The Application of $^1$H NMR-Based Metabolomics to Understand the Biochemical Changes of *Daphnia magna* Exposed to Sub-lethal Levels of Environmental Stress

by

Edward G. Nagato

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of the Department of Physical and Environmental Sciences
University of Toronto

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Doctor of Philosophy Degree, 2016

Graduate Department of the Department of Physical and Environmental Sciences
University of Toronto, Scarborough Campus

Abstract

Traditional tools used in aquatic toxicology are restricted to examinations of lethality and fecundity and provide little biochemical insight into toxicity. This thesis is predicated on providing this insight using metabolomics. For this, various stressors were examined with *Daphnia magna* as a subject. The first study examined the response of *D. magna* to sub-lethal levels of arsenic, copper or lithium and found that copper and lithium responded similarly, suggesting an impairment in sodium-potassium adenosine triphosphatase function. The second study optimized the extraction procedure and dry mass requirements for metabolomics experiments, using a nuclear magnetic resonance (NMR) microprobe. A D$_2$O-based buffer provided the most comprehensive metabolite profile and the best signal to noise ratio. The third study examined the response of *D. magna* to a gradient of sub-lethal concentrations of an organophosphate (diazinon and malathion) or bisphenol-A (BPA). With increasing concentrations, the *D. magna* metabolome response was not linear and responded in a manner that corresponded with the severity of the stressor. The fourth study focused on the response of the *D. magna* to chronic BPA exposure. This was compared to a reproduction test and measurements of 20-hydroecdysone (20HE). While the reproduction test showed no response,
the metabolome responses indicated that energy production was impaired by 14 days, though by 21 days there were signs of adaptation. 20HE levels were unaltered and therefore BPA does not act on the *D. magna* endocrine system. The fifth study examined the stress incurred by *D. magna* when carbon sources were altered. Cyanobacteria induced a toxic response, shown as a compensatory strategy to produce energy. When supplied with dissolved organic carbon, metabolome changes indicated that *D. magna* were undergoing starvation. The work in this thesis demonstrates that NMR-based metabolomics can provide biochemical insight into the response of *D. magna* to environmental stress.
Acknowledgments

I would like to thank my supervisor, Dr. Myrna Simpson, who has assumed a number of roles too numerous to list, but include mentor, confidant, editor and advocate and has always challenged me to be the best I could be. Myrna has been unflinching in her support, in the often thankless task of supervising me and has given me opportunities that I can only repay by giving likewise to others. I would also like to thank my committee members, Dr. André Simpson and Dr. Malcolm Campbell for their advice and guidance through my Ph.D. studies and for also being the role models by which I aim to pattern myself.

I would like to thank the funding sources that have made my graduate studies possible. These include the Ontario Graduate Scholarship and the Krembil Foundation to whom I am indebted for allowing me to conduct my research.

I would like to thank the members of the Simpson labs, both present and departed, for the fun, the frustration and the fatuity that constitutes this strange purgatory known as graduate school. If knowledge moves along a concentration gradient, then I have been the low concentration beneficiary and I thank all of you. In particular I would like to thank Dr. Brian Lankadurai for being there for me when I started and showing me the ropes, for the substantial discussions on sport and the importance of the NFL fourth down and for generally being a cool guy.

I would like to thank Michaela Marshall for being there for me when I went down the Rin path and almost suffered an emotional implosion. It was emotional. Thanks Michaela.

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I would like to thank a number of cellists in the Hart House Chamber Strings who have tolerated me for so many years as a terrible cellist who couldn’t read the tenor clef. This includes Rachel Stapleton, Michael Auduong and Natalie Yeung. Through the struggles in life, we can take comfort knowing that as cellists, there are worse things to be in life (i.e. violinists).

I would like to thank Lily Hou, ‘ayumiashi-sune’ visionary, who among many things, was there for me when the Raptors were swept in the first round and when the Leafs imploded in the third.

I would like to thank my sister, who throughout my life has played various roles, including patient benefactor and impatient scourge, but has always had a disinterested (not uninterested) care for her incorrigible reprobate brother. I would like to thank Andrew Vallance for understanding the mendicant graduate student lifestyle, and for providing me with enough shirts and pants for the next ten years. I would like to thank my parents, who have taken on a beatific zen in their dealings with me, for things too numerous to list. They have been patient and doting, and have afforded me the latitude to become who I am today and it is a profound debt that I owe to them. It is their support that reminds me that I am not done learning and that I owe it to them to become the best person I can be.
Finally I would like to thank all those I have omitted here, through sheer callous neglect, for being part of the cast in this great MMORPG that is life; the power-levellers that helped me through the daily grind and all those graduate students who are driven by curiosity and an internal chaos to learn and to grow. You know who you are.

Finally, I would like to acknowledge that I have been truly privileged, these past five years, to have experienced what I have: to have seen Miku at the Hammerstein, to have learned to fight in a hasso stance, to have fought undergrads to win in intramural ball hockey. For lunch time Tanto Cuore and 7 Wonders. For tennis in the dark. The list is exhaustive and I have been so fortunate, while being so undeserving of all this. I carry an enormous debt.
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<tr>
<td>20HE</td>
<td>20-hydroecdysone</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>AMIX</td>
<td>Analysis of MIXtures</td>
</tr>
<tr>
<td>AMW</td>
<td>Acetonitrile:methanol:water</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BD</td>
<td>Bligh and Dyer</td>
</tr>
<tr>
<td>BPA</td>
<td>Bisphenol-A</td>
</tr>
<tr>
<td>C+DOC</td>
<td>Chlorophyta plus dissolved organic carbon</td>
</tr>
<tr>
<td>CHH</td>
<td>Crustacean hyperglycemic hormone</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlation spectroscopy</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P-450</td>
</tr>
<tr>
<td>DOC</td>
<td>Dissolved organic carbon</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DSS</td>
<td>2,2-dimethyl-2-silapentane-5-sulfonate sodium salt</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FAMEs</td>
<td>Fatty acid methyl esters</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>Fourier transform – ion cyclotron resonance</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>HSQC</td>
<td>$^1$H-\textsuperscript{13}C heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>JRES</td>
<td>J-resolved spectroscopy</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LC\textsubscript{50}</td>
<td>Concentration that causes 50% lethality in a population</td>
</tr>
<tr>
<td>MF</td>
<td>Methyl farnesoate</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>Na\textsuperscript{+}/K\textsuperscript{+} ATPase</td>
<td>Sodium-potassium adenosine triphosphatase</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OECD</td>
<td>Organization for Economic Co-operation and Development</td>
</tr>
<tr>
<td>OP</td>
<td>Organophosphate</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
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<tr>
<td>PLS-DA</td>
<td>Partial least squares – discriminant analysis</td>
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<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>PURGE</td>
<td>Presaturation using relaxation gradients and echoes</td>
</tr>
<tr>
<td>QXI</td>
<td>Quadruple resonance inverse</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal to Noise ratio</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TMS</td>
<td>Trimethylsilane</td>
</tr>
<tr>
<td>TXI</td>
<td>Triple resonance inverse</td>
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Preface

The following contents are the combination of works published in peer review journals (Chapters 2-4) and may contain some overlapping information. Contributions to the various chapters are outlined below.

CHAPTER 1

Introduction: The Application of $^1$H NMR-Based Metabolomics to Understand the Biochemical Changes of *Daphnia magna* Exposed to Sub-lethal Levels of Environmental Stress

Contributions: Written by Edward G. Nagato with critical review from Myrna J. Simpson

CHAPTER 2

$^1$H NMR-Based Metabolomics Investigation of *Daphnia magna* Responses to Sub-lethal Exposure to Arsenic, Copper and Lithium

Published as Nagato, E.G., D’eon, J.C., Lankadurai, B.P., Poirier, D.G., Reiner, E.J., Simpson, A.J., Simpson, M.J. 2013. $^1$H NMR-Based metabolomics investigation of *Daphnia magna* responses to sub-lethal exposure to arsenic, copper and lithium. Chemosphere, 93: 331-337 (modified to include an analysis of variance (ANOVA) with a Bonferroni posthoc test, which replaces the multiple student $t$-tests used in calculating statistical significance for both the principal component analysis (PCA) and the metabolite percent changes. Text in the method and results sections has been modified to account for these changes. Figure 2-3 has also been
modified to reflect the changes in significance. The in house R script used in the ANOVA has been included in Appendix A, as well as the results of the Bonferroni posthoc test).

**Contributions:** Data collection was performed by Jessica D’eon and Dave Poirier. Experimental design was aided by Eric J. Reiner and NMR expertise was provided by André J. Simpson. The data was analyzed and written as a manuscript by Edward G. Nagato with critical review provided by André J. Simpson and Myrna J. Simpson.

CHAPTER 3

**Development of an NMR Microprobe Procedure for High-throughput Environmental Metabolomics of *Daphnia magna***


**Contributions:** The experiment was designed by Edward G. Nagato and Myrna J. Simpson. NMR expertise was provided by Ronald Soong and André J. Simpson. Data collection was performed by Edward G. Nagato and Brian P. Lankadurai. The data was analyzed and the manuscript was written by Edward G. Nagato, with critical review provided by André J. Simpson and Myrna J. Simpson.
CHAPTER 4

Metabolomics reveals energetic impairments in *Daphnia magna* exposed to diazinon, malathion and bisphenol-A

Published as: Nagato, E.G., Simpson, A.J., Simpson, M.J. 2016. Metabolomics reveals energetic impairments in *Daphnia magna* exposed to diazinon, malathion and bisphenol-A. Aquatic Toxicology. 170: 175-186

(Modified to include an ANOVA with a Bonferroni posthoc test, to replace the multiple *t*-tests and false discovery rate that were used in calculating statistical significance in the PCA and metabolite percent changes. Text in the methods and results sections has been modified to reflect these changes. Figures 4-1, 4-2, 4-3, 4-4 have also been modified to reflect the changes in statistical significance after the ANOVA and Bonferroni posthoc test. Results of the ANOVA and Bonferroni posthoc test have also been included in Appendix C).

**Contributions:** The experiment was designed by Edward G. Nagato and Myrna J. Simpson. Data was collected, analyzed and written as a manuscript by Edward G. Nagato. Critical review of the manuscript was provided by André J. Simpson and Myrna J. Simpson.

CHAPTER 5

Chronic bisphenol-A exposures induce metabolomics changes in *Daphnia magna* that are age dependent

**Contributions:** The experiment was designed by Edward G. Nagato and Myrna J. Simpson. Data collection and analysis was performed by Edward G. Nagato. The thesis chapter was written by Edward G. Nagato
CHAPTER 6

$^1$H NMR-based metabolomics shows *Daphnia magna* responds to different carbon resources through a change in energy partitioning.

**Contributions:** The experiment was designed by Brian P. Lankadurai, Michael Arts and Myrna J. Simpson. Data collection was performed by Brian P. Lankadurai and Edward G. Nagato. Fatty acid analysis was performed by Stephanie Hixson. Data analysis and manuscript writing was performed by Edward G. Nagato with critical review from Myrna J. Simpson.

