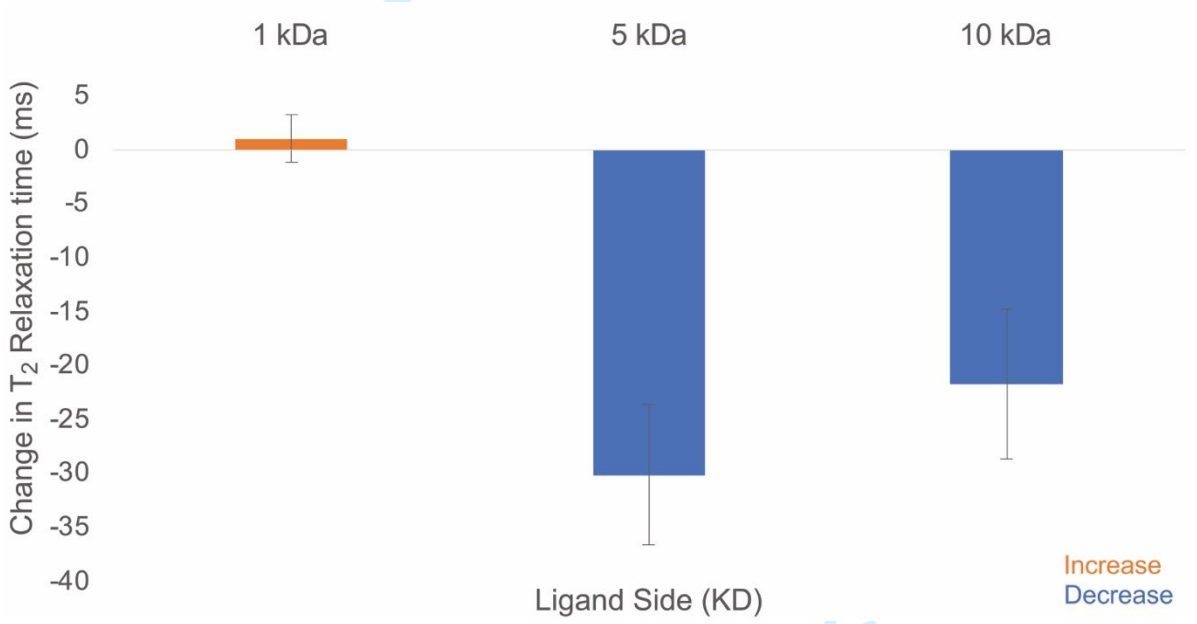

Figure 4. Changes in T_2 relaxation inside dead *Daphnia magna.* Control *Daphnia* were not exposed to nanoparticles and their T_2 relaxation was measured as an average of the whole organism. Relaxation of exposed organisms was then measured, and the change in the relaxation relative to the controls plotted. In the 1 kDa ligand there was no obvious decrease in the relaxation time (orange), indicating no uptake of nanoparticles, whereas in both 5 and 10 kDa there was a decrease. 12 nm:5 kDa nanoparticles were determined to be most impactful due to the highest average decrease in relaxation time (~30 ms) based on uptake and were chosen for further study. Each experiment was conducted in triplicate and error bars represent standard error in the measured changes.

T_1 Weighted Spectroscopy

While imaging can provide a physical overview of SPION locations, the impact of relaxation weighted spectroscopy on SPIONs can be useful in unravelling the chemistry, or the "what" with respect to binding, in the system. Due to their paramagnetic nature, SPIONs can be studied by relaxation weighted spectroscopy, which holds potential to elucidate the types of chemical structures within *Daphnia magna* they interact with. Having exploited the T_2 effect[36] of SPIONs

in imaging experiments, here the T_1 capabilities are applied. Due to the overlap in the ^{1}H NMR of *Daphnia*, ^{1}H-^{13}C HSQC is used to provide additional spectral dispersion to allow identification of chemical structures. To provide T_1 information, a 3rd dimension is acquired where a T_1 storage period is ramped via a delay list. After the processing the 3D cube contains a series of HSQC spectra which are progressively dampened via T_1 relaxation. To visualize the important differences in the data, a heat map in Figure 5 is used to show the changes in the relaxation times of each metabolite inside *Daphnia magna* relative to control organisms. Chemical shifts that are red have the largest changes in T_1, as compared to control (SPION free), where beige shows little to no interaction.

