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Evaluating sub-lethal stress from Roundup® exposure in Artemia franciscana using ^{1}H NMR and GC-MS

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Abstract

Global salinization trends present an urgent need for methods to monitor aquatic ecosystem health and characterize known and emerging stressors for water bodies that are becoming increasingly saline. Environmental metabolomics methods that combine quantitative measurements of metabolite levels and multivariate statistical analysis are powerful tools for ascertaining biological impacts and identifying potential biomarkers of exposure. We propose the use of the saltwater aquatic crustacean, *Artemia franciscana*, as a model organism for environmental metabolomics in saltwater ecosystems. *Artemia* are a good choice for ecotoxicity assays and metabolomics analysis because they have a short life cycle, their hemolymph is rich in metabolites and they tolerate a wide salinity range. In this work we explore the potential of *Artemia franciscana* for environmental metabolomics through exposure to the broad-spectrum herbicide, glyphosate. The LC_{50} for a 48 hr exposure of Roundup® was determined to be 237 ± 23 ppm glyphosate in the Roundup® formulation. *Artemia* cysts were hatched and exposed to sub-lethal glyphosate concentrations of 1.00, 10.0, 50.0, or 100 ppm glyphosate in Roundup®. We profiled 48 hr old *Artemia* extracts using ^{1}H NMR and GC-MS. Dose-dependent metabolic perturbation was evident for several metabolites using univariate and multivariate analyses. Metabolites significantly affected by Roundup® exposure included aspartate, formate, betaine, glucose, tyrosine, phenylalanine, gadusol, and isopropylamine. Biochemical pathway analysis with the KEGG database suggests impairment of carbohydrate and energy metabolism, folate-mediated one-carbon metabolism, *Artemia* molting and development, and microbial metabolism.

Keywords

Artemia franciscana; environmental metabolomics; Roundup; glyphosate; ^{1}H nuclear magnetic resonance; gas chromatography-mass spectrometry

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1. Introduction

Global freshwater salinization is a major threat to aquatic ecosystems, drinking water availability, and recreation (Dugan 2017; Williams 1999, 1993, 2002; Kaushal 2005). Studies predict that salinity levels for Midwestern lakes may exceed the aquatic life threshold for many ecosystems within 50 years (Dugan 2017). Midwestern lakes have long suffered from the effects of nonpoint source agricultural pollution, including eutrophication from fertilizer and animal waste and pesticide run-off (Watson 2016). Rising salinity compounded by agricultural pollution and other stressors presents a growing need for thorough monitoring of ecosystem health to better manage known and emerging stressors.

In addition to monitoring physical and chemical properties of water to identify potential aquatic hazards, ecosystem health and stressor identification may be evaluated through studies with bioindicators. Bioindicators include biological organisms, communities, or processes that can be used to evaluate the state of an ecosystem (Parmar 2016; Holt 2010). *Daphnia magna*, an aquatic microcrustacean, is a commonly used bioindicator for freshwater quality. Established standardized tests from the OECD and the EPA utilize mortality, heart rate, and fecundity as endpoints of assays using bioindicators such as *Daphnia*, but further targeted testing is typically required to identify the toxic mode of action ("Effects on Biotic Systems" 2017; Guilhermino 2000; US EPA 2013).

Studies using metabolomics have shown promise for monitoring metabolite shifts during sublethal stress and identifying toxic modes of action. The field of environmental metabolomics studies changes to an organism's small-molecule metabolite profile before and after exposure to a stressor (Lankadurai 2013; Larive 2015). The biochemical mode of action of the stressor can be elucidated by characteristic metabolite shifts resulting from the exposure. Arthropods, such as *Daphnia*, are ideal candidates for metabolomics analysis because their hemolymph circulates in an open circulatory system that is in constant contact with the organism's tissue and regularly exchanges with the surrounding environment to maintain osmotic balance and homeostasis (Campbell, JW 1970). This hemolymph is abundant in small molecule metabolites that are readily monitored through metabolomics methods.

Many standardized ecotoxicological assays rely on *Daphnia magna* to provide toxicity information for freshwater ecosystems; however, *Daphnia* are highly sensitive to salinity and are not a suitable model for ecosystems with rising chloride levels (Martínez-Jerónimo 2007; Schuytema 1997). Therefore, we propose using *Artemia franciscana* because they are closely related to *Daphnia magna* but can live in environments with a salinity range up to 300 ppm (Nunes 2006; Kerster 1983). *Artemia* fulfill all criteria of a good indicator: they are abundant and common, well-studied, economically important, and they provide measurable responses to environmental stress (Holt 2010).

The genus *Artemia* is an ancient and primitive aquatic crustacean that is found worldwide in inland saltwater lakes. *Artemia* are an interesting biological model due to their unique developmental stages. In favorable environmental conditions, females give birth to live, free-swimming *Artemia* nauplii, but when environmental conditions are poor, encysted gastrula

embryos (cysts) are released (Clegg, J 2002). These cysts are metabolically inactive but hatch once favorable environmental conditions return (Campbell 1970, Warner 2013). This phenomenon makes *Artemia* cysts ideal for ecotoxicity assays because the cysts are inexpensive, have a long shelf-life, and are easy to hatch (Nunes 2006; Gajardo 2012; Libralato 2014).

Artemia also have a short life cycle, allowing life-stage and generation specific testing. For the first few days of life, nauplii utilize internal yolk platelet organelles for energy. *Artemia* begin to feed after depleting their yolk reserves and their digestive system matures. Their naupliar eye guides them towards light to find food. *Artemia* molt through several naupliar stages and then to the juvenile and adult stages (Clegg 2002; GSLEP 2019). Because they do not require feeding and because they have a high concentration of free amino acids, the naupliar stage of development is an ideal model system for environmental metabolomics (Nunes 2006; Nakamura 2007; Helland 2000). The assay in this study was modeled after the EPA 48 hr Freshwater Daphnids Aquatic Invertebrate Acute Toxicity Test using metabolite shift as an endpoint instead of mortality (US EPA 2013). As *Artemia* are not as sensitive to standard mortality tests, the potential for environmental metabolomics application offers a new opportunity for *Artemia* use in ecotoxicity assays for studying stressors in saltwater systems.

This study uses environmental metabolomics to evaluate the effects of sub-lethal stress from the Roundup® herbicide in *Artemia*. Roundup® is a formulation of glyphosate (*N*-(phosphonomethyl) glycine), a broad-spectrum herbicide that is widely applied to actively growing weeds. As a chemical compound, glyphosate is not considered a harmful contaminant because it is minimally toxic to animals, is readily degraded by soil microbes, and has low leach potential. However, due to poor management of this herbicide, it is detected at sublethal levels in many water systems (Battaglin 2005, 2014; US EPA 2015). Roundup® is a well-characterized environmental contaminant and has been thoroughly studied in soil and freshwater systems, but there are few studies on the effects of Roundup® in saltwater lakes (Mercurio 2014; Carlisle 1988). Many studies have found that glyphosate products affect aquatic organisms, including leeches, mussels, and crayfish; therefore, we hypothesize that glyphosate may also impact saltwater organisms such as *Artemia franciscana* (Mercurio 2014; Shahl 2016; Rzymski 2013; Howe 2004).

Glyphosate kills actively growing weeds by inhibiting the shikimate pathway in plants and microorganisms (Schuette 1998; Herrmann 1999). Although the glyphosate molecule is minimally toxic to animals, long-term oral exposure to glyphosate led to reduced body weight, liver toxicity, and loose stool in dogs and rats (Shahl 2016). Recent studies have also shown that it is a possible carcinogen and it perturbs the gut microbiota of honey bees that were fed glyphosate (Shahl 2016; Motta 2018). Toxicity results for aquatic species differ from those of terrestrial species (Rzymski 2013; Schuette 1998, Rodriguez- Gil 2017). Bluegill sunfish and rainbow trout were susceptible to glyphosate exposures below the maximum contaminant level (700 ppm). Also, trace levels of glyphosate affected growth, metabolism, and energy utilization in juvenile crayfish (Avigliano 2014). Several studies have shown that the formulation of glyphosate is more toxic than the active ingredient alone. Roundup® is the Monsanto company's formulation of the active ingredient glyphosate

(Erickson 2015). Other components such as isopropylamine and polyethoxylated tallow amine (POEA) (Figure 1) are added for stabilization of the active ingredient and to facilitate delivery through plant cuticles. POEA and isopropylamine have been reported to be more toxic than glyphosic acid, and POEA variants have been found to be highly toxic to North American frogs and fairy shrimp (Howe 2004; Rodriguez- Gil 2017). An additional aim of this study is to probe the molecular mechanism of the Roundup® formulation and identify which ingredients contribute to measurable metabolic effects. However, we were unable to obtain a commercial source of POEA for this study.

This study uses nuclear magnetic resonance spectroscopy (NMR) and gas chromatography-mass spectrometry (GC-MS) to evaluate changes in *Artemia* metabolite levels in response to exposure to Roundup® herbicide. NMR is a rapid, robust, and quantitative technique that requires minimal sample preparation (Schuhmacher 2013; Barding 2012). GC-MS requires derivatization, but its lower limits of detection and well-established libraries provide greater coverage of the metabolome (Barding 2013; Halket 2005; Strehmel 2008; Nagana 2017). The complementarity of NMR and GC-MS produce a more comprehensive analysis of the *Artemia* metabolome than could be obtained with either method alone.

The aims of this study are to identify the lethal concentration (LC_{50}) of Roundup® in *Artemia*, characterize the metabolite profile of *Artemia* 48 hr after hatching using NMR and GC-MS, characterize the molecular response of *Artemia* to sub-lethal Roundup® exposure, and to better understand how the Roundup® ingredients contribute to the metabolic perturbation. Analysis with principal component analysis (PCA) and univariate statistical methods helped identify metabolite shifts resulting from sub-lethal Roundup® exposure providing insights about the mode of action.

2. Materials and Methods

2.1. Hatching and Exposures

Grade A brine shrimp cysts (Brine Shrimp Direct, Ogden, UT) were hatched in a 1 L hatching vessel (San Francisco Bay Brand, Newark, CA) in 35 g/L Oceanic Natural Sea Salt Mix (Premium Aquatics, Edinburgh, IN) added to ultrapure (EMD Millipore, Burlington, MA) water at 26.7 °C (80 °F) with constant aeration. During the 48 hr hatching period, the cysts were exposed to constant fluorescent light. Hatched nauplii were transferred from the hatching vessel into 50 mL glass jars with fresh salt water and stored in a temperature (26.7 °C) and light (16:8 hr cycle) controlled environment.

Roundup® Weed & Grass Killer Concentrate Plus purchased locally was used as the glyphosate formulation and 96% N-(phosphonomethyl)glycine, purchased from Sigma-Aldrich (St. Louis, MO), was used for the glyphosate (glyphosic acid) standard. The formulation reports 18.00 % glyphosate isopropylamine salt. Roundup® exposures in this study are reported as the concentration of glyphosate isopropylamine in the formulation. Initial range-finding studies using solutions of 0–700 ppm (4.14 mM) Roundup® or glyphosic acid revealed that neither influenced the *Artemia* hatch rate and that glyphosic acid had no effect on *Artemia* mortality over the 48 hr exposure. To determine the LC_{50} of Roundup®, 20 nauplii were transferred into each jar with 10 replicates (jars) per dose

ranging from 0 to 300 ppm (1.77 mM) Roundup®. Solutions were adjusted to pH 8.0 for all dose solutions. The numbers of dead and living *Artemia* in each jar were recorded after 48 hr. The LC_{50} for the Roundup® formulation was determined to be 237 ± 23 ppm (1.40 ± 0.14 mM).