CHAPTER 7

**Conclusions, limitations and future research**

**Contributions:** The chapter was written by Edward G. Nagato, with critical feedback provided by Myrna J. Simpson.
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Figure 3-5. 2D $^1$H-$^{13}$C Heteronuclear Single Quantum Coherence (HSQC) nuclear magnetic resonance spectrum with metabolites labeled.

Figure 4-1. Averaged principal component analysis (PCA) scores plots for diazinon (A), malathion (B) and bisphenol-A (C). Statistically significant separation from the control ($p < 0.05$), based on an analysis of variance with a Bonferroni posthoc test is indicated by an asterisk (*).

Figure 4-2. Percent changes of metabolites showing statistically significant changes after 48 hours of diazinon exposure. An analysis of variance with a Bonferroni posthoc test, was used to determine statistical significance and is indicated by an asterisk (*), n=10.

Figure 4-3. Percent changes of metabolites showing statistically significant changes after 48 hours of malathion exposure. An analysis of variance with a Bonferroni posthoc test was used to determine statistical significance and is indicated by an asterisk (*), n=10.

Figure 4-4. Percent changes of metabolites showing statistically significant changes after 48 hours of bisphenol-A exposure. An analysis of variance with a Bonferroni posthoc test was used to determine statistical significance and is indicated by an asterisk (*), n=10.
Figure 5-1. Reproductive output of *Daphnia magna* exposed to bisphenol-A for 21 days plotted against an unexposed control. Values are based on 10 replicates for each condition (n=10) and statistical differences from the control are calculated using a student’s *t*-test (α = 0.05) and are indicated by an asterisk (*).

Figure 5-2. Averaged principal component analysis scores plots (n = 10) for the four time points tested in this study. Statistical separation between the control and bisphenol-A exposure is indicated by an asterisk (*) and is calculated using a *t*-test (α = 0.05).

Figure 5-3. Loadings plots for the principal component analysis scores plots in Figure 5-2 that account for the buckets that contribute to the separations seen. Each value corresponds to the bucket values associated with the *1*H nuclear magnetic resonance spectra.

Figure 5-4. Metabolite changes expressed as percent differences for identified metabolites. Statistical significance based on a *t*-test (α = 0.05) is indicated with an asterisk (*).

Figure 5-5. 20-hydroxyecdysone levels in *Daphnia magna* throughout the 21 day test period. There was no statistically significant change between the two conditions (α = 0.05) at any of the time points.

Figure 6-1. An averaged principal component analysis scores plot shows that each of the conditions tested induced a separation in the metabolome after 48 hours that was statistically significant. The *Synechocystis* and dissolved organic carbon conditions induced the greatest
separation on PC1. An analysis of variance with a Bonferroni posthoc test was used to determine statistically significant separation ($\alpha = 0.05$) and is indicated by an asterisk (*).

**Figure 6-2.** A loadings plot from the principal component analysis (PCA) scores plot shows which bucket regions are inducing separation in the PCA scores plot.

**Figure 6-3.** Individual metabolite percent changes are shown for each carbon source, relative to the chlorophyll mixture. An analysis of variance with a Bonferroni posthoc test was used to determine statistically significant changes ($\alpha = 0.05$) and is indicated by an asterisk (*).
Chapter 1

Introduction: Metabolomics as an alternative to traditional toxicity bioassays in aquatic toxicology
1.1 Tools used in aquatic toxicology and their limitations

Zooplankton are continually under stress, as their environments are subjected to both anthropogenic disturbances and natural pressures, with consequences that can alter aquatic community composition (Hanazato, 2001). Anthropogenic contaminants pose the predominant threat, as they invariably end up in aquatic systems, creating an imperative to develop efficient procedures for examining the potential toxicities of these contaminants (Carvalho et al., 2003). This is salient given the thousands of contaminants of unknown toxicity that risk assessors and environmental monitors are under pressure to understand (Khangarot and Rathore, 2003). For example, approximately a hundred different types of pharmaceuticals have been detected in sewage effluent, and little is known about their toxicity to fish and other aquatic organisms (Samuelsson et al., 2006). It is not just organic compounds that are problematic; many metals are also common contaminants, and are particularly hazardous, as they cannot be degraded further and therefore accumulate in biota and sediment (Spann et al., 2011). This is to say nothing of the emerging contaminants, which have little to no toxicological data (Hahn, 2011). The field of aquatic toxicology developed largely in response to these types of concerns and has since been evolving to tackle emerging environmental problems, such as the use of DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) in the 1940s, which marked the beginning of aquatic toxicology (Pritchard, 1993). However, focusing on anthropogenic contamination does not take into account the fact that stress can be more broadly defined. For example, climate change is altering a multitude of parameters including food quality and quantity, UV radiation and ambient temperatures in aquatic environments (Altshuler et al., 2011) all of which are additional stressors to aquatic organisms. These stressors then, whether anthropogenic or natural in origin, need to be studied in detail to understand their consequences on environmental organisms. For this reason,
bioassays have been developed that characterize the impact that stressors have on important ecosystem species (Hammers-Wirtz and Ratte, 2000).

Under current protocols for evaluating aquatic ecosystem health, the 48 hour acute toxicity and 21 day reproduction test constitute the major types of assays used globally in regulatory testing (Dang et al., 2012; Hanazato, 2001). They are favoured for use as they are easy to conduct and provide the most direct proxy of an organism’s fitness (Dang et al., 2012; Melvin and Wilson, 2013). Practices and procedures have been codified for interlaboratory consistency by international organizations such as the Organization for Economic Co-operation and Development (OECD) and International Organization for Standardization. In spite of these advantages, they are problematic in several ways. For example the commonly used acute toxicity test gauges how much a toxicant concentration will kill 50% of a population (LC$_{50}$), but contaminants are rarely present at lethal levels (Chen et al., 1999). This test does not provide insight into how toxicity manifests at sub-lethal levels, which has been defined as being 10% of the LC$_{50}$ or less (Ren et al., 2007) and furthermore does not provide insight into the biochemical mode of action. This is important as responses to sub-lethal stressors at the biochemical level generally precede higher order changes such as mortality (Printes and Callaghan, 2004). The other commonly used test, the 21 day reproduction test, measures various reproductive parameters such as intermoult period and sex ratios, but principally total neonatal output (Environment Canada, 2007; OECD, 2012). In an examination of the literature that used the 21 day reproduction test, Dang et al. (2012) found that there were inconsistencies between different laboratories using the same contaminants, concluding that the test is insufficient for evaluating contaminant toxicities. But like the acute toxicity test, it too does not provide any mechanistic biochemical information. For example, using the 21 day reproduction test, Caspers (1998) did
not find that there were any changes in the reproduction of the crustacean Daphnia magna when exposed to bisphenol-A (BPA) and concluded that because there was no reproductive impairment, that BPA toxicity was negligible. However, at the same concentrations another group, using a comet assay, were able to find molecular evidence for DNA strand breaks and molecular indicators of oxidative stress (Park and Choi, 2009), highlighting the insensitivity of the 21 day reproduction test. The use of singular apical endpoints such as reproduction and mortality may underestimate the toxicities of sub-lethal contaminant stressors on aquatic species and so more sensitive biochemical assays of stress are needed (Chen et al., 1999; Spann et al., 2011).

The transition from these tests has already begun, with a number of assays that measure specific biomarkers. For example, vitellogenin is a commonly used biomarker, especially for examining estrogen mimics (Jones et al., 2000), given the importance of this system across many taxa as precursors of yolk proteins (Kato et al., 2004; Matozzo et al., 2008). It has been widely used in aquatic toxicology studies (Matozzo et al., 2008) especially with fish (Harries et al., 1997; Matozzo et al., 2008; Nicolas, 1999). Antioxidant stress has also become a popular focal point of toxicology research as many toxicants induce oxidative stress via the production of reactive oxygen species (Valavanidis et al., 2006). For this, specific assays for biomarkers such as malondialdehyde (Lushchak, 2011), catalase, superoxide dismutase, glutathione peroxidase and glutathione-S-transferase (Kim et al., 2010; Paila et al., 2012) are among many that have been extensively used, for example being used to study D. magna responses to acetylsalicylic acid (Manuel Gomez-Olivan et al., 2014), titanium dioxide nanoparticles (Kim et al., 2010) and arsenic (Fan et al., 2015) among many other studies. But beyond biochemical markers, even behavioural responses, such as swim speed and activity, have seen application in aquatic
toxicology (Melvin and Wilson, 2013). For example it has been used in *Gammarus lawrencianus* studies, with one study finding that they respond to cadmium exposure by limiting vertical and horizontal movements (Wallace and Estephan, 2004). While the biomarkers mentioned are useful and can serve as early indicators of stress, the major limitation is that they do not account for changes that may be occurring in other biochemical pathways as well (Long et al., 2015). For this reason there has been an effort to find alternative tests that are rapid and sensitive to toxicity, but also to move away from the paradigm of looking at one particular biomarker or pathway and looking at organisms as holistic entireties (Garcia-Reyero and Perkins, 2011).

### 1.2 Metabolomics as a tool for examining stress on a biochemical level

Metabolomics is defined as the characterization of an organism’s entire metabolome (Lin et al., 2006); the metabolome being the aggregate low molecular weight metabolites (less than 1000 Daltons) such as amino acids, sugars and fatty acids, found in a sample tissue or organism (Clarke and Haselden, 2008; Viant, 2008). It is the logical extension of the genomics and proteomics that preceded it (Robertson, 2005; Viant et al., 2009). The metabolome is often the first to respond to external stimuli; faster than genes, transcripts and proteins (Clarke and Haselden, 2008) and as such, represents the functional state of an organism (Dunn and Ellis, 2005; Penuelas and Sardans, 2009). While it can be used for the simple characterization of an organism’s metabolome, in practice metabolomics is an analysis of an organism’s response to some type of stressor or stimuli (Clarke and Haselden, 2008). By juxtaposing the metabolomes of stressed and unstressed groups/individuals, the relative changes provide insight into the biochemical mechanisms that are altered in response to stress. Metabolic pathways – specifically, primary metabolism – are well conserved throughout evolutionary history, and so across many taxa, metabolic mechanisms are similar (Keum et al., 2010). Metabolomics holds great promise
as an efficient, cost effective solution for analyzing the sub-lethal toxicity associated with stressors, both anthropogenic and natural in origin, and to provide the biochemical context for stress responses (Biales et al., 2015).

The major advantage of metabolomics is that it is a holistic analysis that is unencumbered by prior assumptions about the mode of toxicity of a stressor, thus providing information that is often novel and unexpected (Robertson, 2005; Samuelsson et al., 2006). Because it is not inherently hypothesis driven, it can be an impartial analysis for examining biochemical pathway perturbations (Biales et al., 2015). In cases where little is known about a species, metabolomics provides an excellent exploratory platform upon which further investigation can be based (Wone et al., 2011). While it is beneficial to examine the entire metabolome, if there is an *a priori* understanding of what pathways one wishes to study, targeted metabolomics – the quantification of specific metabolites – is also an option (Goodacre et al., 2004; Ryan and Robards, 2006). However, since metabolic pathways are interrelated (Ryan and Robards, 2006) it is ultimately best to examine a suite of metabolites to understand how they relate to each other. Like the other ‘omics’ technologies, metabolomics marks the move from looking at singular endpoints (i.e. single molecular bioassays) and understanding that a complex system is best examined as a whole entity and marks the progression from apical endpoints tests, to single biomarkers, to finally a holistic analysis of an organism.