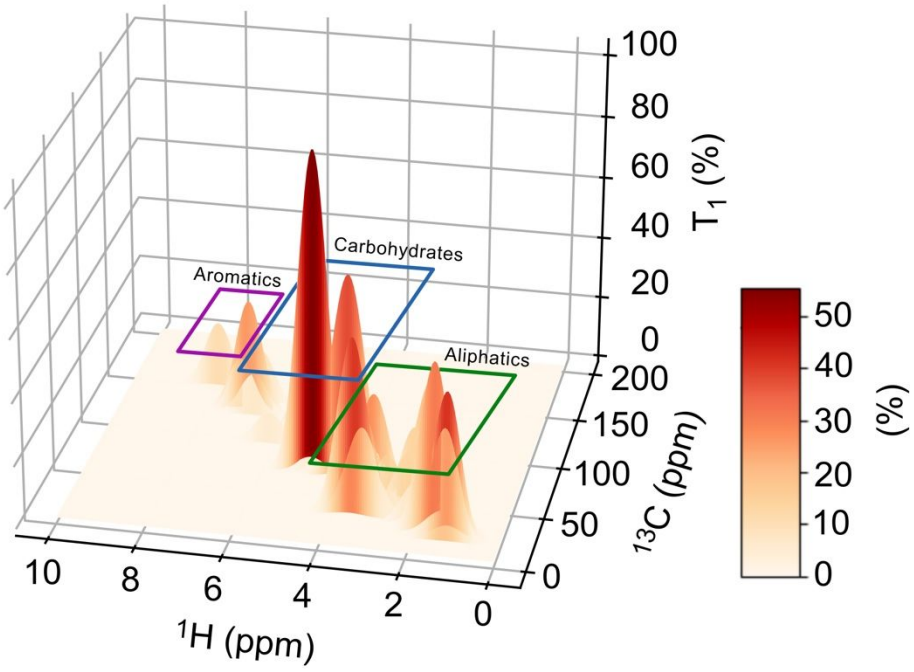

Figure 5. Changes in the T_1 relaxation, relative to the controls, inside *Daphnia magna* (dosed with 5kDa-grafted 12 nm SPIONs) as a heat map with specific regions highlighted of the intact, *ex-vivo* organism re-swollen and unwashed. Aliphatics and aromatics are boxed in green and purple respectively with most signals arising from amino acids, including alanine, asparagine, histidine, leucine, proline and tyrosine. This is most likely as a result of general protein binding. Carbohydrates are contained in the blue box, which is consistent with Chitin, the main component of the organism's carapace (shell).

In *in-vivo* ^{1}H NMR spectra there are three general δ areas, namely: aliphatics, carbohydrates, and aromatics, which can be seen in coloured boxes in Figure 5. Within the aliphatics and aromatics, the largest changes were present in the amino acid region from alanine, leucine, histidine, asparagine, proline, and tyrosine. Not all amino acids can be assigned due to spectral overlap but reduction in the T_1 time of a wide range of amino acids is generally consistent with protein binding. It is well documented that despite their great hydration and rapid dynamics PEG chains can interact with proteins in aqueous solution.[37] Protein – PEG interactions could play an important role in SPION retention and could help explain the preferential uptake of the

5kDa/10kDa coated particles observed with imaging. As imaging supports the high concentrations of SPIONs around the heart, it is possible that the SPIONs in the circulatory system are interacting with hemolymph proteins. Such interactions are well documented when high molecular weight PEG grafts are present.[38]