Since mortality was not affected by unformulated glyphosic acid exposure, we focused on the impact of Roundup® for the remainder of the study. To assess the metabolic impacts of sub-lethal Roundup® exposure, cysts (1 oz) were hatched and nauplii evenly distributed into fifty individual 50 mL jars for dose-response exposures at Roundup® concentrations of 0 ppm, 1.00 ppm (5.91 μM), 10.0 ppm (59.1 μM), 50.0 ppm (296 μM), and 100 ppm (591 μM). The highest tested concentration was 100 ppm because this exposure led to minimal mortality and the specimens were not visibly stressed. After a 48 hr exposure, living *Artemia* from each jar were collected in pre-weighed 2 mL microvials and flash frozen in liquid nitrogen. The samples were thawed, and the dose solution was exchanged three times by pipetting off the supernatant and replacing it with 1.00 mL ultrapure water. The samples were lyophilized, weighed, and stored in −80 °C. The sample mass ranged from 0.50 to 6.00 mg All samples were prepared concurrently.

To assess the effect of Roundup® exposure and explore how the formulation ingredient isopropylamine contributes to toxicity, in separate experiments *Artemia* were exposed for 48 hr to a control, 50.0 ppm isopropylamine (Acros Organics, Morris, NJ) and 50.0 ppm Roundup®. Samples were collected as described above.

2.2. Metabolite Extraction

Polar metabolites were extracted from lyophilized *Artemia* samples using a methanol: deuterated water: chloroform method (Theodoridis 2012). To each sample 640 μL cold methanol (Fisher Scientific), 240 μL cold D_2O (Sigma Aldrich), and 600–750 μL Zirconia beads (Biospec Products, Bartlesville, OK) were added. The samples were mechanically homogenized using a ThermoMixer C vortex mixer (Eppendorf, Hauppauge, New York) at 4 °C for 1 min. An additional 240 μL cold D_2O and 640 μL chloroform (Macron Fine Chemicals, Center Valley, PA) were added to each sample followed by centrifugation at 15700 g for 20 min at 4 °C (Eppendorf). The top layer of the supernatant was divided into samples for analysis by NMR (800 μL) and GC-MS (100 μL) and evaporated at room temperature using a Savant SC110 speedvac equipped with a refrigerator vapor trap (RVT400) (ThermoFisher Scientific, Waltham, MA). The dried samples were stored at −80°C until analysis.

2.3. NMR Acquisition and Processing

Dried metabolite extracts were reconstituted in 200 μL 50 mM phosphate buffer (pD 7.45) in D_2O (D, 99.9%) (Cambridge Isotope Laboratories, Tewksbury, MA) containing 0.4 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonic acid-d_6 (DSS-d_6) and 0.2 mM ethylenediaminetetraacetic acid-d_{16} (EDTA) (Cambridge Isotope Laboratories, Tewksbury, MA). ^{1}H NMR spectra were acquired with a Bruker Avance III NMR spectrometer (Billerica, MA) equipped with a 5 mm TCI CryoProbe operating at 700.23 MHz. ^{1}H NMR spectra were measured with the 1D NOESY pulse sequence (noesypr1d) with presaturation

during the 120 ms mixing time and 2 s relaxation delay. Spectra were acquired at 25°C with 256 FIDs coadded, 32 dummy scans, and a 3.0 s acquisition time. A spectral width of 11.6808 ppm was used with 32768 complex data points acquired using digital quadrature detection.

Spectra were processed using Bruker Topspin 3.2 to manually phase and reference each spectrum to the resonance of DSS-d_6 (0 ppm). Spectral deconvolution and line fitting were performed using MestReNova 11 (Mestrelab Research, Escondido, CA). FIDs were apodized by multiplication with an exponential function equivalent to 1.5 Hz line broadening, zero-filled to 131072 points, and baseline corrected using a Whittaker Smoother function set to autodetect. Peak fitting was conducted using a generalized Lorentzian peak shape, a lower width constraint of 0 Hz, an upper width constraint of 30 Hz, position constraint within ± 5%, maximum number of fine iterations of 100, and local minima filter of 0. The peak fitting results were exported to Excel (Microsoft Office 2017) and the results of each spectrum were normalized to the sum of the total area between 0.8–9.0 ppm, excluding the region between 4.6–5.2 ppm. One well-resolved resonance for each metabolite was used for statistical analysis.

Metabolites were identified in the ^{1}H NMR profile of naupliar *Artemia* extracts using the metabolomics databases, Chenomx (Chenomx Inc, Edmonton, Alberta) and the Human Metabolome Database, and by comparison to spectra of authentic standards in our in-house library (Wishart 2007, 2013, 2018). Two-dimensional NMR experiments, including ^{1}H-^{1}H TOCSY, and ^{1}H-^{1}H COSY were performed to verify the assignments. At 600 MHz, the double quantum filtered COSY spectra (cosygpprqf) were measured with a 45° pulse, while the TOCSY spectra (mlevgpphw5) were measured using a mixing time of 120 ms. Both experiments were performed with 32 scans and 16 dummy scans, with 2048 points acquired in F2 and 512 in F1. The TOCSY spectra are apodized using a cosine function in both dimensions, while the COSY spectra were apodized with a sine bell function.

2.4. GC-MS Acquisition and Processing

The protocols for the derivatization, GC separation, and MS analysis were taken from Barding et al. 2013 with minor modifications. GC-MS aliquots were derivatized prior to analysis by adding 20 μL of 20 mg/mL methoxyamine (Sigma-Aldrich, St. Louis, MO) in pyridine (Thermo Scientific, Bellefonte, PA) and mixing at 300 rpm for 90 min at 37°C. A 90 μL aliquot of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) containing 1% trimethylchlorosilane (TMCS) (Thermo Scientific, Bellefonte, PA) was added to each sample and reacted for 30 min at 37°C. A 2 μL aliquot of a fatty acid methyl ester (FAMES) standard containing 0.8 mg/mL C8, C9, C10, C12, C14, and C16 and 0.4 mg/mL C18, C20, C22, C24, C26, C28, and C30 was added to each sample to reference retention times and immediately sealed with a crimp cap. Chloroform blank samples and quality control samples containing a 2 μL FAME aliquot in 110 μL chloroform were also prepared. Twenty experimental samples, a blank, and a FAMEs standard were prepared for each day of GC-MS analysis.

A blank and a FAMEs standard was run before 20 experimental samples. The ten replicate samples for each dose were analyzed sequentially as a batch. Samples were injected in

pulsed splitless mode on an Agilent J&W DB-5MS UI 30 m × 0.25 mm × 0.25 μm column (Santa Clara, CA) using an Agilent 7890A gas chromatograph coupled to a Waters GCT Premier mass spectrometer. Samples were introduced at initial oven temperature of 60 °C held for 1 min, ramped at 10 °C/min to a final temperature of 320 °C with a final 5-min hold. The injector, transfer line, and source were maintained at 230°C, 320°C, and 220°C, respectively, and the liner was changed after every 25 injections. Instrument operations were controlled by Waters MassLynx software version 4.1 (Waters Corporation, Milford, MA).

The data were collected in the Waters file format (*.raw) and converted to NetCDF (*.cdf) for compatibility with the Automated Mass Spectral Deconvolution and Identification System (AMDIS, NIST, Gaithersburg, MD). Deconvolution parameters were set to a component width of 17 scans, high resolution, high sensitivity, and medium shape. Retention indices (RIs) were calculated for each sample by AMDIS using an internal standard library and calibration standard library. Experimental samples were calibrated to the FAMEs standard run on the same day of analysis. Compounds were identified using the Golm Metabolome Database (Max Planck Institute of Molecular Plant Physiology, Potsdam, Germany) and NIST 2017 Mass Spectral Library (National Institute of Standards, Gaithersburg, MD).

MarkerLynx XS (Waters Corporation) was used for data preprocessing to collect integration values for identified metabolites. Peaks were detected without smoothing from an initial retention time of 7.00 min and a final retention time of 32.00 min, with a low-mass cutoff of 73.5 Da, a high-mass cutoff of 600 Da, and a mass accuracy of 0.10 Da. A peak-to-peak baseline noise value of 1.0, a marker intensity threshold of 25 counts, and a mass and retention time window of 0.1 Da/min, with 3.0 noise elimination were used. The results were exported to Excel where the retention times and mass were matched with identified metabolites. One mass–retention time pair with the corresponding area for each metabolite was taken for data normalization and statistical analysis. For metabolites with more than one silylation product, the most abundant mass-retention time pair for each retention time was summed to obtain one value for each metabolite. The mass–retention time pair with the highest relative abundance was chosen to represent each metabolite and this value was normalized to the total spectral area.

2.5. Data Analysis

Statistical analysis and data visualization was performed using SIMCA 14.1 (Umetrics, Malmo, Sweden) and the muma R package in R Studio (v 1.0.136) (Gaude 2013). NMR and GC-MS results for each sample were combined in SIMCA for multiblock PCA. Principal Component Analysis (PCA) is an unsupervised exploratory dataset analysis that identifies the variation between groups.

Univariate statistical analysis was performed using the muma R package. Univariate analysis was used for pairwise comparisons between doses to identify significant variables in the dataset. A significant variable for our purpose is defined as a metabolite that has a measured fold change (fc) $1.2 > fc < 0.8$ and p-value < 0.05 when comparing two conditions, in this case control versus Roundup® dose (Gaude 2013). The Welch’s t-test, Wilcoxon-Mann Whitney U Test, the Shapiro Wilk’s Test, and an outlier test are performed on each

metabolite (Gaude 2013; De Livera 2013). Volcano plots were constructed to visualize the significant variables. In these plots, the fold change and p-value for each dose vs the control group comparison are depicted, and the significant variables are shown furthest from the center of the plot.

The R package PAPi was used for pathway analysis of *Artemia* metabolites and their measured responses to Roundup® exposure (Aggio 2014). Spreadsheets were constructed according to the specifications of the PAPi package with metabolite identity, 2 sample treatments (control and 100 ppm Roundup®), and sample replicates in separate columns. PAPi queries the Kyoto Encyclopedia of Genes and Genomes database (KEGG, Kyoto University, Japan) to first convert metabolite names to KEGG codes and then to extract biochemical pathway information. An activity score is calculated from the number of metabolites identified from each pathway and the relative abundance of each metabolite in a sample. A line graph of the activity score of significant pathways ($p < 0.05$) is plotted with the control group and Bonferroni corrected p-values set as a reference. This Bonferroni correction is automatic in the PAPi calculation, it adjusts p-values to account for experimental error.