1.2.1 General methodology used in metabolomics studies

One of the main advantages of metabolomics is that the fundamental approach easily translates to different experimental designs and can use an array of species. Therefore, scientists knowledgeable in metabolomics can apply this technique to test a host of hypotheses. The basic metabolomics study is comprised of: organism/tissue selection, the preparation of experimental
and control samples, the measurement of metabolite content, and a statistical analysis of the findings. This has been described succinctly as a “collect, grind, measure and analyze” method (Bundy et al., 2009). These are methods that are becoming increasingly standardized (Aliferis and Jabaji, 2011) and for example, a general protocol for use in plant metabolomics (Kim et al., 2010) and in reporting mammalian experiments (Griffin et al., 2007) exist. This standardization will allow for greater interlaboratory consistency and is a step towards metabolomics being accepted into regulatory programs.

1.2.2 Metabolite sample preparation

Prior to extraction, samples need to be preserved for them to remain representative of the metabolome at a particular time. For this purpose, samples are frozen with liquid nitrogen to cease enzymatic and further biochemical activity (Fiehn, 2002; Macel et al., 2010). They are subsequently freeze dried, as the absence of water will ensure that enzymes and transporters are unable to function (Fiehn, 2002). Also, grinding samples into a powder increases metabolite yield during extraction processes (Cevallos-Cevallos et al., 2009).

The metabolite extraction procedure is an important element in any metabolomics experiment, and can be a source of variation between labs (Liebeke and Bundy, 2012). The degree of solvent polarity dictates what can be extracted, acting under the “like dissolves like” principle (Kim and Verpoorte, 2010). For example, a solvent such as water or methanol is used for extracting polar metabolites, while chloroform is used for nonpolar metabolites (Dunn and Ellis, 2005). There is no single solvent that can extract all the metabolites in a sample and often a combination of solvents is necessary, depending on the types of metabolites that are being targeted (Choi and Verpoorte, 2014; Sardans et al., 2011).
A number of studies have focused on examining polar metabolites (Brown et al., 2008; Bundy et al., 2009; Donarski et al., 2008; Lankadurai et al., 2011) because they are sensitive to various types of external stressors and have been linked to specific biochemical pathways (Lankadurai et al., 2011; Li et al., 2014). An extraction procedure based solely on using an aqueous buffer can be a simple and effective method for extracting a wide range of metabolites (Fasulo et al., 2012; Lankadurai et al., 2013). The use of a buffer can help control for pH and a phosphate buffer has been shown to offer better resolution with nuclear magnetic resonance (NMR) than an acetate buffer (Kim et al., 2006). While there are a number of extraction methods that have been tested, one study concluded that methanol/chloroform/water extractions, based on the popular Bligh and Dyer biphasic extraction procedure (Bligh and Dyer, 1959), yielded the best results in terms of extracting a wide range of both polar and nonpolar metabolites (Wu et al., 2008). The introduction of multiple solvents does however have the consequence of being both time and labor intensive (Lamichhane et al., 2015). Also, if using NMR, a biphasic separation method such as the one proposed by Wu et al. (2008) requires the drying and reconstitution of a sample into an appropriate solvent (Kim et al., 2010) which adds an extra layer of work and potential variability. An interesting study by Liebeke et al. (2012) tested, among a number of extraction procedures, an acetonitrile/methanol/water extraction procedure and found it to be an excellent balance of metabolite yield and reproducibility (Liebeke and Bundy, 2012), showing an interesting use of three miscible solvents. Another component of an extraction procedure will be in quenching metabolites and being able to release them from the matrices to which they are attached (Choi and Verpoorte, 2014). For this, ultrasound and microwave can allow for quicker solubility and increase metabolite yield (Choi and Verpoorte, 2014; Kim and Verpoorte, 2010).
1.2.3 Analytical platforms used in metabolomics

In selecting an analytical modality for metabolomics studies, it is necessary to have a platform that can be sensitive enough to detect metabolites of interest and yet be accessible (Fiehn, 2002). The most commonly used analytical platforms are NMR and mass spectrometry (MS)-based techniques and there is debate over which platform is better, with many factors to consider (Nagana Gowda and Raftery, 2015; Robertson, 2005). For example, NMR is seen as particularly useful for non-targeted analyses and MS-based methods are more apt for situations when there are targeted metabolites as they are selective in the metabolites that they can detect (Clarke and Haselden, 2008; Goodacre et al., 2004). While both platforms are seeing continual improvement, initial costs for both are high, though once acquired, the per sample costs are low (Zhang et al., 2012).

Many metabolomics studies use methods based on MS, which ionizes and identifies compounds by separating ions based on their mass to charge ratio, which are then compared to a standard to give quantitative information on metabolite content (Dunn and Ellis, 2005; Macel et al., 2010). With MS-based methods, it is more usual for analysis to be preceded by separation techniques such as gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (Dunn and Ellis, 2005). The sensitivity of MS-based methods is a particular strength, as for example with GC-MS detection limits are in the nmol or pmol concentrations (Dunn and Ellis, 2005). It has been found to be particularly useful in targeted metabolomics studies. For example GC-MS has been used in a targeted in vivo study of the volatile compounds from a petunia flower, being sensitive enough to identify the circadian rhythm by which benzenoid compounds are emitted (Verdonk et al., 2003). While GC-MS is appropriate for targeting volatile and low molecular weight metabolites, non-volatile higher molecular weight
metabolites cannot be detected, though derivatization can be used for some compounds to make metabolites volatile (Dunn and Ellis, 2005; Zhang et al., 2012). With GC-MS there are also data deconvolution steps incurred by peak overlaps that add more work to the process (Brown et al., 2005a), though Ralston-Hooper et al (2008), have successfully used the multiseparation GCxGC time of flight-MS to measure and analyze the metabolome of *Diporeia* to help resolve this problem. While GC-MS is applicable to the analysis of volatile compounds, LC-MS avoids the need for derivatization and is appropriate for analyzing polar/semi-polar compounds (Zhang et al., 2012). For example, it has been used to characterize the metabolites that are released by a red alga in response to grazing pressure, finding that eicosanoids play a key role in their defense systems (Nylund et al., 2011). Also, the use of multiple reaction monitoring with tandem quadrupole MS has been used in the targeted metabolite profiling of 100 metabolites from various plant species (Sawada et al., 2009). The lower detection limits of these techniques, relative to NMR, combined with a greater affordability make this platform appealing for many researchers (Aliferis and Chrysayi-Tokousbalides, 2011).

It is also possible to not use chromatographic separation and have a direct injection into MS. For example fourier transform ion cyclotron resonance (FT-ICR) precludes the necessity for separation, while detecting a broad range of compounds (Heeren et al., 2004; Macel et al., 2010). However, while it provides the greatest resolution of all the MS-based methods (Brown et al., 2005b), the information gleaned is primarily the elemental composition of the metabolites, and therefore presents the difficulty in confidently identifying metabolites (Macel et al., 2010).

While powerful, MS-based methods raise the issue of efficiency, with GC-MS and LC-MS being hampered by intensive sample preparation, deconvolution steps and potential matrix effects (Dunn and Ellis, 2005). The separation before MS can introduce analytical variability and
can be time consuming (Pan and Raftery, 2007) and also column performance changes with age, adding another layer of variability to experiments (Verpoorte et al., 2007).

NMR is a popular platform for environmental metabolomics because it is able to provide a broad overview of many metabolites at once (Kim et al., 2010; Viant, 2008). It is high throughput, requires little sample preparation, and is non-selective (Goodacre et al., 2004; Nagana Gowda and Raftery, 2015; Reo, 2002). It is able to detect many metabolites and provides quantitative and structural information on these metabolites as well (Moco et al., 2007; Wu and Wang, 2010). NMR uses strong magnetic fields, combined with radio frequency pulses, to examine the resonance of half spin atomic nuclei (Dunn and Ellis, 2005). The absorbed radio frequency energy will shift the energy level of the nuclei to a higher state and this will be relaxed; the subsequent resonance frequency is detected after the pulse (Dunn and Ellis, 2005). The energy released upon this relaxation is detected and used to identify the compound and the resulting spectrum is unique to each molecule (Dunn and Ellis, 2005). The nuclei that are most commonly used include: $^1$H, $^{13}$C, $^{15}$N and $^{31}$P, whether alone or as part of a multidimensional analysis (Keum et al., 2010; Reo, 2002; Verpoorte et al., 2007). However, since most metabolites contain hydrogen and $^1$H has a high natural presence at over 99.9% (Akkanen et al., 2001; Moco et al., 2007), $^1$H is the most commonly used nuclei in metabolomics. This is in contrast with nuclei such as $^{13}$C and $^{15}$N, which have much lower natural abundances of 1.1 and 0.4% respectively (Keum et al., 2010) and therefore present issues, though isotope labelling can help improve signal quality (Kikuchi et al., 2004). An example $^1$H NMR spectrum is shown in Figure 1-1 and illustrates the way in which resonances can help identify metabolites.
Figure 1-1. A sample $^1$H nuclear magnetic resonance spectrum taken from a smolt muscle extract. Characteristic peaks can help determine the metabolite content shown in the spectra. Reproduced with permission from Tjeerdema, (2008).

The large amount of data produced by NMR needs to be pattern matched to identify the compounds represented within the data (Snape et al., 2004). For this, there are programs such as the Bruker BioSpin AMIX software and databases such as the Madison Metabolomics Consortium Database, which is a spectral database of standard metabolites that serve as a reference for identifying compounds. The metabolites found are matched to the metabolic pathways they are associated with to determine what aspect of metabolism is altered. For this purpose the Kyoto Encyclopedia of Genes and Genomes provides an excellent repository of metabolite pathway information (Kanehisa et al., 2014).
One of the major advantages of NMR metabolomics is the high level of reproducibility between labs, given that the chemical shift of a metabolite is a physical property (Yoshida et al., 2014). This has been demonstrated by Viant et al (2009), who had 7 separate laboratories across the world examine European flounder liver extracts from contaminated and uncontaminated sites. The results (Figure 1-2) showed a consistency within the seven labs and demonstrated the reproducibility and reliability of data from $^1$H NMR environmental metabolomics (Viant et al., 2009), giving further confidence to the use of NMR as an analytical platform.