However, the regions with the largest percent T_1 changes were the carbohydrates. These regions most likely arise from chitin, which is the predominant structure in the carapace.[39–41] Interactions were not seen with the outside of the shell in imaging due to the washing step. However, here the intact but dead organisms are re-swollen and unwashed, thus the reduction in the T_1 times of chitin-like signals are consistent with the carapace playing an important role in SPION binding in nature, as seen with other nanoparticles in the environment.[9–11] Unfortunately, it is not possible to determine the binding mechanism, but the fact that washing removes the nanoparticles suggests the interaction is relatively weak, with one possibility being that nanoparticles are able to be partially trapped in the relatively porous chitin structure.[42]

Determining where nanoparticles bind is important to their potential uptake, retention, and impact in the organisms. However, it provides little information as to the sub-lethal toxicity. To determine how the organisms respond to the SPIONs presence, *in-vivo* metabolomics is highly complementary.

In-Vivo Spectroscopy

Thus far, imaging has provided information on the physical location of SPIONs, and relaxation weighted 2D has provided clues as to their molecular interactions. However, one missing aspect is the toxic-mode-of-action of SPIONs, or in simple terms, “how” SPIONs are toxic. *In-vivo* spectroscopy is ideal to address this gap. To our knowledge, nanoparticle impacts have not been studied using *in-vivo* NMR in *Daphnia*, but previous *in-vitro* assays have shown a decrease in energy creating metabolites, including glucose, glutamate, glutamine, tyrosine, histidine, and ATP production as a mechanism to increase molting, a documented response of the organism to the presence of externally interacting nanoparticles.[10,11]

In the current study, organisms are first allowed to acclimatize to the conditions in the flow system as per previous protocols[43] without the presence of SPIONs. After 6 hours the SPIONs are introduced and monitored for an additional 18 hours, resulting in a 24-hour total experiment time. HSQC detection is used to provide spectral dispersion required for metabolite assignment. All cross peaks were screened using manual peak-picking, and assignments were confirmed as previously described.[22] The metabolites with the most significant changes are plotted in Figure 6. As in previous reports, decreases in relative concentration of glycine, glutamine, and histidine were seen. These are key metabolites related to increased energy demand and oxidative stress.[11,16] It has also been shown in previous work that the disturbance of nanoparticles increased lactate because of anaerobic metabolism.[11] However, similar trends were not seen here, likely due to the low nanoparticle concentration. It is hypothesized that sustained exposure would eventually cause a shift to anaerobic metabolism as energy stores are depleted, and food and oxygen uptake slow due to aggregation of nanoparticles on the outside the organisms.

An additional metabolite methionine, not previously reported in nanoparticle studies on *Daphnia,* was seen to cycle with larger impact, relative to control organisms. Methionine is a key metabolite, which is replenished in the TCA cycle, indicating that energy production is being significantly impacted due to SPION exposure.[17] This could be due to oxidative stress caused from a build-up of electrons in the electron transport chain due to the new energy requirements. The increased electrons create reactive oxygen species (ROS) in the organisms, which have been directly implicated in cell aging and death.[10,44] L-glutathione was also measured to decrease relative to the control. This metabolite is used to help remove ROS by creating a protein which aids in their removal from the body.[45] This decrease indicates that both processes mentioned above may be taking place simultaneously in the organism.