3. Results and Discussion

The *Artemia* metabolome has not previously been characterized through metabolomics studies, but much is known about their biological makeup due to its importance as a model for cellular development and as a food source for aquaculture. Most of the identified metabolites in these specimens are universal in the animal kingdom, such as the amino acids, and sugars. However, less common metabolites were also identified, namely homarine and gadusol. The *Artemia* metabolome reported herein consists of high concentrations of free amino acids, sugars, organic acids, and nucleic acid derivatives that are important for hatching, osmoregulation, detoxification, and molting (Campbell 1970; Clegg, 2002; Warner 2013). Metabolome characterization and metabolomics analysis of sublethal exposure to Roundup® identified seven endogenous and one exogenous metabolite significantly affected by exposure. Additionally, pathway analysis of the metabolite shifts suggested biochemical pathways that may be perturbed by Roundup® exposure.

3.1. Profiling the metabolite extracts of naupliar *Artemia*

The *Artemia* metabolome was characterized from a pooled sample of *Artemia* exposed to 100 ppm Roundup® to achieve improved detection of low abundance metabolites for both 1D and 2D NMR analysis. The metabolites reported in ^{1}H NMR spectra include amino acids, osmolytes, sugars, polyamines, nucleic acids, and one exogenous compound (Figure 2). The presence of these molecules was verified by resonance assignments using the 2D COSY and TOCSY spectra as well as by GC-MS (Figure SI-1) for some metabolites. Only polar metabolites were identified due to the methanol:water:chloroform extraction protocol. This was optimal for ^{1}H NMR analysis because the chloroform cleanup step removed broad lipid peaks that obscure other primary metabolites such as amino acids.

NMR and GC-MS combined identified 43 metabolites in the naupliar *Artemia* extracts (Table 1). These metabolites are classified as amino acids, sugars, osmolytes, nucleic acids,

and polyamines. Many of these same primary metabolites, especially amino acids, have been identified in *Daphnia* by ^{1}H NMR, GC-MS, and LC-MS; however, several metabolites including gadusol, trehalose, and homarine were unique to *Artemia* (Nagato 2015; Taylor 2008; Zhang 2017). Alanine, betaine, formate, lactate, taurine, homarine, glycerophosphocholine, phosphocholine, methanol, guanosine, uridine, cytidine, and gadusol were only detected by NMR. These low molecular weight compounds were not detected by GC-MS because they are not volatile or derivatized, eluted in the solvent delay of our GC method, or extensively fragmented under electron impact ionization preventing their conclusive identification and quantification. L-DOPA, cholesterol, linolenic acid, urea, spermidine, putrescine, uracil, and myo-inositol were only detected by GC-MS. These compounds are present in quantities too low to be quantified by our NMR method or are obscured by overlap in the ^{1}H NMR spectrum. Sugars, such as glucose, were better resolved in GC-MS but were identified by both techniques.

Several GC-MS peaks and/or NMR resonances were not identified in public metabolomics libraries. Isopropylamine, homarine, and gadusol were identified after a literature search (Grant 1985; Shick 2002; Tikunov 2010; Cartigny 2008). Isopropylamine and homarine were verified from available NMR spectra and standards after they were identified in literature. Isopropylamine is an exogenous compound that is an important ingredient in the Roundup® formulation, and is therefore, not considered part of the *Artemia* metabolome. Homarine has not previously been reported in *Artemia* but it has been reported in other saltwater organisms, such as the American oyster (Tikunov 2010). Homarine has been determined to be an important methyl donor and possibly serves as a methyl group reservoir (Netherton 1982; Berking 1987). Gadusol has been reported in *Artemia* and zebrafish and is an interesting compound with a UV-protective function, however, gadusol (3.502 ppm) was challenging to identify in our extracts because its NMR resonances were masked by overlap with more abundant species and because an authentic standard was not available (Shick and Dunlap 2002). The homonuclear multiple bond correlation (HMBC) spectrum aided in the structural elucidation of gadusol by revealing long range ^{1}H-^{13}C couplings (Figure SI-2). The presence of gadusol in our extracts was confirmed by mass spectroscopy (Figure SI-3) (Wishart 2007).

3.2. Dose-dependent metabolite changes

3.2.1. Roundup® exposures—Mortality studies for a 48 hr exposure were conducted to identify lethal concentrations of Roundup®. Seven concentrations ranging from 0 to 500 ppm Roundup® were evaluated with 10 replicate tanks per dose each containing 20 nauplii. Greater variance in mortality was observed at 0 ppm compared to 1 – 100 ppm Roundup®. It is unclear why the variance is greater for the control exposure, with mortality ranging from 5% to 60% within ten replicates. The lethal concentration for 50 % of the starting population of nauplii (LC_{50}) was determined to be 237 ± 23 ppm Roundup® (Figure 3). This value was used as a starting point for testing the sublethal effects in metabolomics studies and the remainder of the Roundup® exposures were conducted at approximately half the LC_{50}.

The lethal concentration determined for Roundup® is lower than the LC_{50} of glyphosate for *Daphnia magna* (930 ppm, 48 hr) and higher than the LC_{50} reported in sunfish (78 ppm, 96 hr) and trout (38 ppm, 96 hr) (Schuette 1998; Folmar 1979). We were unable to determine a lethal concentration for glyphosic acid in *Artemia* because no mortality was observed at the solubility limit (400 ppm). Saltwater organisms may be less susceptible to Roundup® due to the solubility limits of glyphosate, especially in saltwater lakes where salinity levels can reach over 300 ppm (Williams 1993). The glyphosate formulation of Roundup® had toxic effects in naupliar *Artemia* so this was the focus of the remaining exposures.

Metabolomics analysis was conducted on *Artemia* exposed to 0, 1.00, 10.0, 50.0, and 100 ppm Roundup® concentrations to elucidate the sublethal dose-dependent metabolic impacts. Using multiblock multivariate analysis, the results for NMR and GC-MS were combined for each sample to simplify the visualization/interpretation of the data in the whole dataset and confirm the identification of metabolites that contribute to the variation between groups (Figure 4). The metabolites reported in the *Artemia* metabolome (Table 1) were not all considered for statistical analysis because larger pooled sample quantities were used for profiling than were possible in the exposure experiments. The metabolites that were measurable by the experimental procedure are reported in Table SI-1.

Multiblock-PCA indicates that 35.6 % of variance can be explained by PC 1 (23.2 %) and PC 2 (12.4 %). Our results suggest dose-dependent trajectory, considerable overlap is evident among the control, 1.00 ppm and 10.0 ppm doses suggesting a similar metabolite profile for these samples. The higher Roundup® doses (50.0 ppm and 100 ppm) have greater separation from the control and lower Roundup® doses (1.0 and 10.0 ppm) indicating greater metabolic perturbation.

The PCA loading plot (Figure 4b) shows which metabolites most contribute to each component. In the score plots, component 1 contributes to the separation between control and 100 ppm and component 2 contributes to the separation between control and 50 ppm Roundup®. The PCA loading plot shows that tyrosine, aspartate, formate, glutamate, lysine, and arginine are plotted furthest from the origin in PC1. Phenylalanine, GPC, homarine, betaine, and gadusol are plotted furthest from the origin in PC2. Loading plots can be challenging to decipher when comparing multiple groups, so in order to better understand how individual metabolite levels change with exposure, univariate analyses were performed for each metabolite.

Univariate analysis was conducted with the muma package to determine which metabolites change significantly with Roundup® exposure (Table SI-2). P-values calculated between the control and treatment are reported for each dose. Differences between the results obtained by NMR and GC-MS are expected due to sample preparation, sensitivity, limits of detection, reproducibility, and data processing. For example, tryptophan significantly increased in several treatment groups but is not always observed across both instruments due to higher limits of quantitation in NMR spectra. Since the p-values vary between the techniques, volcano plots were generated to identify robust metabolite biomarkers.

The fold change and p-value for each metabolite in control and 100 ppm Roundup® samples were plotted in a volcano plot. These plots allow visualization of the metabolites with the most significant deviations as a result of exposure. As shown in Figure 5, several of the metabolites identified in both GC-MS and NMR, such as aspartate and tyrosine, are significant in both datasets. The volcano plots indicate that much of the difference between the high dose (100 ppm Roundup®) and the control can be attributed to formate, aspartate, glucose, betaine, phenylalanine, tyrosine, and gadusol. When comparing the control and 50 ppm Roundup® exposure, many of the same metabolites are affected in addition to homarine for NMR and myoinositol, proline, and arginine for GC-MS (Figure SI-5). Between 50 and 100 ppm, aspartate increases significantly by both methods, but tyrosine, arginine, L-Dopa, glucose, alanine, valine, leucine, proline, myoinositol, and urea change significantly by GC-MS (Figure SI-6).

Dose-response box and whisker plots of ^{1}H NMR and GC-MS normalized spectral area for each significant variable are plotted in Figure 6. According to ^{1}H NMR, aspartate, betaine, gadusol, and tyrosine significantly decrease with Roundup® exposure while formate levels increase. The GC-MS data trend for aspartate and tyrosine is consistent with the ^{1}H NMR results but the error bars indicate greater response variability in the GC-MS data. Threonine, glucose, phenylalanine, and isoleucine were quantified by both GC-MS and NMR, but changes were only identified as significant by GC-MS. The difference in the detection of these metabolites is likely a result of resonance overlap which made quantification by NMR less reliable. Although other metabolites were affected by Roundup® exposure, we focused our attention on the significant variables because they contribute strongly to the metabolic variation according to PCA loading plots and volcano plots.

3.3. Contribution of Roundup ingredients to metabolic perturbation

The Roundup® formulation of glyphosate contains a polyethoxylated tallow amine (POEA) surfactant and isopropylamine stabilizing salt. Isopropylamine (Figure 2A), was identified in the ^{1}H NMR spectrum of *Artemia* exposed to 50.0 ppm and 100 ppm Roundup® (Cartigny 2008). An exposure of *Artemia* to 50.0 ppm isopropylamine and 50.0 ppm Roundup® was performed to identify the effects of the Roundup® ingredients. The PCA score plot (Figure SI-8) shows variation among the isopropylamine and Roundup® exposures, but many of the same metabolites are affected, indicating that some of the impact observed in exposure to formulated Roundup® can be attributed to isopropylamine toxicity (Tsui 2003). An assessment of the environmental impact quotient of active and inert pesticide ingredients found that POEA and isopropylamine have higher dermal toxicity than glyphosate for human, avian, fish, and arthropods (Surgan 2010). POEA is essential for facilitating the uptake of glyphosate by plants and was found to be toxic in certain frog, bacteria, algae, protozoa, and crustacea species (Howe 2004; Tsui 2003). We were unable to identify a commercial source of POEA; therefore, it is unclear in this study whether POEA contributes to *Artemia* toxicity by facilitating the uptake of glyphosate and isopropylamine or because the surfactant is toxic on its own.

3.4. Biological interpretation of endogenous metabolic perturbations

Pathway activity profiling was used to provide biological insights for metabolomics results using the PAPi R package. Global metabolite shifts from the 100 ppm Roundup® exposure were correlated with metabolic pathway information found in the Kyoto Encyclopedia of Genes and Genomes (KEGG) to predict biochemical pathways and compare their activity between the dosed and control experimental conditions (Aggio 2014). A negative activity score (AS) indicates the pathway is down-regulated compared to the control group and a positive AS indicates the pathway is up-regulated. Between GC and NMR, 87 unique pathways may have been significantly altered from Roundup® exposure with 10 pathways identified in both NMR and GC metabolome (SI-7, SI-8). Many of the pathways are not likely directly relevant to the *Artemia* metabolome, such as alcoholism and nicotine addiction, since the KEGG includes the genome for many different organisms.