![Figure 1-2](image_url)

**Figure 1-2.** Seven separate laboratories were given six metabolite mixtures (S1 through to S6). Using $^1$H nuclear magnetic resonance, they were able to demonstrate that there was consistency in the analysis of these samples that could be discerned using a principal component analysis, where the closeness of the points to each other indicated group similarities. Reproduced with permission from Viant et al. (2009).
One of the oft reiterated criticisms of NMR is the low sensitivity, detecting compounds in the micro-molar range, and potentially not detecting metabolites below this threshold (Pan and Raftery, 2007; Zhang et al., 2012). However, technological developments such as cryogenically cooled probes can improve sensitivity by up to five-fold (Sardans et al., 2011) and the use of microprobe technology can help improve sensitivity as well (Grimes and O'Connell, 2011). For example, the amount of sample required for a standard solution state 5 mm NMR probe can be up to 100 mg of dry mass in 600 μL of solvent (Viant, 2007), which is a problem especially with mass limited samples. The use of a microprobe, such as a 1.7 mm microprobe, can reduce the required sample volumes to 30 μL and thus substantially reduce the amount of biomass used in an experiment. It can provide more sensitivity as well, given the closer physical proximity a sample has to the coil (Grimes and O'Connell, 2011), thus allowing for a better signal to noise ratio. A microprobe has found use in the analysis of secretions of a walking stick insect, a difficult sample to extract, with analyses conducted on approximately 1 μL of insect secretion (Dossey et al., 2006).

The use of two dimensional (2D) NMR analyses can also greatly impact the quality of data as signals are dispersed into a second dimension and allows for better identification of metabolites (Lindon et al., 2004). These include homonuclear methods such as J-resolved NMR, correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) and heteronuclear single quantum coherence spectroscopy (HSQC; Sardans et al., 2011). The use of a heteronuclear method such as HSQC is particularly interesting as it uses the $^{13}$C axis, which is able to disperse signals into a wider dimension than in $^1$H and provides direct $^1$H-$^{13}$C correlations (Reynolds and Enriquez, 2002). While able to increase NMR sensitivity, a heteronuclear 2D acquisition does
have the disadvantage of requiring much greater acquisition time than does a homonuclear spectrum (Reynolds and Enriquez, 2002).

Another issue that is common to NMR spectra is the overwhelming signal from water that is the result of the large water content in biofluids (Dunn and Ellis, 2005). Given that signal intensity is a function of concentration, water can appear at an intensity that is several orders of magnitude more than the signal from other molecules (Giraudeau et al., 2015). As a result, the water signal can saturate the receiver which causes baseline irregularities and improper digitization of the other metabolite peaks and therefore necessitates solvent suppression (Giraudeau et al., 2015). For this, there have been a number of techniques that have been developed and include presaturation, variations on WATERGATE and combinations of both (Kim et al., 2010) as well as Presaturation Using Relaxation Gradient and Echoes (PURGE; Simpson and Brown, 2005). In a study comparing the various solvent suppression techniques it was concluded that PURGE provided the best suppression, while requiring less effort in optimization than other methods (McKay, 2009).

Whether NMR of MS is used, there is inevitably going to be some bias towards a particular compound type, and therefore no single technique can measure all the metabolites in a sample (Weckwerth and Morgenthal, 2005; Zhang et al., 2012). Ultimately, the laboratory with access to various analytical platforms will be most poised for a successful metabolomics experiment (Robertson, 2005). In any case, it has been advances in these analytical modalities that has allowed for the recent development of metabolomics (Zhang et al., 2012).

1.2.4 Data processing and analysis of metabolomics data

Metabolomics studies often produce large data sets that can include hundreds of individual NMR spectra. A typical $^1$H NMR spectrum is divided into discrete sections – termed
‘bins’ – which are then integrated (Reo, 2002). Between spectra, the signal intensities of these bins are compared, and the relative intensities can discern the extent of change in metabolite concentrations (Reo, 2002). However, the many spectra and bins that are produced in a metabolomics experiment create a large amount of data that require analysis beyond rudimentary univariate statistics. This necessitates the need for pattern recognition and multivariate statistical processing software to make the data coherent (Miller, 2007; Prince and Pohnert, 2010). Specifically, the use of both principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) multivariate statistical models has become a standard of metabolomics studies and are ways to take large complex datasets and reduce them to fewer dimensions and easily understood with visual plots (Worley et al., 2013).

The PCA is an unsupervised statistical method, where no prior assumptions about the systems are made and is a fixture of metabolomics studies (Reo, 2002). Spectra are incorporated into a single matrix and the PCA determines which variables in a sample have more weight and reduces the data into fewer dimensions; with usually two or three being sufficient to account for most of the variation (Giansante et al., 2003). The PCA provides plots that are linear combinations with weighting factors of the input variables and these are called principal components (Reo, 2002; Viant et al., 2003a; Weckwerth and Morgenthal, 2005). By reducing the number of dimensions in a dataset, it is possible to visualize differences and similarities between datasets (Viant et al., 2003a), and these are represented in two dimensions, plotting one PC against another. An example PCA scores plot is shown in Figure 1-3 and shows that a PCA can differentiate plant species based on metabolite profiles but that the PCA scores plot is also a visual tool that is easily understood.
Figure 1-3. Based on the clustering pattern, with each point representing a nuclear magnetic resonance spectrum, the principal component analysis is able to distinguish the difference in metabolome profiles of four different plant species. Reproduced with permission from Roessner and Bowne (2009).

In the PCA scores plot, each point represents an NMR spectra (Robertson, 2005), and points that are close together indicate a similarity between the spectra and therefore the metabolite profiles (Hines et al., 2007). If there are clear visual clusters of data points that appear in the PCA, these can then be subjected to statistical tests, such as the student $t$-test or multiple analysis of variance to discern whether there is a statistically significant separation between the metabolome clusters (Fiehn, 2002).
Alternatively, there are supervised statistical tests such as the PLS or the PLS-DA. A supervised statistical test such as the PLS-DA incorporates prior knowledge about the system into the statistical model; i.e. the knowledge of a diseased and control group (Brown et al., 2005a) and the PLS-DA in particular is used extensively in all the omics fields (Gromski et al., 2015). The PLS-DA functions as a statistical tool that uses an X table of bin values like the PCA, but also a Y table that indicates class membership and maximizes the separation between different groups (Gromski et al., 2015). These types of analyses provide a tighter clustering and hence better separation between groups (Reo, 2002). They are however models that are prone to overfitting and therefore require model validation procedures (Gromski et al., 2015). Among the two major multivariate statistics methods however, PCA has emerged as the favoured tool in metabolomics studies, in giving both statistical coherence and visual clarity to the data (Weckwerth and Morgenthal, 2005).

1.3 Environmental metabolomics

The origins of metabolomics can be traced back to the medical sciences (Nicholson et al., 1999; Tuffnail et al., 2009), where it has gained significant traction and utility in, for example, detecting early signs of developmental disorders (Issaq et al., 2008) or in the detection of cancer biomarkers (Spratlin et al., 2009). However, the use of metabolomics in the environmental sciences is more recent and less developed. Environmental metabolomics is more specifically defined as the metabolic changes that are wrought by environmental stressors and anthropogenic pollution on biological systems (Miller, 2007; Størseth and Hammer, 2007; Viant, 2008). In environmental systems, ‘stress’ is a larger umbrella term that encompasses both biotic and abiotic stressors such as disease, predation, exposure to chemical contamination, changes in pH, temperature, nutrient quality and salinity (Lin et al., 2006; Macel et al., 2010; Viant, 2008).
practice, most environmental metabolomics studies are examinations of anthropogenic pollution, where test organisms are subjected to sub-lethal amounts of chemical contamination, to provide mechanistic information that is absent in traditional toxicity assays (Poynton et al., 2011; Størseth and Hammer, 2007). While there is hope that it can be incorporated into environmental monitoring regimes eventually, it will require extensive research and validation before this can occur (Biales et al., 2015; Van Aggelen et al., 2010). In the meantime, metabolomics, and the ‘omics’ in general, will supplement the tests currently used, as part of a broader ‘weight of evidence’ strategy in risk assessments (Biales et al., 2015). A number of elements need to be considered in experimental design. For example, chemical dosages and chronic toxicity needs to be examined, since many chemicals do not give a linear response to toxicity, though this is commonly assumed (Biales et al., 2015; Vandenberg, 2014). Moreover, there is a need to understand how the metabolome differs between acute and chronic toxicity tests and whether organisms can adapt to perturbations over time. These are among some of the challenges that are presented in environmental metabolomics.

1.3.1 Metabolomics studies using aquatic organisms

Studies using aquatic organisms are particularly important given the myriad of contaminants that aquatic environments receive (Santos et al., 2010; Størseth and Hammer, 2007). Because many metabolic and physiological functions are similar to those found in vertebrates (Santos et al., 2010), aquatic organisms such as fish are believed to be appropriate for understanding human toxicology as well. While in principle metabolomics can be used to analyze any species, hitherto studies have focused on established model species in aquatic toxicology studies (Bundy et al., 2009).
Fish such as the rainbow trout (Baumgarner and Cooper, 2012; Kullgren et al., 2010; Samuelsson et al., 2006), goldfish (Jordan et al., 2012; Li et al., 2014; Liu et al., 2015) and fathead minnow (Baumgarner and Cooper, 2012; Collette et al., 2010; Ekman et al., 2007; 2008) comprise a large number of the environmental metabolomics studies. Viant et al (2003), in an early use of metabolomics in environmental science, examined steelhead trout responding to elevated temperatures. They found that the change seen in metabolomics could be correlated with changes in heat shock proteins, a commonly used biomarker, and the comparison to a known biomarker provides important validation for the metabolomics method (Viant et al., 2003b). A more unusual study with fish includes using the urine of fathead minnows and finding biomarkers to discriminate between territorial and non-territorial males, finding that bile and volatile amines could be used as potential biomarkers to distinguish them from each other (Martinovic-Weigelt et al., 2012). This demonstrates an interesting use of metabolomics in chemical ecology and in understanding the natural history of a commonly used aquatic toxicology species.

Mussels are a popular family of organisms used in metabolomics studies (Cubero-Leon et al., 2012; Ellis et al., 2014; Roznere et al., 2014; Tuffnail et al., 2009), as they are sessile, accumulate toxins and are appropriate for field studies since they can be easily transplanted into other sites where they are exposed to local environmental conditions (Fasulo et al., 2012; Roznere et al., 2014; Spann et al., 2011). For example, *Mytillus galloprovincialis* were placed along contaminated and non-contaminated sections on the Augusta coast of Italy (Fasulo et al., 2012). Mussels in an industrial area were seen to have altered energetic pathways such as lipid metabolism and gluconeogenesis when compared to those in an uncontaminated area (Fasulo et
al., 2012). By not using lab reared organisms the use of mussels presents an interesting opportunity for field based metabolomics studies.

In freshwater studies, aquatic invertebrates are important as they are commonly used as indicators of aquatic ecosystem health (Hodkinson, 2005) but remain understudied in metabolomics. Some aquatic invertebrates used in metabolomics studies include the amphipods *Hyalella Azteca* (Ralston-Hooper et al., 2011; Ralston-Hooper et al., 2008), and *Diporia spp.* (Maity et al., 2012; Maity et al., 2013) as well as the blackworm (Agbo et al., 2013). One interesting study examined the stonefly response to varying degrees of oxygen and temperature, as their thermal limits are dictated by available oxygen (Verberk et al., 2013). Using metabolomics the progression into anaerobic metabolism was observed as seen by increased lactate and succinate levels, which were insufficient to offset the loss from reduced aerobic respiration (Verberk et al., 2013).