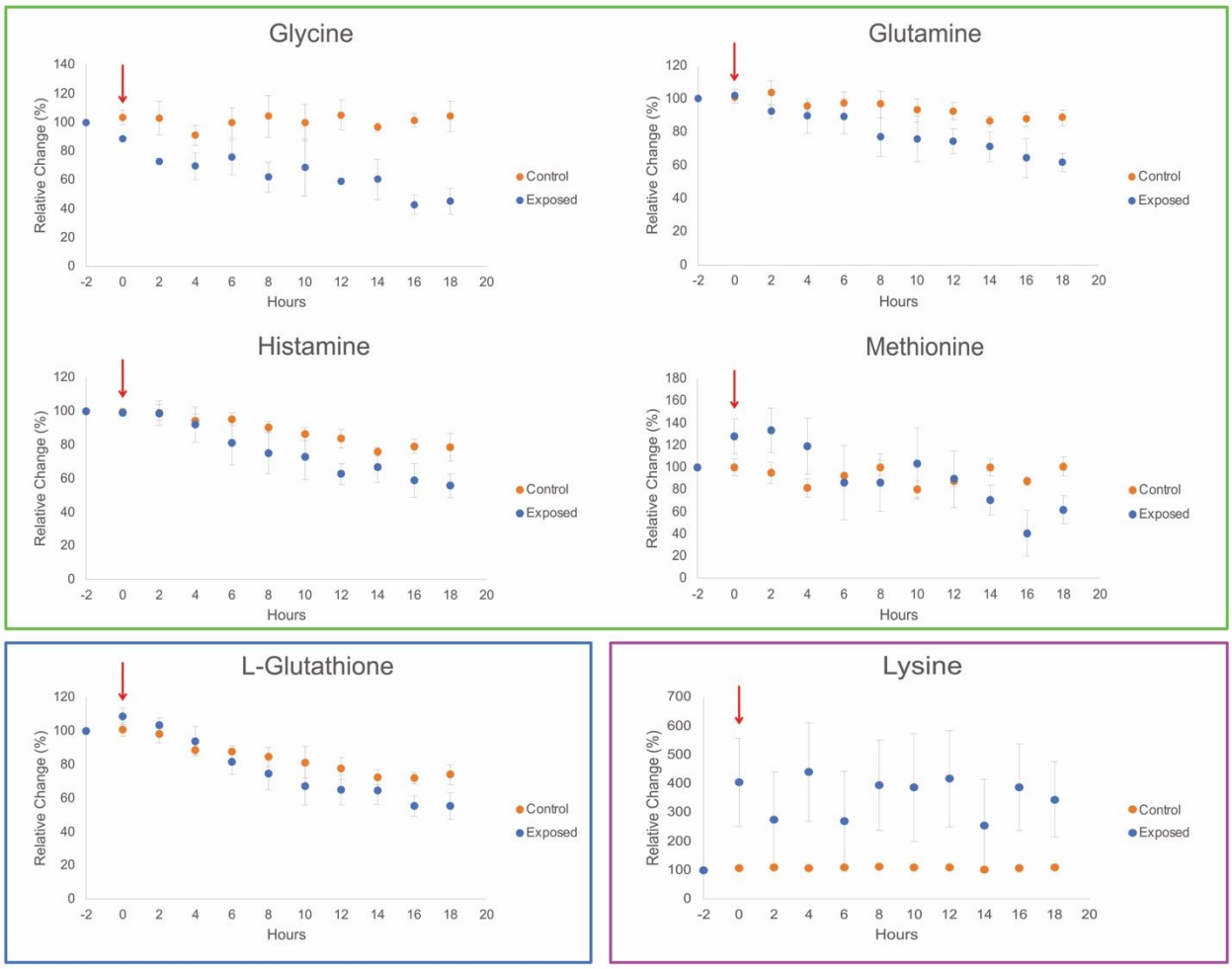

Figure 6. Metabolite responses in *Daphnia magna* due to nanoparticle exposure *in-vivo.* The upper green box shows the down regulation of metabolites resulting from increased energy metabolism, likely due to the nanoparticles both aggregating to the outside of the organisms resulting in increased molting requirements, and through consumption impacting the circulatory system. The lower left blue box indicates the down regulation of L-glutathione, an important metabolite in the removal of ROS. A decrease in this metabolite indicates it is likely being utilized to counteract ROS created from the increased energy requirements. Loss of this metabolite introduces DNA damage caused by cell aging and death. The lower right purple box indicates an increase in lysine. In each graph blue represents exposed *Daphnia,* and orange represents the control. Results are plotted as percent change relative to the first control point. The X-axis shows one point prior to exposure (-2 hours). Point 0 represents when SPIONs were exposed (also represented by a red arrow). Each experiment was conducted in triplicate and error bars represent standard error.