The metabolites that were significantly affected ($p < 0.05$) by Roundup® exposure (100 ppm vs control) include aspartate, betaine, tyrosine, phenylalanine threonine, isoleucine, formate, gadusol, and glucose. PAPi analysis indicates that Roundup® exposure may significantly alter pathways related to these metabolites, including amino acid metabolism, carbohydrate metabolism, energy metabolism, vitamin metabolism, and biosynthesis of secondary metabolites.

Glucose levels increased significantly with Roundup® exposure. PAPi analysis predicted down regulation of pathways involved in carbohydrate metabolism, such as glycolysis. Glucose has been identified as a cryoprotectant in earthworm species (Bundy 2003) and in *Artemia franciscana* (Clegg 2002), our observations show that large amounts of glucose accumulate in cold-stressed naupliar *Artemia*, therefore it may protect internal structures from damage from other xenobiotic stressors or adverse environmental conditions (Teets 2013). In the encysted *Artemia* embryo, energy is stored in the form of trehalose. As the embryo emerges and develops through the naupliar stages, trehalose is converted to glucose with the help of the proteolytic trehalase enzyme (Clegg 2002; Teets 2013; Yancey 2005). Trehalase and the other proteolytic enzymes involved in *Artemia* development are highly sensitive to environmental conditions, such as temperature, oxygen levels, and pH (Yancey 2005; Ezquieta 1985). Glucose accumulation may reflect suspended development and increased energy storage in response to the poor environmental conditions induced by the Roundup® exposure.

Amino acids are readily identified by NMR and GC-MS and they are abundant in *Artemia*, therefore it is reasonable that many of the pathways identified by PAPi are related to amino acid metabolism. Many of these pathways were suggested to be down-regulated at the high dose Roundup® exposure, which may indicate a protective stress response. Cysteine proteases (CP) are essential enzymes in *Artemia* for yolk utilization and growth in the early stages of pre- and post-emergence development. (Clegg 2002; Ezquieta 1985; James 1998). When *Artemia* are under stress from unfavorable environmental conditions, these proteolytic enzymes may be inhibited to prevent nutrient and energy loss. Inhibition of CP may account for the overall observed decrease in metabolite concentrations at higher Roundup® doses and for the down-regulation in pathways related to amino acid metabolism.

The negative activity score assigned to the degradation of aromatic compounds metabolic pathway and the positive activity score assigned to tyrosine metabolism may be related to the decreasing levels of tyrosine with Roundup® exposure (Figure 6). Tyrosinase is an enzyme located in crustacean hemolymph that is active during crustacean molt cycles and is important for converting tyrosine into *N*-acetyldihydroxyphenylalanine, which ultimately forms the chitin exoskeleton (Clegg 2002; Chang 1993). The developing *Artemia* nauplii are expected to undergo two molt cycles within the timeframe of the experiment (48 hr hatch, 48 hr exposure) (Clegg 2002; Gajardo 2012; GSLEP 2019). Perturbations of tyrosine metabolism suggests that some of the *Artemia* exposed to Roundup® may have been in the process of molting when they were sacrificed for the experiment. This could indicate either faster or slower development compared to the control group. Tyrosinase is also an important enzyme for wound healing in arthropods. Early juvenile crayfish exposed to glyphosate had elevated aspartate transaminase to alanine transaminase levels (ASAT:ALAT) (Avigliano 2014). Elevated ASAT:ALAT is an indicator of tissue damage. ASAT converts aspartate and α-ketoglutarate into glutamate and oxaloacetate. ALAT converts alanine and α-ketoglutarate into glutamate and pyruvate. Therefore, decreased aspartate levels and increased glutamate levels in *Artemia* exposed to Roundup® may correlate to elevated ASAT:ALAT which would necessitate upregulation of tyrosinase activity to counter tissue damage. Tissue damage may be a result of isopropylamine accumulation and the reported dermal toxicity of isopropylamine and POEA (Surgan 2010).

Gadusol and betaine levels decreased with Roundup® exposure. Gadusol is an interesting metabolite prevalent in marine organisms as a sunscreen-like molecule that absorbs UV radiation. (Shick 2002; Grant 1985; Khosravi 2013). A study on *Artemia* in Lake Urmia determined that the bioaccumulation of mycosporine-like amino acids, such as gadusol, is affected by salinity and UV radiation, suggesting that gadusol also contributes to osmoregulatory function (Khosravi 2013). Considering that betaine is an osmolyte and gadusol has a similar dose-dependent response, Roundup® or POEA exposure may affect osmoregulation.

The concentration of formate increases significantly with the increasing Roundup® concentration. Formate is an essential intermediate in folate-mediated one-carbon metabolism. Formate has been identified as a possible biomarker for deficiency in folate and vitamin B_{12} and downregulation of one-carbon metabolic processes (Lamarre 2013; Fox 2008). One-carbon metabolism involves metabolic processes where methyl groups from donors, such as serine, choline, glycine, betaine, and methionine, are interchanged and transferred using folate cofactors (Fox 2008; Bailey 2015; Locasale 2013). Thiamine, nicotinamide, vitamin B_{12}, pantothenate, riboflavin, pyridoxine, folic acid, biotin, and inositol have been identified as necessary vitamins for successful *Artemia* cultures (Campbell 1970). Formic acid is a toxic metabolite so accumulation resulting from vitamin deficiency from Roundup® exposure may cause delayed development and mortality (Lamarre. 2013; Fox 2008).

Many of the pathways identified through PAPi are related to plant and bacteria metabolic pathways. Carbon fixation in prokaryotes, porphyrin and chlorophyll metabolism, and microbial metabolism in diverse environments were suggested to be down-regulated while

bacterial chemotaxis, puromycin biosynthesis, and novobiocin biosynthesis were upregulated. These pathways are correlated to the changes measured in aromatic amino acids, such as tyrosine, L-DOPA, and phenylalanine and also gadusol. *Artemia* either obtain gadusol from the consumption of algae, from gut microbes, or through a biosynthetic pathway that has been identified in zebrafish (Brotherton 2015; Osborn 2015; Grant 1985; Carreto 2005). Microbes synthesize gadusol through the shikimate pathway, which is the targeted pathway for the herbicidal activity of glyphosate so reduced expression of gadusol with increased Roundup® exposure may indicate an effect on the microbial community (Motta 2018). Gut microbes play an important role in the *Artemia* life cycle and the genome of this community has been sequenced (Riddle 2013; Nougue 2015). However, the literature about the impact of Roundup® on the soil and gut microbiome has produced inconsistent and contradictory results. Some studies point to an increase in bacterial diversity and abundance, other studies report that prolonged Roundup® exposure causes a shift in bacterial community composition towards glyphosate-tolerant species, and some studies report no effect on gut or soil microbes (Imparato 2016; Pizarro 2016; Allegrini 2017; Newman et al. 2016; Riede et al. 2016; Zabaloy et al. 2016). Further studies on the gut microbes in *Artemia* are necessary to elucidate the impact of Roundup® exposure.

4. Conclusion

The metabolome provides important insight into how an organism responds to its environment and metabolomics is proving to be a promising method for understanding external stressors. We believe that *Artemia* is a great model organism for metabolomics studies because the hemolymph metabolites are readily studied by analytical methods and they are highly responsive to environmental cues. It is a versatile model system and by studying the naupliar stage of *Artemia* development, we were able to identify an impact of Roundup® on developmentally important metabolites, such as trehalose. *Artemia* have many different metabolites that work to maintain the osmotic balance, and we have also shown through metabolomics how these osmolytes respond to Roundup® stress.

Forty-three polar metabolites were identified by NMR and GC-MS in this study, and they were carefully characterized and verified. We found that these methods are complementary and provide good coverage of metabolite classes. NMR is especially useful for identifying uncommon metabolites, such as gadusol and homarine, because multiple experiments can be run on each sample and the spectra are highly reproducible. Using multiblock-PCA, we were able to combine the datasets, show that they are in agreement, and look at the big picture effects on dose-dependent metabolite shifts.

Roundup® was chosen for this study because there is ample literature on the effects of this compound on nontarget organisms, but there has not been a study on *Artemia* and few studies for saltwater organisms; there is also no scientific consensus on the safety of exposure to this compound. Although we determined that environmentally relevant concentrations of Roundup® are not a major concern to *Artemia*, using metabolomics we were able to identify potential pathways and biomarkers of toxicity. There is a growing body of literature that points to Roundup® impacting the gut microbiome. Our results also suggest that this may be the case for *Artemia*; however, this was not verified conclusively in

our study. Future work will involve the use of *Artemia* metabolomics to characterize the sublethal mode of action for other aquatic stressors that have been identified as emerging environmental contaminants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
COSY	Correlation Spectroscopy
GC-MS	Gas Chromatography – Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
PCA	Principal Component Analysis
PLS-DA	Partial Least Squares – Discriminant Analysis
POEA	Polyethoxylated Tallow Amine
TOCSY	Total Correlation Spectroscopy
HMBC	Heteronuclear multiple bond correlation spectroscopy

5. References

Aggio Raphael B. M. 2014 “Pathway Activity Profiling (PAPi): A Tool for Metabolic Pathway Analysis” In Yeast Metabolic Engineering, 233–50. Methods in Molecular Biology. Humana Press, New York, NY 10.1007/978-1-4939-0563-8_14.

Allegrini Marco, Elena del V. Gomez, and María Celina Zabaloy. 2017 “Repeated Glyphosate Exposure Induces Shifts in Nitrifying Communities and Metabolism of Phenylpropanoids.” Soil Biology and Biochemistry 105 (Supplement C): 206–15. 10.1016/j.soilbio.2016.11.024.

Avigliano L, Fassiano AV, Medesani DA, De Molina MC Ríos, and Rodríguez EM. 2014 “Effects of Glyphosate on Growth Rate, Metabolic Rate and Energy Reserves of Early Juvenile Crayfish, Cherax Quadricarinatus M.” Bulletin of Environmental Contamination and Toxicology 92 (6): 631–35. [PubMed: 24584268]

Bailey Lynn B., Stover Patrick J., McNulty Helene, Fenech Michael F., Gregory Jesse F., Mills James L., Pfeiffer Christine M., et al. 2015 “Biomarkers of Nutrition for Development—Folate Review.” The Journal of Nutrition 145 (7): 1636S–1680S. 10.3945/jn.114.206599. [PubMed: 26451605]

Barding Gregory A., Barding Gregory A., Béni Szabolcs, Fukao Takeshi, Julia Bailey-Serres, and Larive Cynthia K.. 2013 “Comparison of GC-MS and NMR for Metabolite Profiling of Rice Subjected to Submergence Stress.” Journal of Proteome Research 12 (2): 898–909. 10.1021/pr300953k. [PubMed: 23205590]

Barding Gregory A., Salditos Ryan, and Larive Cynthia K.. 2012 “Quantitative NMR for Bioanalysis and Metabolomics.” Analytical and Bioanalytical Chemistry 404 (4): 1165–79. 10.1007/s00216-012-6188-z. [PubMed: 22766756]

Battaglin William A. 2014 “Glyphosate and Its Degradation Product AMPA Occur Frequently and Widely in U.S. Soils, Surface Water, Groundwater, and Precipitation.” JAWRA Journal of the American Water Resources Association 50 (2): 275–90. 10.1111/jawr.12159.