1.3.2 Metabolomics studies using *Daphnia magna* as a subject

Hitherto work with *D. magna* has focused largely on the response to exogenous chemical contamination (Kovacevic et al., 2016; Li et al., 2015; Poynton et al., 2011; Taylor et al., 2009; 2010; 2016a; 2016b), with one focusing on nutritional quality of foods and natural stressors such as increased salt and bacterial infection (Wagner et al., 2015). A brief summary of these studies is provided in Table 1-1.
<table>
<thead>
<tr>
<th>Authors</th>
<th>Analytical Instrumentation</th>
<th>Environmental Stressor</th>
<th>Major Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taylor et al. (2009)</td>
<td>FT-ICR</td>
<td>Copper</td>
<td>• Reductions in amino acids as well as indications of oxidative stress markers such as glutathione and ophthalmic acid</td>
</tr>
<tr>
<td>Taylor et al. (2010)</td>
<td>FT-ICR</td>
<td>Cadmium, fenvelerate, dinitrophenol and propranol</td>
<td>• Whole organism extracts were more predictive of toxicity than hemolymph</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Propranol decreases fatty acid metabolism and eicosanoid synthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Fenvelerate induces aminosugar disruptions</td>
</tr>
<tr>
<td>Poynton et al.</td>
<td>FT-ICR and NMR</td>
<td>Cadmium</td>
<td>• Decreases in amino acids and fatty acids in the hemolymph, indicating disruptions in their production</td>
</tr>
<tr>
<td>(2011)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Vandenbrouck et</td>
<td>NMR and GC-MS</td>
<td>Pyrene and fluoranthene</td>
<td>• Disturbances in aminosugar metabolism with greater responses seen at higher concentrations</td>
</tr>
<tr>
<td>al. (2010)</td>
<td></td>
<td></td>
<td>• Pyrene and fluoranthene co-exposures result in changes that are more than additive</td>
</tr>
<tr>
<td>Wagner et al.</td>
<td>NMR</td>
<td>Differing diet quality and quantity</td>
<td>• Increases in amino acids in response to poor food conditions and low quantity conditions</td>
</tr>
<tr>
<td>(2015)</td>
<td></td>
<td></td>
<td>• Higher salinity induced decreases in amino acids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Pathogenic bacteria did not induce changes in the metabolome</td>
</tr>
<tr>
<td>Kovacevic et al.</td>
<td>NMR</td>
<td>Triclosan, ibuprofen and carbamazepine</td>
<td>• Carbamazepine induced decreases in amino acids across a gradient of exposure concentrations, as well as an increase in glucose</td>
</tr>
<tr>
<td>(2016)</td>
<td></td>
<td></td>
<td>• Triclosan induced increases in amino acids and a decrease in glucose</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Ibuprofen caused non-monotonic changes in amino acids and glucose levels</td>
</tr>
<tr>
<td>Li et al. (2015)</td>
<td>NMR</td>
<td>Silver nanoparticles and silver ions</td>
<td>• Silver nanoparticles and silver ions induced similar perturbations, with changes in 3-hydroxybutyrate,</td>
</tr>
</tbody>
</table>
arginine, lysine and glucose

- Changes in metabolites indicated disturbances in energy metabolism, oxidative stress and the induction of anaerobic metabolism

<table>
<thead>
<tr>
<th>Study</th>
<th>Method</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garreta-Lara et al. (2016)</td>
<td>GC-MS</td>
<td>Increases in salinity, temperature and hypoxic conditions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- All three conditions induced changes in amino acids and energy molecules that suggest impairments in energy metabolism</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Salinity specifically impacted glycerol and trehalose</td>
</tr>
<tr>
<td>Taylor et al. (2016a)</td>
<td>FT-ICR</td>
<td>Copper and nickel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Metabolomics profiles could be used to predict reproductive impairment in <em>D. pulex-pulicaria</em>, using partial least squares models</td>
</tr>
<tr>
<td>Taylor et al. (2016b)</td>
<td>FT-ICR</td>
<td>Copper</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- The addition of dissolved organic carbon was able to mitigate copper toxicity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- This protection from copper toxicity did not extend to the next generation, as they had depletions in amino acid reserves, as well as in glutathione</td>
</tr>
</tbody>
</table>

The earliest of these studies used FT-ICR-MS to examine a gradient of copper toxicities (Taylor et al., 2009). They found that FT-ICR-MS could be a sensitive platform for discerning changes to increasing copper toxicity, with decreases in amino acids that could signify their induction into cellular repair mechanisms (Taylor et al., 2009). They also found that depletions in glutathione and the similarly acting ophthalmic acid likely signified their use in response to oxidative stress caused by copper exposure (Taylor et al., 2009). A similar vein of research was also conducted using FT-ICR-MS to understand the toxicity of cadmium, 2,4-dinitrophenol, propranolol and fenvelerate and also examined the differing responses between whole organisms and hemolymph (Taylor et al., 2010). Propranolol induced increases in a number of fatty acids and eicosanoids, while fenvelerate caused increases in a number of metabolites involved in amino sugar production (Taylor et al., 2010). Most interesting however is the finding that whole
organisms were more predictive of toxicity than *D. magna* hemolymph, which has pragmatic benefits, as there are limitations to extracting hemolymph from organisms so small, being extractable from only adults (Taylor et al., 2010). However, hemolymph samples were also analyzed by Poynton et al. (2011), who examined the changes incurred after 24 hours of cadmium exposure. This particular study used both FT-ICR-MS and NMR as analytical platforms and also incorporated a transcriptomics component to the study. Amino acids and fatty acids were seen to decrease and were related to the changes seen in the transcripts of a decrease in lipid transport (Poynton et al., 2011). The combination of ‘omics’ technologies has also been performed by Vandenbrouck et al. (2010), who studied the toxicity of pyrene and fluoranthene and mixtures of both, using a combination of NMR and GC-MS metabolomics as well as transcriptomics. The results were related to a 21 day reproduction test, where total reproduction, growth and survival were examined. While reproduction was impaired at the highest concentrations, the metabolomics analysis showed impairments in aminosugar metabolism. Further studies on contaminants include pharmaceuticals such as triclosan, ibuprofen and carbamazepine, finding indicators of oxidative stress in response to triclosan and reduced energy expenditures in response to carbamazepine (Kovacevic et al., 2016).

More recent studies have examined elements that are more reflective of what is found in the natural environment. For example, Taylor et al. (2016b) used metabolomics to demonstrate that dissolved organic carbon (DOC) does mitigate copper toxicity in a concentration dependant manner (Taylor et al., 2016b). Another study was based specifically on non-contaminant stressors and investigated the changes that differing diet quality would have on *D. magna*, comparing this nutritional stress against other environmental stressors such as salinity and bacterial infection (Wagner et al., 2015). They found that *D. magna* grown under low food
conditions had more amino acids, likely a response to a greater need to create energy. This is in contrast with a stressor such as higher saline content, which induced an opposite response, with large decreases in a number of amino acids which is unusual given that many organisms compensate for osmotic stress by releasing more amino acids to create osmotic balance (Wagner et al., 2015).

In aggregate, the studies described show a progression in metabolomics, where increasingly there is a trend to amalgamate other elements (such as a reproduction tests and transcriptomics) into studies, as well as broadening stress from contaminants, to looking at actual ecosystem elements such as DOC and food quality. These studies provide not just the groundwork that is necessary for the acceptance of metabolomics into environmental monitoring regimes, but also simultaneously provides information on the biochemical natural history of *D. magna* as well. In this vein, the focus of this thesis will be to examine *D. magna* in response to stressors that can be found in the environment. These include both anthropogenic contaminants such metals, organophosphates (OPs) and the endocrine disruptor BPA but also responses to a change in allochthonous and authochthonous carbon sources.

### 1.4 Copper, arsenic and lithium

Anthropogenic activities such as mining and manufacturing have resulted in trace metal contamination of water systems and these are particularly problematic as unlike organic contaminants, metals cannot be degraded and therefore persist in the environment (Haferburg and Kothe, 2010). They also differ from organic contaminants in that many of them are requirements for healthy physiological function. One of these metals is copper, which is released into the environment from industries such as mining and fossil fuel combustion (Khangarot and Rathore, 2003). It is simultaneously a required micronutrient but also toxic to organisms,
requiring a certain optimum that is between deficiency and excess (Bossuyt and Janssen, 2005; Santos et al., 2010). For example, copper is required for energy production and oxygen transport enzymes (Santos et al., 2010) and for this reason its levels are regulated by aquatic organisms (Bossuyt and Janssen, 2005). However, in excess copper has been shown to induce stress as copper(II) can be reduced to copper(I) which in turn can result in radical hydroxyl formation which results in oxidative stress and DNA damage (Craig et al., 2007; Paila et al., 2012; Santos et al., 2010). In *D. magna* it alters hemoglobin levels in a dose dependant matter at low microgram levels (Dave, 1984). However, while toxic in excess, it appears that *D. magna* do have a limited capacity to deal with excess copper through increased metallothionein activity or through sequestration in copper granules (De Schamphelaere and Janssen, 2004).

While copper has been well studied, lithium has seen much less attention as a contaminant, even though it has a multitude of industrial uses that emerged from military exigency but also includes use in the ceramics, batteries and was even used as a salt substitute until it was deemed toxic (Corbella and Vieta, 2003; Kszos and Stewart, 2003). It is also used as a treatment for bipolar disorders and so is known to act on the neurological system (Tkatcheva et al., 2007). Unlike copper, lithium is not a physiological requirement for organisms though it is found throughout the human body (Aral and Vecchio-Sadus, 2008). Because it is a monovalent cation, it is thought to act as a substitute for sodium (Kszos and Stewart, 2003) and in a study on rainbow trout has been shown to reduce sodium-potassium adenosine triphosphatase (Na\(^+\) K\(^+\)-ATPase) activity, though lithium was likely not directly inhibiting it, but rather acting on lipid levels instead which acted on Na\(^+\) K\(^+\)-ATPase activity (Tkatcheva et al., 2007). One study on the Ramshorn snail assesses a gradient of lithium exposures which resulted in increased mucus
production and cilia length (Sawasdee et al., 2011). However, beyond this, lithium has received little attention in aquatic toxicology studies and requires further examination.

Like lithium, arsenic is also not known to have any biological function but is found in trace levels in all life (Phillips, 1990; Wang and Mulligan, 2006). Its inputs into the environment can be from the natural weathering of rocks, to the anthropogenic input from mining, wood preservation and arsenic based pesticides, with anthropogenic inputs being three times the rate of natural inputs (Phillips, 1990; Wang and Mulligan, 2006). Because it is long lived in the environment, its mobilization into the surrounding environment has been inevitable (Feng et al., 2005). Arsenic can take a number of oxidative states (-III, 0, III and V), and even organic forms such as arsenobetaine and arsenosugars, but the inorganic forms arsenite and arsenate are thought to be more toxic (Caumette et al., 2012; Chen et al., 1999; Phillips, 1990). While studies with Daphnia spp. have been performed to understand arsenic toxicity, many of them have been studies using endpoints such as mortality and reproduction in order to understand the toxicity of the various forms it can take in the environment (Chen et al., 1999; He et al., 2009; Hoang et al., 2007; Shaw et al., 2007; Theegala et al., 2007). Since metals are long lived and persist in the environment, it is important that their toxicities are understood beyond gauging concentrations that alter mortality and fecundity and instead provide more mechanistic information on the changes that are incurred to sub-lethal exposure. Given the important industrial uses of the three metals discussed, and the general paucity of mechanistic data, these metals were selected for study in this thesis and form the basis for Chapter 2.