One additional component to note is lysine. Previous studies have indicated that lysine decreases as a response to oxidative stress response,[11] however, in this study it increased significantly in the presence of SPIONs relative to the control. As can be seen in Figure 6 (purple box), the response was quite variable and thus harder to quantify in the presence of the nanoparticles. This may be because lysine is known to bind strongly to PEG.[46,47] Historically, the combination of PEG and lysine have been used to help keep biological implants from being rejected due to their stability and ease of binding. This binding would impact the TCA pathway which has potential impacts with respect to DNA repair and protein degradation due to oxidative stress.[11,48] These results indicate that even at sub-lethal concentrations, SPIONs cause a large impact to *Daphnia magna* from which they may be unable to recover.

Conclusion

This research describes a three-pronged approach to examine toxicity in living organisms, which provide holistic understanding of how contaminants interact in complex organisms. Using all three NMR experiments together was critical to gain a complete understanding of the toxic-mode-of-action, answering "why" SPIONs are toxic. Confirmation of SPION uptake was provided by MRI, and accumulation in the area of the heart was consistent with SPIONs entering the *Daphnia's* hemolymph. Relaxation weighted spectroscopy supported two main "targets" of interaction. One with the chitin in the carapace and the other with protein, likely within the hemolymph. The organism's response manifested as an increase in energy and oxidative stress related metabolites. The results suggest that even at sub-lethal concentrations, SPIONs cause a large negative impact to *Daphnia magna*. Continued study on the longer-term impacts of SPIONs is recommended given the present environmental concentrations and projected future increases. Combining different magnetic resonance techniques provides insight into any changes in macroscopic structural characteristics and molecular processes induced by exposure to SPIONs. Similar approaches may be of value for evaluating the impact of the wide range of paramagnetic and ferromagnetic nanoparticles, and metal-organic complexes that are being released into the environment.

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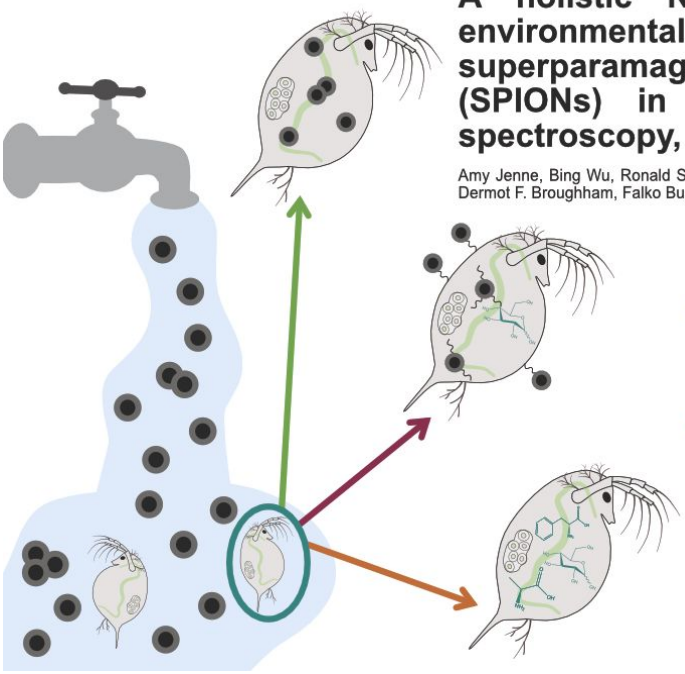

A holistic NMR framework to understand environmental impact: Examining the impacts of superparamagnetic iron oxide nanoparticles (SPIONs) in *Daphnia magna* via imaging, spectroscopy, and metabolomics

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A three-tiered approach targeting the where, what, and how of the sub-lethal toxicity of SPIONs is conducted. By using experiments in combination, we can gain a holistic understanding of **why** SPIONs are toxic to living organisms. SPIONs impact energy creating metabolites due to oxidative stress and aggregation to the organisms carapace.