Battaglin William A. 2005 “Glyphosate, Other Herbicides, and Transformation Products in Midwestern Streams, 20021.” JAWRA Journal of the American Water Resources Association 41 (2): 323–32. 10.1111/j.1752-1688.2005.tb03738.x.

Berking S 1987 “Homarine (N-Methylpicolinic Acid) and Trigonelline (N-Methylnicotinic Acid) Appear to Be Involved in Pattern Control in a Marine Hydroid.” Development (Cambridge, England) 99 (2): 211–20.

Brotherton Carolyn A. 2015 “Biochemistry: Shedding Light on Sunscreen Biosynthesis in Zebrafish.” ELife 4 (5): e07961 10.7554/eLife.07961.

Bundy Jacob G., Hans Ramløv, and Martin Holmstrup. 2003 “Multivariate Metabolic Profiling Using 1H Nuclear Magnetic Resonance Spectroscopy of Freeze-Tolerant and Freeze-Intolerant Earthworms Exposed to Frost.” Cryoletters 24 (6): 347–58. [PubMed: 14671686]

Campbell JW, ed. 1970 “Nitrogen Metabolism in Non-Insect Arthropods” In Comparative Biochemistry of Nitrogen Metabolism, 299–372. Academic Press INC.

Carlisle SM 1988 “Glyphosate in the Environment.” Water, Air, and Soil Pollution 39 (3–4): 409–20. 10.1007/BF00279485.

Carreto José. 2005 A High-Resolution Reverse-Phase Liquid Chromatography Method for the Analysis of Mycosporine-like Amino Acids (MAAs) in Marine Organisms. Vol. 146 10.1007/s00227-004-1447-y.

Cartigny Bernard, Azaroual Nathalie, Imbenotte Michel, Mathieu Daniel, Parmentier Erika, Vermeersch Gaston, and Lhermitte Michel. 2008 “Quantitative Determination of Glyphosate in Human Serum by 1H NMR Spectroscopy.” Talanta 74 (4): 1075–78. 10.1016/j.talanta.2007.07.029. [PubMed: 18371753]

Chang Ernest S. 1993 “Regulation of Crustacean Molting: A Multi-Hormonal System.” Integrative and Comparative Biology 33 (3): 324–29. 10.1093/icb/33.3.324.

Clegg J 2002 “Physiological and Biochemical Aspects of Artemia Ecology” In Artemia Basic and Applied Biology. Vol. 1 Biology of Aquatic Organisms. Kluwer Academic Publisher.

Livera De, Alysha M, Moshe Olshansky, and Terence P. Speed. 2013 “Statistical Analysis of Metabolomics Data” In Metabolomics Tools for Natural Product Discovery, edited by Roessner Ute and Dias Daniel Anthony, 291–307. Methods in Molecular Biology 1055. Humana Press 10.1007/978-1-62703-577-4_20.

Dugan Hilary A., Bartlett Sarah L., Burke Samantha M., Doubek Jonathan P., Krivak-Tetley Flora E., Skaff Nicholas K., Summers Jamie C., et al. 2017 “Salting Our Freshwater Lakes.” Proceedings of the National Academy of Sciences 114 (17): 4453–58. 10.1073/pnas.1620211114.

“Effects on Biotic Systems.” 2017 In OECD Guidelines for the Testing of Chemicals. OECD Publishing http://www.oecd-ilibrary.org/.

Erickson B 2015 “Rocky Road for Roundup.” Chemical & Engineering News 93 (37): 10–15.

Ezquieta Begoña, and Vallejo Carmen G.. 1985 “The Trypsin-like Proteinase of Artemia: Yolk Localization and Developmental Activation.” Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 82 (4): 731–36. 10.1016/0305-0491(85)90517-6.

Folmar LC 1979 “Toxicity of the Herbicide Glyphosphate and Several of Its Formulations to Fish and Aquatic Invertebrates.” Archives of Environmental Contamination and Toxicology 8 (3): 269–78. [PubMed: 507937]

Fox Jennifer T. 2008 “Chapter 1 Folate- Mediated One- Carbon Metabolism” In Vitamins & Hormones, 79:1–44. Folic Acid and Folates. Academic Press 10.1016/S0083-6729(08)00401-9. [PubMed: 18804690]

Gajardo Gonzalo M. 2012 “The Brine Shrimp Artemia: Adapted to Critical Life Conditions.” Frontiers in Physiology 3 (6). 10.3389/fphys.2012.00185.

Gaude Edoardo, Chignola Francesca, Spiliotopoulos Dimitrios, Spitaleri Andrea, Ghitti Michela, Garcia-Manteiga Jose M., and Mari Silvia and Musco Giovanna. 2013 “Muma, An R Package for Metabolomics Univariate and Multivariate Statistical Analysis.” Current Metabolomics. 3 31, 2013. http://www.eurekaselect.com/107837/article.

Grant Patrick T., Middleton Colin, Plack Peter A., and Thomson Ronald H.. 1985 “The Isolation of Four Aminocyclohexenimines (Mycosporines) and a Structurally Related Derivative of Cyclohexane-1:3-Dione (Gadusol) from the Brine Shrimp, Artemia.” Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 80 (4): 755–59. 10.1016/0305-0491(85)90457-2.

Barding Gregory A. Jr. 2013 “Comparison of GC-MS and NMR Metabolite Profiling of Rice Subjected to Submergence Stress,” no. 12: 898–909.

GSLEP. 2019 “Brine Shrimp: Parthenogenesis and Shrimp Genetics.” Great Salt Lake Ecosystem Program. 2019. http://wildlife.utah.gov/gsl/brineshrimp/parthenogenesis.php.

Guilhermino L, Diamantino T, Silva MC, and Soares AM. 2000 “Acute Toxicity Test with Daphnia Magna: An Alternative to Mammals in the Prescreening of Chemical Toxicity?” Ecotoxicology and Environmental Safety 46 (3): 357–62. 10.1006/eesa.2000.1916. [PubMed: 10903834]

Halket John M., Waterman Daniel, Przyborowska Anna M., Patel Raj K. P., Fraser Paul D., and Bramley Peter M.. 2005 “Chemical Derivatization and Mass Spectral Libraries in Metabolic Profiling by GC/MS and LC/MS/MS.” Journal of Experimental Botany 56 (410): 219–43. 10.1093/jxb/eri069. [PubMed: 15618298]

Helland S, Triantaphyllidis GV, Fyhn HJ, Evjen MS, Lavens P, and Sorgeloos P. 2000 “Modulation of the Free Amino Acid Pool and Protein Content in Populations of the Brine Shrimp Artemia Spp.” Marine Biology 137 (5–6): 1005–16. 10.1007/s002270000409.

Herrmann Klaus M., and Weaver Lisa M.. 1999 “THE SHIKIMATE PATHWAY.” Annual Review of Plant Physiology and Plant Molecular Biology 50 (6): 473–503. 10.1146/annurev.arplant.50.1.473.

Holt E 2010 “Bioindicators: Using Organisms to Measure Environmental Impacts” 3 (10): 8.

Howe Christina M., Berrill Michael, Pauli Bruce D., Helbing Caren C., Werry Kate, and Veldhoen Nik. 2004 “Toxicity of Glyphosate-Based Pesticides to Four North American Frog Species.” Environmental Toxicology and Chemistry 23 (8): 1928–38. 10.1897/03-71. [PubMed: 15352482]

Imparato Valentina, Santos Susana S., Johansen Anders, Geisen Stefan, and Winding Anne. 2016 “Stimulation of Bacteria and Protists in Rhizosphere of Glyphosate-Treated Barley.” Applied Soil Ecology. http://agris.fao.org/..

James Margaret O, and Sean M Boyle. 1998 “Cytochromes P450 in Crustacea.” Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 121 (1–3): 157–72. 10.1016/S0742-8413(98)10036-1.

Kaushal Sujay S., Groffman Peter M., Likens Gene E., Belt Kenneth T., Stack William P., Kelly Victoria R., Band Lawrence E., and Fisher Gary T.. 2005 “Increased Salinization of Fresh Water in the Northeastern United States.” PNAS. 102 (38): 13517–20. 10.1073/pnas.0506414102. [PubMed: 16157871]

Kerster Harold W. 1983 “Brine Shrimp (Artemia Salina) Nauplii as a Teratogen Test System.” Ecotoxicology and Environmental Safety 7 (3): 342–49. 10.1016/0147-6513(83)90079-9. [PubMed: 6872918]

Khosravi Sanaz, Khodabandeh Saber, Agh Naser, and Bakhtiarian Mahdieh. 2013 “Effects of Salinity and Ultraviolet Radiation on the Bioaccumulation of Mycosporine-like Amino Acids in Artemia from Lake Urmia (Iran).” Photochemistry and Photobiology 89 (2): 400–405. 10.1111/j.1751-1097.2012.01245.x. [PubMed: 22998644]

Lamarre Simon G., Morrow Gregory, Macmillan Luke, Brosnan Margaret E., and Brosnan John T.. 2013 “Formate: An Essential Metabolite, a Biomarker, or More?” Clinical Chemistry and Laboratory Medicine 51 (3): 571–78. 10.1515/cclm-2012-0552. [PubMed: 23241677]

Lankadurai Brian P. 2013 “Environmental Metabolomics: An Emerging Approach to Study Organism Responses to Environmental Stressors.” Environmental Reviews 21 (3): 180–205. 10.1139/er-2013-0011.

Larive Cynthia K. 2015 “NMR Spectroscopy for Metabolomics and Metabolic Profiling.” Analytical Chemistry 87 (1): 133–46. 10.1021/ac504075g. [PubMed: 25375201]

Libralato G 2014 “The Case of Artemia Spp. in Nanoecotoxicology.” Marine Environmental Research 101 (10): 38–43. 10.1016/j.marenvres.2014.08.002. [PubMed: 25195085]

Locasale Jason W. 2013 “Serine, Glycine and the One-Carbon Cycle: Cancer Metabolism in Full Circle.” Nature Reviews. Cancer 13 (8): 572–83. 10.1038/nrc3557. [PubMed: 23822983]

Martínez-Jerónimo Fernando, and Laura Martínez-Jerónimo. 2007 “Chronic Effect of NaCl Salinity on a Freshwater Strain of Daphnia Magna Straus (Crustacea: Cladocera): A Demographic Study.” Ecotoxicology and Environmental Safety 67 (3): 411–16. 10.1016/j.ecoenv.2006.08.009. [PubMed: 17055052]

Mercurio Philip, Flores Florita, Mueller Jochen F., Carter Steve, and Negri Andrew P.. 2014 “Glyphosate Persistence in Seawater.” Marine Pollution Bulletin, 7th International Conference on Marine Pollution and Ecotoxicology, 85 (2): 385–90. 10.1016/j.marpolbul.2014.01.021.