1.5 Malathion and diazinon

OPs are a group of insecticides that are used in the management of pests and to a lesser extent used as an herbicide (Maroni et al., 2000). They are a less persistent alternative to the
organochlorine pesticides that preceded them (Barata et al., 2004; Printes and Callaghan, 2004; Ren et al., 2007) and are not regarded as being liable to accumulate through trophic levels (Barata et al., 2004). As a result, since the 1930s they have seen widespread use globally with both agricultural and domestic uses (Guilhermino et al., 1996; Jaga and Dharmani, 2003). The general structure of OPs is as seen in Figure 1-4 where R1 and R2 are ethyl or methyl groups and the R3 group is what differentiates different OPs from each other (Maroni et al., 2000).

Figure 1-4. The general structure of an organophosphate (OP) is shown on the left, where the phosphorous double bond can be to either oxygen or sulfur. It is the R3 group that primarily differentiates OPs from each other.

Two of the major OPs manufactured are malathion and diazinon. Malathion is used to control mosquito populations but also other agricultural pests and is regarded as being less toxic to humans than other OPs (Maroni et al., 2000; Rassoulzadegan and Akyurtlakli, 2002). Similarly, diazinon is also used in the control of agricultural insect pests and is also used directly on humans as a treatment for head lice (Maroni et al., 2000; Sanchez et al., 2000). In spite of their chemical similarities, OPs differ from each other in their chemical fate and behaviour in aquatic ecosystems. For example, malathion and diazinon have log octanol-water partition
coefficients ($K_{ow}$) of 2.75 (Printes and Callaghan, 2004) and 3.81 (Kretschmann et al., 2011) respectively and this will dictate the extent to which they accumulate within an organism. Both OPs are known to degrade quickly, with 95% of diazinon degrading within 2 days and 30% for malathion (Bavcon et al., 2003). The major route of entry for OPs into the environment is through storm water runoff, with rain events creating pulses of exposure that can last from days to weeks and results in the continual exposure to aquatic organisms (Kuivila and Foe, 1995; Scholz et al., 2000). This is a concern because OPs lack target specificity and aquatic organisms are particularly sensitive to exposure, entering organisms through dermal, respiratory and digestive routes (Ren et al., 2007). For example, the LC$_{50}$ for $D. magna$ is in the low μg range for both (Ren et al., 2007; Toumi et al., 2015) and at concentrations as low as 1 μg/L has been shown to alter the alarm responses of the chinook salmon (Scholz et al., 2000).

OPs function as acetylcholinesterase (AChE) inhibitors, which is the enzyme responsible for breaking acetylcholine into acetate and choline (Maroni et al., 2000; Sparling and Fellers, 2007). The accumulation of acetylcholine results in the inability to cease the firing of nerve impulses, resulting in hyperactivity, paralysis and eventually death (Maroni et al., 2000; Ren et al., 2007; Sparling and Fellers, 2007). OPs such as malathion and diazinon are more potent when they have been desulfurated by mixed-function oxidases and transformed into their more toxic oxon analogues (Sparling and Fellers, 2007). The recovery of AChE inhibited by the oxon analogue is slow, and as a result, recovery is primarily through de novo AChE synthesis (Barata et al., 2004). With mammals, the inhibition of AChE can lead to asphyxiation at high concentrations, but in aquatic organisms, the toxicity is less clear (Barata et al., 2004). Studies with $D. magna$ and OPs have been largely mortality/reproduction tests (Sanchez et al., 2000) or studies of AChE inhibition (Barata et al., 2004; Day and Scott, 1990; Guilhermino et al., 1996;
Jemec et al., 2007; Printes and Callaghan, 2004). There is however, some suspicion that OPs can act on systems other than AChE inhibition that are currently unknown, as AChE inhibition may not necessarily be linked to acute toxicity (Printes and Callaghan, 2004). In other studies it has been shown to cause oxidative stress (Lushchak, 2011; Wu et al., 2011) and potentially acts on the endocrine system of the closely related D. pulex (Barry, 2002). OPs may even exert their toxicity by inhibiting feeding behaviours in D. magna (Fernandez-Casalderrey et al., 1994). That the nature of OP toxicity is ambiguous makes an untargeted investigation using metabolomics particularly interesting and formed the basis for acute toxicity studies in Chapter 4.

1.6 Bisphenol-A

BPA has a multitude of industrial uses, though 95% is used as material for polycarbonate plastics and epoxy resins, making it one of the most produced chemicals in the world (Chen et al., 2002; Mu et al., 2005; Richardson et al., 2014). Its ubiquity has resulted in widespread contamination of aquatic systems, with point sources such as sewage and landfill discharge (Crain et al., 2007). In rivers, BPA has been demonstrated to fully degrade by 8 days, though its intermediary degradation products can last even after two weeks (Suzuki et al., 2004). For humans, the major route of exposure is through the hydrolysis of plastic products, allowing the migration of BPA into foods in the low ppb range (Biles et al., 1998). The widespread use of BPA is rife with controversy, as it has been shown to have a mode of action similar to that of estradiol, binding to the estrogen receptors and thus interfering with the human endocrine system (Crain et al., 2007; Jordão et al., 2016; Rubin, 2011; Wolstenholme et al., 2011). As a result, it has drawn much attention and has seen extensive study in mammalian systems (Crain et al., 2007; Flint et al., 2012; Vandenberg et al., 2007). Structurally, it is the phenol group (Figure 1-5).
that is believed to give it properties similar to estradiol and is able to interact with the estrogen receptor (Nishihara et al., 2000).

![Figure 1-5](image)

**Figure 1-5.** The structure of bisphenol-A is compared to estradiol. It is the phenol group that is thought to act on the estrogen receptors the way estradiol does.

But in addition to altering mammalian endocrine function it has been shown to alter the neurobehaviour in children (Mustieles et al., 2015), as well as acting as an obesogen (Wang et al., 2013).

Though in vertebrate systems BPA toxicity has received widespread attention (Crain et al., 2007), there is less known about how BPA toxicity to aquatic invertebrates. Studies that have been performed with aquatic invertebrates have been largely studies that employ traditional assays such as the LC$_{50}$ acute toxicity test and reproduction tests (Marcial et al., 2003; Mihaich et al., 2009; Sieratowicz et al., 2011; Ura et al., 2002; Watts et al., 2001). One study with Ramshorn snails found superfeminization in response to BPA exposure (Oehlmann et al., 2006) and another found that the gonad of female mussels were sensitive to BPA (Ji et al., 2014), suggesting that there may be an estrogenic capacity even in invertebrates. Studies using cladocerans have also largely employed apical endpoint tests (Brennan et al., 2006; Caspers, 1998; Chen et al., 2002; Klecka et al., 2001; Mansilha et al., 2013). Two studies have found that in the low mg/L range there is no change reproductive output in *D. magna* when subjected to the 21 day reproduction test (Brennan et al., 2006; Caspers, 1998), yet these tests may be insensitive to understanding the
full extent of BPA toxicity. For example at similar concentrations, oxidative stress markers, such as glutathione S-transferase and catalase have confirmed the oxidative stress capacity of BPA (Jemec et al., 2012; Park and Choi, 2009) and as such warrants further investigation at the molecular level.

Because of the widely reported endocrine disrupting potential of BPA, there has been effort to understand whether the hormone system of *D. magna* is altered in response to BPA exposure (Mu et al., 2005). In arthropods, these are controlled by ecdysteroids, which are hormones that are responsible for molting, growth and reproduction (Mu et al., 2005). In *D. magna* the predominant ecdysteroid is 20-hydroxyecdysone (20HE; Martin-Creuzburg et al., 2007) and altered levels of 20HE impair growth and reproduction (Mu et al., 2005). One study investigated whether BPA altered 20HE levels by looking at reproduction parameters and found that an increased intermoult period and altered embryonic development were suggestive of ecdysteroid interference (Mu et al., 2005), though direct analyses of 20HE were not performed. It is difficult to determine if there are direct inhibitions on 20HE or if the reproductive impairment is the result of other mechanisms. While a method to extract and analyze 20HE levels in *D. magna* has been pursued by others (Martin-Creuzburg et al., 2011), examinations of 20HE in response to BPA have not been made yet and it remains difficult to conclude whether BPA acts on the ecdysone axis of *D. magna*.

Given the paucity of biochemical information on the response of *D. magna* to BPA, this compound was selected for analysis using metabolomics. However, while it was also part of the acute toxicity test (Chapter 4), because of the potential endocrine disruption of BPA, it was also selected for examination using chronic toxicity test over 21 days (Chapter 5). This is because it will be important to monitor both neonatal output and to monitor 20HE levels over a longer time.
span, to be able to determine if indeed BPA acts on the hormonal system of *D. magna* and if these changes can be related to changes in the metabolome. For the analysis of 20HE, LC-MS/MS will be used as the primary analytical platform as it has been shown to be amenable for ecdysone analysis (Zhou et al., 2011).

1.7 Autochthonous and allochthonous carbon sources

While the focus of environmental metabolomics has been largely restricted to studies of contaminant toxicity, stress can be more inclusive to include environmental elements such as salinity (Wu et al., 2013), temperature flux (Boroujerdi et al., 2009) and even predator/prey interactions (Stitz et al., 2011). One of these environmental elements is the changing sources of carbon that exist in an aquatic environment and the stress that this can have on zooplankton. Where aquatic trophic transfer was once viewed as a system where photosynthetic primary production was the only food resource for zooplankton, an emerging view is that there are other means by which zooplankton accumulate carbon (Rautio et al., 2011). This has been demonstrated by whole lake additions of $^{13}$C that showed that primary production could not support aquatic food webs and that terrestrial sources offset this imbalance (Pace et al., 2004). This allochthonous carbon, namely terrestrially derived DOC, can be assimilated by bacteria which are then consumed by zooplankton (Bouchnak and Steinberg, 2013), or more controversially, directly assimilated by zooplankton (Hofmann et al., 2012; Speas and Duffy, 1998). In any case, it is clear then that zooplankton derive their nutrients from both autochthonous primary production and allochthonous secondary production, and the consumption of these serves as the conduit through which carbon enters a trophic system (Jansson et al., 2007; Taipale et al., 2014). However, the quality of the carbon sources cannot be said to be the same, especially given that essential fatty acids are found in phytoplankton and far
less in allochthonous sources (Rautio et al., 2011). Sources such as cyanobacteria and bacteria are poor carbon sources as they are generally lacking in fatty acids as well (Brett and Muller-Navarra, 1997; Taipale et al., 2014). This is problematic as the composition of a lake can change seasonally. For example, in early fall there is a larger proportion of allochthonous carbon sources entering water sources and less contribution from autochthonous sources (Taipale et al., 2007). These are essentially then, challenges that D. magna face, as the quality of the water around them is subject to seasonal flux. While there have been studies that have examined the zooplankton response to these changes, they have used traditional ecotoxicity assays, particularly reproduction tests (Bouchnak and Steinberg, 2010; Brett et al., 2009; Burns et al., 2011; Wacker and Martin-Creuzburg, 2007) and do not provide information on the biochemical changes incurred by these changes. For this reason, studying the response of D. magna to differing autochthonous and allochthonous carbon sources that reflect these changes will help understand how D. magna respond to the stress of a changing environment around them. This will form the basis for the study in Chapter 6.

1.8 Objectives and hypotheses

The overarching objective of the work in this thesis is to apply \(^1\)H NMR-based metabolomics in the analysis of the metabolome of D. magna responding to various stressors. For this, a number of stressors were chosen to encompass a wide breadth of what would constitute environmental stress. This research also entailed the development of a more refined and efficient method for metabolomics procedures and then the application of the procedure in \(^1\)H NMR-based metabolomics studies. Two types of contaminants were selected for this purpose: metals such as copper, lithium and arsenic and organic contaminants such as malathion, diazinon and BPA. Examining both malathion and diazinon allows for the analysis of two presumably
similarly acting OPs and a much different endocrine disruptor in BPA. These are contaminants that have received little biochemical study and thus warrant examination using metabolomics.

Furthermore, the response of \emph{D. magna} to autochthonous and allochthonous carbon sources will provide insight into the stress incurred by changing carbon sources.

Objective 1: To examine the metabolite changes incurred by exposure to two common metal contaminants (copper and arsenic) and another that has received little attention (lithium) to neonates, over 48 hours of exposure. This objective is the focus of Chapter 2.

Objective 2: To examine various extraction procedures and dry masses to optimize the use of \emph{D. magna} in metabolomics studies using an NMR microprobe. A number of extraction procedures will be tested to gauge which provides the most consistent, comprehensive metabolite coverage using \textsuperscript{1}H NMR. This objective is addressed in Chapter 3.

Objective 3: To investigate the use of \textsuperscript{1}H NMR-based metabolomics in understanding the toxicity of malathion, diazinon and BPA over 48 hours. For this purpose, \emph{D. magna} will be exposed to a gradient of contaminant concentrations in order to examine whether responses are necessarily dose dependant. This objective is addressed in Chapter 4.

Objective 4: To investigate how the metabolome of \emph{D. magna} changes in response to chronic BPA exposures. This will be a 21 day test, mimicking the 21 day reproduction test, which will also be performed for comparison. Also, an examination of 20HE levels will be made as well,
using LC-MS/MS to address whether the changes incurred are necessarily hormonal or not, given the endocrine disrupting nature of BPA. This objective is addressed in Chapter 5.

Objective 5: To determine whether changes in the *D. magna* metabolome can be detected when they are subjected to a change in carbon quality. For this they will be raised on differing authochthonous and allochthonous carbon sources that will be represented by cyanobacteria (*Synechocystis* spp.), DOC and differing chlorophyta concentrations (*Chlorella vulgaris* and *Raphidocelis subcapitata*). This objective is addressed in Chapter 6.

There are several hypotheses tested in this thesis:

1) It is hypothesized that NMR based metabolomics will be sensitive enough to detect distinct changes in *D. magna* responding to one of copper, lithium or arsenic over 48 hours of exposure (tested in Chapter 2).

2) As OPs are known to have similar modes of action, it is hypothesized that they will induce a similar dose dependant response in *D. magna* to a gradient of sub-lethal exposures. They are predicted to be distinct from what will be seen with BPA exposure (tested in Chapter 4).

3) As more BPA accrues within *D. magna*, it is hypothesized that chronic BPA toxicity (over 21 days) will result increasingly in changes to metabolome that become distinctly different with time. This will be reflected in the 20HE levels as there is further endocrine disruption of *D. magna* (Tested in Chapter 5).
4) It is hypothesized that *D. magna* will respond to changes in carbon quality by inducing a compensatory change in energetics that allows it to adjust to a new carbon diet quality. These are changes that should manifest in response to the presence of a cyanobacteria or DOC, given their poor quality as carbon substrates (tested in Chapter 6).

Overall, the objectives outlined in this thesis provide the basis for using $^1$H NMR-based metabolomics in understanding the mechanisms of toxic stress in a sentinel ecosystem species. Through achieving these objectives and testing these hypotheses, there are two major contributions that can be made: the development of a tool that can be used by ecosystem monitors and the further understanding of the biochemistry of how *D. magna* cope with environmental stress.
1.9 Thesis summary

Chapter 1: Metabolomics as an alternative to traditional toxicity bioassays in aquatic toxicology

Chapter 2: $^1$H NMR-based metabolomics investigation of *Daphnia magna* responses to sub-lethal exposure to arsenic, copper and lithium

This chapter has been published in the journal Chemosphere and addresses objective 1. *D. magna* neonates were exposed to sub-lethal levels of one of three metals: arsenic, copper or lithium for a 48 hour period. It was found that lithium and copper showed a mode of toxicity that was similar and suggested Na$^+/K^+$ATPase disfunction. For arsenic there were few changes seen in the metabolome but minor changes that indicated impairment in energy metabolism. This study demonstrated that $^1$H NMR-based metabolomics could be used to examine sub-lethal stress in *D. magna*.

Chapter 3: Development of an NMR microprobe procedure for high-throughput environmental metabolomics of *Daphnia magna*

This chapter has been published in the journal Magnetic Resonance in Chemistry (special issue) and addresses objective 2. After testing various extraction procedures, it was concluded that a D$_2$O buffer extraction procedure yielded the most metabolites while maintaining the highest signal to noise ratio. It was also found that 1-1.5 mg of *D. magna* dry mass in 40 μL of solvent provided the optimal range for *D. magna* metabolomics experiments using a 1.7 mm NMR microprobe. This allowed for greater sample parsimony in an already mass limited sample. The optimization of extraction procedures and dry mass requirements was able to set a foundation for the subsequent experiments in Chapters 4-6.
**Chapter 4:** Metabolomics reveals energetic impairments in *Daphnia magna* exposed to diazinon, malathion and bisphenol-A

This chapter has been published in the journal Aquatic Toxicology and addresses objective 3. Adult *D. magna* were exposed to a gradient of concentrations of one of diazinon, malathion or BPA in order to understand dose dependence. It was found that for OPs, there were two distinct patterns that could be discerned that were characteristic of the severity of the stressor. For moderate stress, amino acids were used in gluconeogenesis and anaerobic metabolism was also induced. For severe stress, there is a cessation in metabolism that is manifested as an increase in amino acids and a decrease in energy molecules. For BPA exposures, the highest concentrations tested induced a response that was characteristic of severe stress. By conducting a dose dependent experiment, it was found that toxicity is not always linear and that there can be distinct responses that would otherwise be missed in single dose experiments.

**Chapter 5:** Chronic bisphenol-A exposures induced metabolomics changes in *Daphnia magna* that are age dependent

This chapter addresses objective 4. *D. magna* were exposed to a sub-lethal level of BPA over 21 days. It was found that after 21 days there were no changes in total reproductive output of *D. magna*. However, at 14 days a metabolomics examination showed that there were changes in a number of amino acids that were suggestive of a compensatory energy strategy. At 21 days of exposure, there were no changes in the *D. magna* metabolome, suggesting an adaptive capacity of *D. magna*. Analyses of 20HE indicated that BPA does not alter circulating levels of this hormone and therefore BPA may not act on the endocrine system of *D. magna*. 
Chapter 6: $^1$H NMR-based metabolomics shows *Daphnia magna* responds to different carbon resources through a change in energy partitioning

This chapter addresses objective 5. *D. magna* were reared on a mixture of two chlorophyta (*R. subcapitata* and *C. vulgaris*) and switched to alternative carbon sources that would mimic what is found in the natural environment. For this, a cyanobacteria, DOC, one of the two chlorophyta or a mixture of chlorophyta and DOC were examined as different carbon sources. It was found that cyanobacteria and DOC induced major changes in the metabolome. Cyanobacteria induced a response indicative of toxic stress, where energy was being produced to maintain a homeostatic balance. DOC induced a response that was indicative of starvation and showed that DOC is a poor source of carbon, if it can be assimilated at all. This study demonstrated that changes in carbon sources, even if only short term, can induce stress on the metabolome of *D. magna*.

Chapter 7: Conclusions, limitations and future research.
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Chapter 2

$^1$H NMR-based metabolomics investigation of *Daphnia magna* responses to sub-lethal exposure to arsenic, copper and lithium


(modified to include an analysis of variance (ANOVA) with a Bonferroni posthoc test, which replaces the multiple student $t$-tests used in calculating statistical significance for both the principal component analysis (PCA) and the metabolite percent changes. Text in the method and results sections has been modified to account for these changes. Figure 2-3 has also been modified to reflect the changes in significance. The in house R script used in the ANOVA has been included in Appendix A, as well as the results of the Bonferroni posthoc test).

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2.1 Abstract

Metal and metalloid contamination constitutes a major concern in aquatic ecosystems. Thus it is important to find rapid and reliable indicators of metal stress to aquatic organisms. In this study, we tested the use of $^1$H nuclear magnetic resonance (NMR)-based metabolomics to examine the response of *Daphnia magna* neonates after a 48 hour exposure to sub-lethal concentrations of arsenic (49 µg/L), copper (12.4 µg/L) or lithium (1150 µg/L). Metabolomic responses for all conditions were compared to a control using principal component analysis (PCA) and metabolites that contributed to the variation between the exposures and the control condition were identified, and quantified. The PCA showed that copper and lithium exposures result in statistically significant metabolite variations from the control. Contributing to this variation was a number of amino acids such as: phenylalanine, leucine, lysine, glutamate, glycine, alanine, methionine and glutamine as well as the nucleobase uracil and osmolyte glycerophosphocholine. The similarities in metabolome changes suggest that lithium has an analogous mode of toxicity to that of copper, and may be impairing energy production and ionoregulation. The PCA also showed that arsenic exposure resulted in a metabolic shift in comparison to the control population but this change was not statistically significant. However, a significant change in alanine was observed, suggesting that energy metabolism is indeed disrupted. This research demonstrates that $^1$H NMR-based metabolomics is a viable platform for discerning metabolomic changes and mode of toxicity of *D. magna* in response to metal stressors in the environment.
2.2 Introduction