Motta Erick V. S. 2018 “Glyphosate Perturbs the Gut Microbiota of Honey Bees.” PNAS. 115 (41): 10305–10. 10.1073/pnas.1803880115. [PubMed: 30249635]

Nagana Gowda GA, and Daniel Raftery. 2017 “Recent Advances in NMR-Based Metabolomics.” Analytical Chemistry 89 (1): 490–510. 10.1021/acs.analchem.6b04420. [PubMed: 28105846]

Nagato Edward G., Lankadurai Brian P., Soong Ronald, Simpson André J., and Simpson Myrna J.. 2015 “Development of an NMR Microprobe Procedure for High-Throughput Environmental Metabolomics of Daphnia Magna.” Magnetic Resonance in Chemistry: MRC 53 (9): 745–53. 10.1002/mrc.4236. [PubMed: 25891518]

Nakamura Kaworu, Iwaizumi Koichi, and Yamada Shoji. 2007 “Hemolymph Patterns of Free Amino Acids in the Brine Shrimp Artemia Franciscana after Three Days Starvation at Different Salinities.” Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 147 (1): 254–59. 10.1016/j.cbpa.2007.01.008.

Netherton JC, and Gurin S. 1982 “Biosynthesis and Physiological Role of Homarine in Marine Shrimp.” The Journal of Biological Chemistry 257 (20): 11971–75. [PubMed: 7118923]

Newman Molli M., Lorenz Nicola, Hoilett Nigel, Lee Nathan R., Dick Richard P., Liles Mark R., Ramsier Cliff, and Kloepper Joseph W.. 2016 “Changes in Rhizosphere Bacterial Gene Expression Following Glyphosate Treatment.” The Science of the Total Environment 553 (5): 32–41. 10.1016/j.scitotenv.2016.02.078. [PubMed: 26901800]

Nougue Odrade, Gallet Romain, Chevin Luis-Miguel, and Lenormand Thomas. 2015 “Niche Limits of Symbiotic Gut Microbiota Constrain the Salinity Tolerance of Brine Shrimp.” American Naturalist 186 (3): 390–403. 10.1086/682370.

Nunes Bruno S., Carvalho Félix D., Guilhermino Lúcia M., and Gilbert Van Stappen. 2006 “Use of the Genus Artemia in Ecotoxicity Testing.” Environmental Pollution (Barking, Essex: 1987) 144 (2): 453–62. 10.1016/j.envpol.2005.12.037.

Osborn Andrew R, Almabruk Khaled H, Holzwarth Garrett, Asamizu Shumpei, LaDu Jane, Kean Kelsey M, Karplus P Andrew, Tanguay Robert L, Bakalinsky Alan T, and Mahmud Taifo. 2015 “De Novo Synthesis of a Sunscreen Compound in Vertebrates.” ELife 4 Accessed January 22, 2018 10.7554/eLife.05919.

Parmar T 2016 “Bioindicators: The Natural Indicator of Environmental Pollution” 9 (2). https://www.tandfonline.com/.

Pizarro H, Vera MS, Vinocur A, Pérez G, Ferraro M, Menéndez Helman RJ, and Dos Santos Afonso M. 2016 “Glyphosate Input Modifies Microbial Community Structure in Clear and Turbid Freshwater Systems.” Environmental Science and Pollution Research International 23 (6): 5143–53. 10.1007/s11356-015-5748-0. [PubMed: 26552793]

Riddle Misty R. 2013 “Molecular Identification of Microorganisms Associated with the Brine Shrimp Artemia Franciscana.” Aquatic Biosystems 9: 7 10.1186/2046-9063-9-7. [PubMed: 23497541]

Riede S, Toboldt A, Breves G, Metzner M, Köhler B, Bräunig J, Schafft H, Lahrssen-Wiederholt M, and Niemann L. 2016 “Investigations on the Possible Impact of a Glyphosate-Containing

Herbicide on Ruminal Metabolism and Bacteria in Vitro by Means of the 'Rumen Simulation Technique.'" Journal of Applied Microbiology 121 (3): 644–56. 10.1111/jam.13190. [PubMed: 27230806]

Rodriguez- Gil Jose L., Prosser Ryan, Poirier David, Lissemore Linda, Thompson Dean, Hanson Mark, and Solomon Keith R.. 2017 "Aquatic Hazard Assessment of MON 0818, a Commercial Mixture of Alkylamine Ethoxylates Commonly Used in Glyphosate-Containing Herbicide Formulations. Part 1: Species Sensitivity Distribution from Laboratory Acute Exposures." Environmental Toxicology and Chemistry 36 (2): 501–11. 10.1002/etc.3559. [PubMed: 27421089]

Rzymski Piotr, Klimaszyk Piotr, Kubacki Tomasz, and Poniedziałek Barbara. 2013 The Effect of Glyphosate-Based Herbicide on Aquatic Organisms – a Case Study. Vol. 13 10.2478/limre-2013-0024.

Schuette J 1998 "Environmental Fate of Glyphosate." Department of Pesticide Regulation. http://www.cdpr.ca.gov/docs/emon/pubs/fatememo/glyphos.pdf.

Schuhmacher Rainer, Krska Rudolf, Weckwerth Wolfram, and Goodacre Royston. 2013 "Metabolomics and Metabolite Profiling." Analytical and Bioanalytical Chemistry 405 (15): 5003–4. 10.1007/s00216-013-6939-5. [PubMed: 23591644]

Schuytema GS, Nebeker AV, and Stutzman TW. 1997 "Salinity Tolerance of Daphnia Magna and Potential Use for Estuarine Sediment Toxicity Tests." Archives of Environmental Contamination and Toxicology 33 (2): 194–98. [PubMed: 9294248]

Shahl PV 2016 "Toxicological Monograph on Glyphosate" In Pesticide Residues in Food-2016, 89–296. Geneva, Switzerland: JMPR.

Shick M, and Dunlap W. 2002 "Mycosporine-Like Amino Acids and Related Gadusols: Biosynthesis, Accumulation, and UV-Protective Functions in Aquatic Organisms | Annual Review of Physiology." 2002. http://www.annualreviews.org/.

Strehmel Nadine, Hummel Jan, Erban Alexander, Strassburg Katrin, and Kopka Joachim. 2008 "Retention Index Thresholds for Compound Matching in GC-MS Metabolite Profiling." Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences 871 (2): 182–90. 10.1016/j.jchromb.2008.04.042. [PubMed: 18501684]

Surgan Michael. 2010 "Pesticide Risk Indicators: Unidentified Inert Ingredients Compromise Their Integrity and Utility." Environmental Management 45 (4): 834–41. 10.1007/s00267-009-9382-9. [PubMed: 20180121]

Taylor Nadine S., Weber Ralf J. M., Southam Andrew D., Payne Tristan G., Hrydziuszko Olga, Arvanitis Theodoros N., and Viant Mark R.. 2008 "A New Approach to Toxicity Testing in Daphnia Magna: Application of High Throughput FT-ICR Mass Spectrometry Metabolomics." Metabolomics 5 (1): 44–58.

Teets Nicholas M., Kawarasaki Yuta, Lee Richard E., and Denlinger David L.. 2013 "Expression of Genes Involved in Energy Mobilization and Osmoprotectant Synthesis during Thermal and Dehydration Stress in the Antarctic Midge, Belgica Antarctica." Journal of Comparative Physiology B 183 (2): 189–201. 10.1007/s00360-012-0707-2.

Theodoridis Georgios, Gika Helen, Franceschi Pietro, Caputi Lorenzo, Arapitsas Panagiotis, Scholz Mattias, Masuero Domenico, Wehrens Ron, Vrhovsek Urska, and Mattivi Fulvio. 2012 "LC-MS Based Global Metabolite Profiling of Grapes: Solvent Extraction Protocol Optimisation." Metabolomics 8 (2): 175–85. 10.1007/s11306-011-0298-z.

Tikunov Andrey P., Johnson Christopher B., Lee Haakil, Stoskopf Michael K., and Macdonald Jeffrey M.. 2010 "Metabolomic Investigations of American Oysters Using 1H-NMR Spectroscopy." Marine Drugs 8 (10): 2578–96. 10.3390/md8102578. [PubMed: 21116407]

Tsui Martin T. K. 2003 "Aquatic Toxicity of Glyphosate-Based Formulations: Comparison between Different Organisms and the Effects of Environmental Factors." Chemosphere 52 (7): 1189–97. 10.1016/S0045-6535(03)00306-0. [PubMed: 12821000]

US EPA, OW. 2013 "Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms." 2013. http://water.epa.gov/scitech/methods/cwa/wet/disk2_index.cfm.

US EPA. 2015 “National Primary Drinking Water Regulations.” Overviews and Factsheets. 11 30, 2015. https://www.epa.gov/ground-water-and-drinking-water/national-primary-drinking-water-regulations.

Warner. 2013 Cell and Molecular Biology of Artemia Development. Springer Science & Business Media.

Watson Susan B., Miller Carol, Arhonditsis George, Boyer Gregory L., Carmichael Wayne, Charlton Murray N., Confesor Remegio, et al. 2016 “The Re-Eutrophication of Lake Erie: Harmful Algal Blooms and Hypoxia.” Harmful Algae 56 (6): 44–66. 10.1016/j.hal.2016.04.010. [PubMed: 28073496]

Williams WD 1993 “Conservation of Salt Lakes.” Hydrobiologia 267 (1–3): 291–306. 10.1007/BF00018809.

Williams WD 1999 “Salinisation: A Major Threat to Water Resources in the Arid and Semi-Arid Regions of the World.” Lakes & Reservoirs: Research & Management 4 (3–4): 85–91. 10.1046/j.1440-1770.1999.00089.x.

Williams WD 2002 “Environmental Threats to Salt Lakes and the Likely Status of Inland Saline Ecosystems in 2025.” Environmental Conservation null (02): 154–167. 10.1017/S0376892902000103.

Wishart David S., Feunang Yannick Djoumbou, Marcu Ana, Guo An Chi, Liang Kevin, Vázquez-Fresno Rosa, Sajed Tanvir et al. 2018 “HMDB 4.0: The Human Metabolome Database for 2018.” Nucleic Acids Research 46 (D1): D608–17. 10.1093/nar/gkx1089. [PubMed: 29140435]

Wishart David S., Jewison Timothy, Guo An Chi, Wilson Michael, Knox Craig, Liu Yifeng, Djoumbou Yannick, et al. 2013 “HMDB 3.0--The Human Metabolome Database in 2013.” Nucleic Acids Research 41 (Database issue): D801–807. 10.1093/nar/gks1065. [PubMed: 23161693]

Wishart David S., Tzur Dan, Knox Craig, Eisner Roman, Guo An Chi, Young Nelson, Cheng Dean, et al. 2007 “HMDB: The Human Metabolome Database.” Nucleic Acids Research 35 (Database issue): D521–526. 10.1093/nar/gkl923. [PubMed: 17202168]

Yancey Paul H. 2005 “Organic Osmolytes as Compatible, Metabolic and Counteracting Cytoprotectants in High Osmolarity and Other Stresses.” Journal of Experimental Biology 208 (15): 2819–30. 10.1242/jeb.01730. [PubMed: 16043587]

Zabaloy María C, Carné Ignacio, Viassolo Rodrigo, Gómez Marisa A, and Gomez Elena. 2016 “Soil Ecotoxicity Assessment of Glyphosate Use under Field Conditions: Microbial Activity and Community Structure of Eubacteria and Ammonia-Oxidising Bacteria.” Pest Management Science 72 (4): 684–91. 10.1002/ps.4037. [PubMed: 25960311]

Zhang Bo, Zhang Hui, Du Chunlei, Qin Xiang Ng, Chaoyang Hu, He Yiliang, and Choon Nam Ong. 2017 “Metabolic Responses of the Growing Daphnia Similis to Chronic AgNPs Exposure as Revealed by GC-Q-TOF/MS and LC-Q-TOF/MS.” Water Research 114 (5): 135–43. 10.1016/j.watres.2017.02.046. [PubMed: 28237781]

Highlights

- GC-MS and ^{1}H NMR identified 43 metabolites in *Artemia* extracts
- Multivariate analysis revealed dose-dependent differences in metabolite expression from Roundup® exposure
- Isopropylamine in the Roundup® formulation contributed to the metabolic perturbation
- Roundup® exposure affected one-carbon, carbohydrate, microbial, and energy metabolism

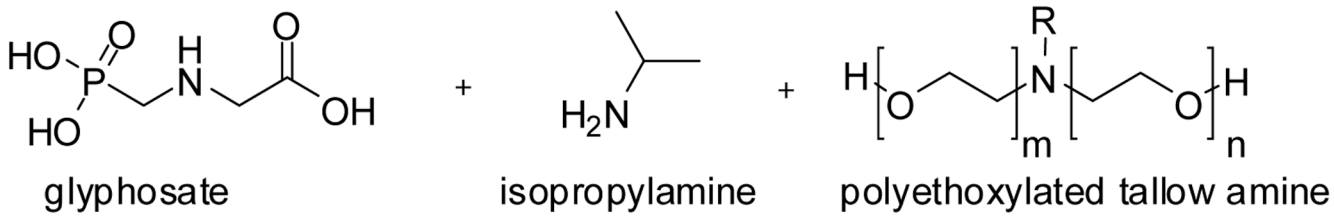

Figure 1.

Ingredients for the Roundup® formulation: the active ingredient glyphosate, the stabilizing salt isopropylamine, and the adjuvant polyethoxylated tallow amine (POEA)

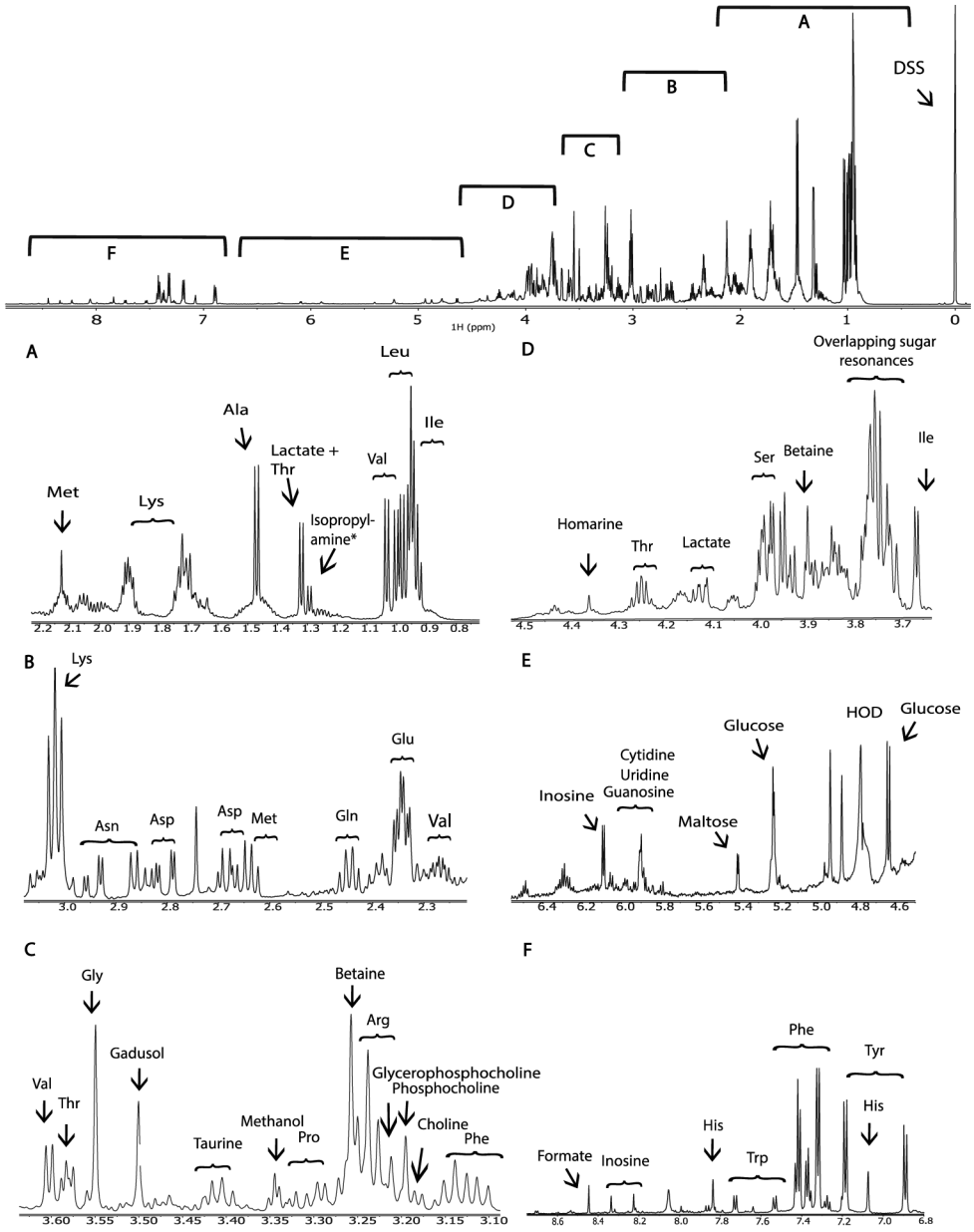

Figure 2.

^{1}H NMR spectra of naupliar *Artemia* extracts labeled with identified metabolites. Refer to Table 1 for full metabolite name. The chemical shift reference DSS at 0 ppm is shown in the full spectrum, A, with individual spectral regions expanded to show spectral detail. The chemical shift range for each inset include: A. 0.8 to 2.2 ppm, B. 2.3 to 3.1 ppm, C. 3.1 to 3.7 ppm, D. 3.7 to 4.5 ppm, E. 4.5 to 6.5 ppm, F. 6.8 to 8.7 ppm.

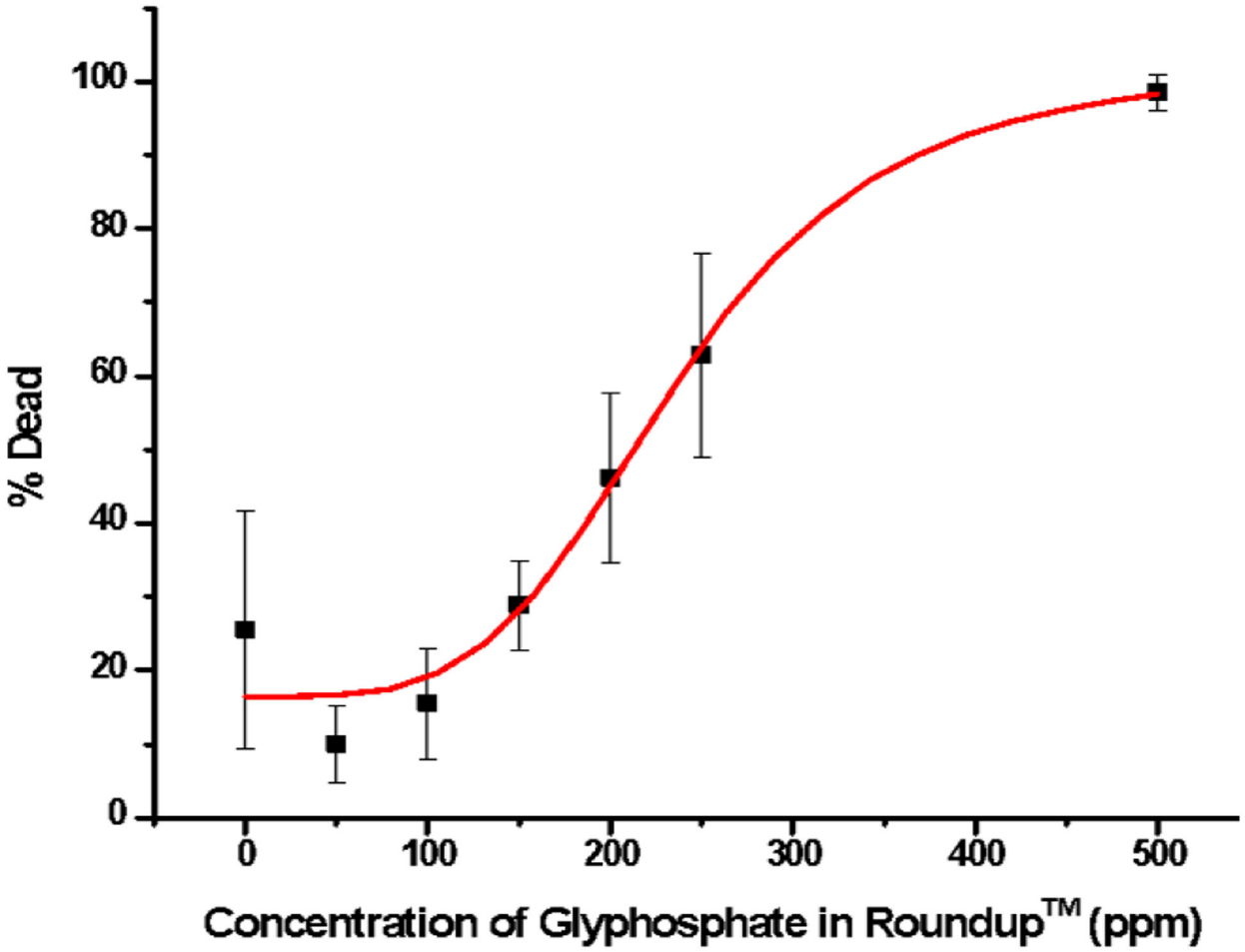

Figure 3.

Dose-Response Plot for *Artemia* mortality with exposure to Roundup®. The LC_{50} is determined to be 237 ± 23 ppm Roundup®.

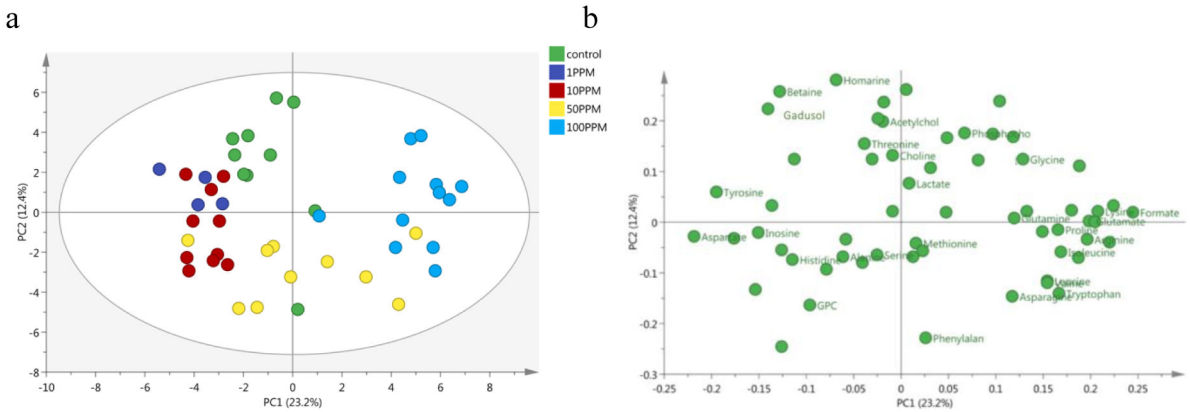

Figure 4.

Multivariate analysis of GC-MS and NMR dose-dependent results compiled in SIMCA. (a) Multiblock-PCA score plot with PC1 = 23.2% and PC2 = 12.4% explained variance for control (green), 1.00 ppm (purple), 10.0 ppm (red), 50.0 ppm (yellow), and 100 ppm (blue) Roundup®. (b) Loading plot indicating how metabolites from GC-MS and NMR contribute to the variance in PC1 and PC2.

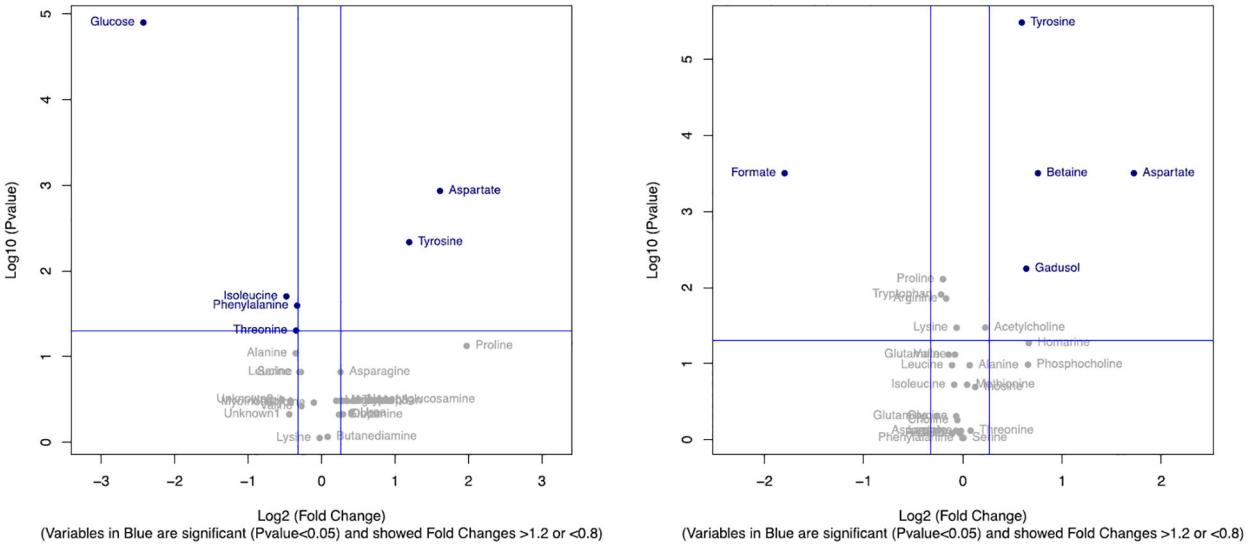

Figure 5.

Volcano plots for control vs 100 ppm Roundup® exposure for metabolites identified by GC-MS (left) and NMR (right). Variables in blue are significant (p-value < 0.05) and exhibit fold changes > 1.2 or < 0.8. On the x-axis, negative values represent metabolites with a positive fold change (increased concentration) compared to the control.

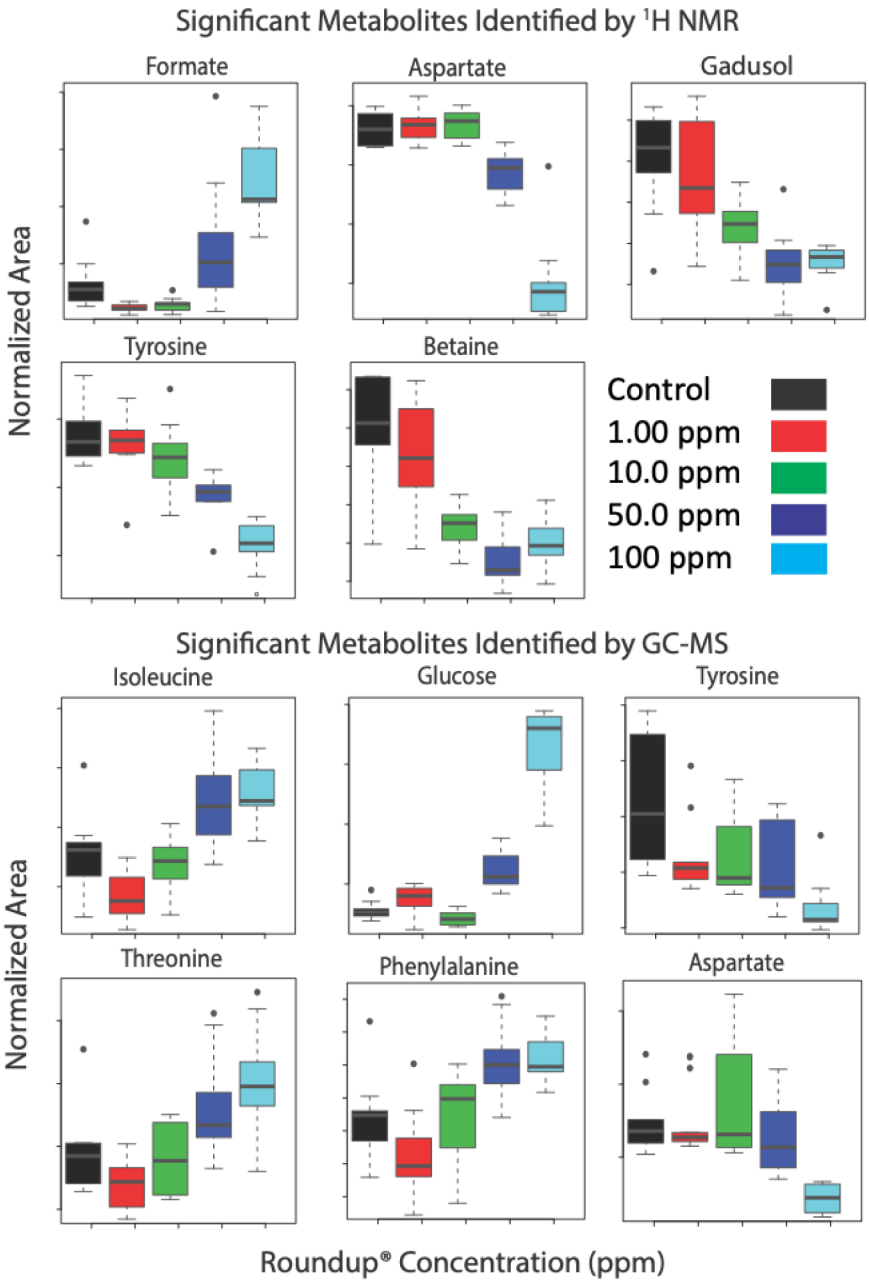

Figure 6.

Box and whisker plots of significant variables for each Roundup® dose in ^{1}H NMR and GC-MS analysis. The box represents the interquartile range, the bar represents the median of the dataset, the whiskers extend to the highest and lowest observations, and black circles represent statistical outliers.

Table 1.

Metabolites identified by ^{1}H NMR and GC-MS spectra of *Artemia* extracts.

Metabolites	^{1}H Chemical Shift (ppm)	GC Retention Index
Amino Acid		
Alanine (Ala)	1.464d, 3.77*	
Arginine (Arg)	1.900m, 3.238m	1821.6
Asparagine (Asn)	2.840m, 2.943dd, 3.99*	1496.9; 1589.2
Aspartate (Asp)	2.667m, 2.802dd, 3.89*	1511.8
Glutamate (Glu)	2.051m, 2.139m, 2.342m	1615.4
Glutamine (Gln)	2.123m, 2.440q, 3.76*	1723.1; 1769
Glycine (Gly)	3.556s	1303
Histidine (His)	3.103m, 3.23*, 3.98*, 7.073s, 7.837s	1914.2
Isoleucine (Ile)	0.929m, 1.002d, 1.252m, 1.461m	1180.3; 1290.5
Leucine (Leu)	0.952t, 1.708m, 3.72*	1159.7
Lysine (Lys)	1.470m, 1.714m, 1.907m, 3.018m, 3.75*	1702; 1916
Methionine (Met)	2.126m, 2.637t, 3.840*	1511.4
Phenylalanine (Phe)	3.117m, 3.276, 3.983*, 7.318d, 7.372t, 7.418t	1621.3
Proline (Pro)	2.027m, 2.347m, 3.330m, 3.414, 4.119dd	1295.4; 1573
Serine (Ser)	3.83*, 3.952m	1356.8
Threonine (Thr)	1.323d, 4.245m	1381.4
Tryptophan (Trp)	7.189t, 7.276t, 7.534d, 7.724d	2204.7
Tyrosine (Tyr)	3.037*, 3.182*, 3.927*, 6.885d, 7.184d	1933.4
Valine (Val)	0.982d, 1.034d, 2.264m, 3.603*	1213.5
Osmolyte		
Betaine	3.258s, 3.894s	
Choline	3.185s, 3.51*, 4.052m	
Formate	8.445s	
Glycerol	3.550m, 3.641m	
Glycerophosphocholine	3.212s, 3.40*, 3.47*, 3.75*, 3.98*	
Homarine	4.357s, 8.534dd, 8.77*	
Lactate	1.322d, 4.124m	
Methanol	3.345s	
Phosphocholine	3.195s, 3.58*, 4.17*	
Taurine	3.417m, 3.33*	1660.9
Sugar		
Gadusol	2.379d, 2.686d, 3.502s, 3.567, 3.718, 4.107s	
Glucose	3.24*, 13.40*, 3.44*, 4.635d, 5.223d	1887.4
Maltose	4.636d, 5.223d, 5.406d	
Myo-inositol		2072.8
N-acetylglucosamine	5.196d	2059.2
Trehalose	5.196d	
Nucleic Acid		

Metabolites	^{1}H Chemical Shift (ppm)	GC Retention Index
Cytidine	5.899d, 6.093d	
Guanosine	5.899d, 7.899s	
Inosine	4.428m, 6.093d, 8.224s, 8.329s	2566.5
Uracil	5.794d, 7.528d	1334.1
Uric acid		2085.1
Uridine	3.85*, 4.12*, 4.22*, 5.902m, 7.855d	
Polyamine		
Ornithine	3.053t	1610.5;1811.7
Ornithine-1,5-lactam		1452.6
Putrescine		1729.1
Urea		1240.6
Other		
L-DOPA		2081.6
Glycyl-proline		1973.8
Pyroglutamate	2.03*, 2.39*, 2.501m, 4.168dd	
Exogenous		
Isopropylamine	1.294d, 3.48*	

Asterisks (*) indicate resonances identified in COSY or TOCSY NMR spectra. These values are reported to two decimal places due to the reduced resolution of 2D spectra. Resonance multiplicity are indicated by singlet (s), doublet (d), triplet (t), doublet of doublets (dd), and multiplet (m).