Aquatic environments are the catchments for many contaminants, both natural and anthropogenic (Komjarova and Blust, 2008; Soetaert et al., 2007; Tomasik and Warren, 1996). Metal contamination is particularly problematic since metals cannot be degraded, and thus accumulate in biota or sediment (Spann et al., 2011), rendering them highly persistent in the environment. Many metals are by-products of mining activity, most notably via acid mine drainage; a process where heavy metals become soluble and consequently contaminate aquatic ecosystems (Altshuler et al., 2011; Gerhardt et al., 2005; Haferburg and Kothe, 2010; Yim et al., 2006). Studies are further confounded by the varying toxic mechanisms exhibited by metals. For example, copper, though widely known as essential for proper physiological function (Atienzar et al., 2001; Bossuyt and Janssen, 2005; Komjarova and Blust, 2008; Santos et al., 2010), in excess is suspected to manifest toxicity by interrupting the activity of the enzyme Na+/K+ ATPase, thus disrupting the intake of the sodium necessary for ionoregulation (Bianchini and Wood, 2008; Grosell et al., 2002; Santore et al., 2001). Copper also serves as a strong redox cycler and may create the potentially harmful superoxide radical via Fenton type reactions (Barata et al., 2005; Lushchak, 2011; Wu and Wang, 2010). By contrast, arsenic is a non-essential metalloid that is assumed to be toxic at low exposure concentrations because it can serve as a surrogate for essential elements in biological processes, namely by replacing phosphorous in phosphatase production (Tomasik and Warren, 1996). This in turn may disrupt energy production (Hoang et al., 2007). The toxic mode of action is known for some metals but there is still a gap in our understanding for some metals and how these may impart sub-lethal stress on aquatic organisms (Bossuyt and Janssen, 2005; De Schamphelaere and Janssen, 2004). For example, lithium has not been studied widely in spite of its known neurotoxic and
teratogenic properties to a wide array of organisms (Corbella and Vieta, 2003; Tkatcheva et al., 2007). It is currently hypothesized that, as with copper, lithium interferes with the action of sodium because monovalent cations may substitute for each other in cellular activities (Kszos and Stewart, 2003), thus potentially interfering in the electrochemical gradient created by Na⁺/K⁺ ATPase. The lack of ecotoxicological understanding of lithium is one example of the thousands of chemicals that have not been adequately screened for toxicity (Khangarot and Rathore, 2003), and as such, there is a need to develop a method to rapidly and reliably test the ecotoxicity of these contaminants. This is a pursuit that will aid in the discovery of novel responses to metal stress and in turn aid in the development of more efficient risk assessment procedures.

Furthermore, many studies in aquatic toxicology have been traditionally rooted in endpoints such as mortality and decreased fecundity (De Coen and Janssen, 1998; Khangarot and Rathore, 2003; Taylor et al., 2009). Though mortality is an obvious indicator of chemical toxicity, sub-lethal stress is more difficult to measure and study (Chen et al., 1999; Spann et al., 2011) and this will aid in the early detection of aquatic ecosystem contamination.

Since organisms quickly respond to toxic stress at the metabolic level, metabolomics, the broad characterization of an organism’s endogenous low molecular weight metabolite content (Clarke and Haselden, 2008; Poynton et al., 2011), is emerging as a sensitive method for detecting sub-lethal stress. Metabolomics is a holistic analysis that allows for the examination of multiple metabolic pathways concurrently (Taylor et al., 2010). Through this, it affords the ability to discern differences between healthy and stressed organisms (Santos et al., 2010). The use of proton nuclear magnetic resonance (¹H NMR) as a platform in metabolomics studies has the potential to provide an efficient and indiscriminant assessment of organism health and aid in novel biomarker identification. As a platform, ¹H NMR is rapid, quantitative and requires little
sample preparation (Clarke and Haselden, 2008; Spann et al., 2011) and as such provides a powerful tool in the rapid screening of many contaminants. Given the advantages of metabolomics, in tandem with $^1$H NMR, further investigation with this method is warranted in aquatic ecotoxicology.

The microcrustacean *Daphnia magna* is a ubiquitous fixture in aquatic ecosystems, where it occupies an intermediate position in the food chain, making it important in the trophic transfer of contaminants (Altshuler et al., 2011; Gillis et al., 2005; Lampert, 2006; Tsui and Wang, 2007). *D. magna* are short lived (approximately 40 days), accessible for culturing and sensitive to toxic contamination (Anderson and Jenkins, 1942; Bianchini and Wood, 2008; Harris et al., 2012; Soetaert et al., 2007; Tsui and Wang, 2007; Vandenbrouck et al., 2010). For these reasons *D. magna* is a widely studied keystone species in ecotoxicology (Lampert, 2006; Soetaert et al., 2007). Research with *D. magna* using $^1$H NMR metabolomics are not as prevalent as with other model organisms but include a study of the toxic mode of action of cadmium (Poynton et al., 2011) and the polycyclic aromatic hydrocarbons, pyrene and fluoranthene (Vandenbrouck et al., 2010). In addition, two studies have employed Fourier Transform Ion Cyclotron Resonance Mass Spectrometry to study the toxicity of copper, cadmium and a number of organic compounds (Taylor et al., 2009; Taylor et al., 2010) and found changes in metabolic pathways such as fatty acid metabolism, eiconasoid synthesis and changes in amino acid concentration (Taylor et al., 2009; Taylor et al., 2010). They have demonstrated that metabolomics techniques are viable with *D. magna*, however, there are many contaminants that remain untested and their mechanisms poorly understood.

In the current study, we tested the efficacy of $^1$H NMR-based metabolomics methods for detecting metabolic shifts in *D. magna* after exposure to sub-lethal concentrations of arsenic,
copper or lithium over a 48 hour period. As mentioned previously, there have only been two studies that employ $^1$H NMR-based metabolomics to ecotoxicity studies of *D. magna*. Thus, part of our objective is to determine the suitability of this emerging technique for monitoring sub-lethal metal stress in aquatic ecosystems. In addition to this, we use the changes in the metabolic fingerprint to ascertain the potential toxic modes of action of arsenic, copper or lithium and to identify potential metabolite markers of sub-lethal metal stress. This information will provide further insight into the mode of action of metal toxicity at the molecular-level.

2.3 Materials and methods

2.3.1 Daphnia culturing

*D. magna* used were from an established, 25 year old culture maintained at the Ontario Ministry of the Environment (Toronto, Ontario). Cultures were maintained at 20°C, a water hardness of 124 mg CaCO$_3$/L (consistent with local freshwater conditions) and were kept under a 16:8, light to dark photoperiod. They were fed an algal diet of *Pseudokirschneriella subcapitata* and *Chlorella fusca* at a 50:50 ratio. Only *D. magna* neonates (<24 hours hold) were used, since they are still feeding on the maternal yolk (Goulden et al., 1987), and therefore eliminating the potential changes in the metabolome that may arise from variations in feeding pattern.

2.3.2 Daphnid exposure to sub-lethal concentrations of arsenic, copper and lithium

*D. magna* were exposed for 48 hours to either: arsenic (added as NaAsO$_2$), copper (added as CuSO$_4$) or lithium (added as LiCl) at sub-lethal concentrations. To ensure that exposure concentrations were at sub-lethal levels, the lethal concentrations that results in 50% mortality of the population (LC$_{50}$ values) were determined (Environment Canada, 2000) and observed to be 490 μg/L, 24.8 μg/L, and 2300 μg/L for arsenic, copper, and lithium, respectively. For copper
and lithium, *D. magna* were exposed to half of the LC$_{50}$ values (12.4 µg/L and 1150 µg/L respectively), but because of the acute response observed in *D. magna* during preliminary arsenic exposure experiments, only 1/10th of the LC$_{50}$ value (49 µg/L) was used. For each exposure, 8 populations comprised of 250 individuals (~10 mg of dry mass) were used for a total of 2000 individuals. 8 groups of 250 individuals that were unexposed control populations were also included and served as the reference to which each exposed population was compared. Preliminary experiments found that ~10 mg of dry mass is required for extraction to obtain $^1$H NMR data.

2.3.3 Sample preparation, extraction and $^1$H nuclear magnetic resonance analysis

After exposure, daphnids were flash frozen with liquid nitrogen and lyophilized. The metabolites were extracted with 750 µL of a D$_2$O 0.2 M phosphate buffer (NaH$_2$PO$_4$ •H$_2$O, 99.3%, Fisher Canada) adjusted to pD=7.4 with NaOD (30% w/w in 99.5% D$_2$O, Cambridge Isotope Laboratories Inc.; (Brown et al., 2008; Lankadurai et al., 2011). The buffer also contained 0.1% w/v sodium azide as a preservative (99.5% purity, Sigma Aldrich) and 10 mg/L of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, 97% Sigma Aldrich) as an internal reference standard (Brown et al., 2008; Lankadurai et al., 2011). Each sample was vortexed for 30 seconds and then sonicated for 10 minutes. Samples were then centrifuged for 20 minutes at 14,000 rpm (~15,000 g) and the supernatant was decanted and placed directly into 5mm High Throughput$^\text{plus}$ NMR tubes (Norell Inc., NJ, USA) for $^1$H NMR analysis. Samples were analyzed using a Bruker Avance III 500 MHz spectrometer equipped with a $^1$H-$^{19}$F-$^{15}$N-$^{13}$C 5mm Quadruple Resonance Inverse (QXI) probe. Water suppression was performed with the Presaturation Using Relaxation Gradients and Echoes (PURGE) pulse program (Simpson and Brown, 2005). NMR data were collected using 128 scans with a relaxation time of 3 seconds and
64k time domain points. Spectra were apodized via multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectra, and a zero filling factor of 2 (Lankadurai et al., 2011). The resulting spectra were manually phased and calibrated to the trimethyl silyl group of DSS (δ = 0.00 ppm).

2.3.4 Statistical analysis, metabolite identification and percent change calculations

Data analysis was performed with AMIX software (v. 3.9.7, Bruker BioSpin). Spectra were divided into segments (or buckets) of 0.02 ppm, between 0.5 and 10 ppm, resulting in a total of 475 buckets. The spectral region between 4.7 and 4.9 ppm (corresponding to the residual water signal) was omitted. The buckets were normalized to the total sum of intensities (Lankadurai et al., 2011) and the integrated areas for the buckets were used in principal component analysis (PCA). PCA is an unsupervised data reduction tool that allows the data to be represented as simpler, uncorrelated variables that represent the greatest variation in the dataset (Kemsley et al., 2007; Trygg et al., 2007). In instances when this variation in the dataset is due to variability in responses to exposure to different toxicants, the classes representing these exposures can be discriminated and the metabolite changes driving these class differences identified. For example, a PCA scores plot was used to assess whether the metabolite composition of each exposed daphnid group showed a variation from the control group. The data from PCA was then subjected to an analysis of variance (ANOVA) with a Bonferroni posthoc test using an in house R script (Appendix A, A2-1), to determine if the observed variations in metabolome for exposed daphnids were statistically significant (p < 0.05) from the control. The mean PCA was also plotted with the associated standard error values as an indicator of variation. As the PCA only indicates that there is a variation between exposure groups, a loadings plot is
used to identify the specific buckets that are contributing to the variation (Bundy et al., 2002). PCA loadings plots were obtained using AMIX software (v. 3.9.7, Bruker BioSpin).

NMR spectral regions highlighted in the PCA loadings plots that differed with metal exposure were compared to metabolite resonances using the AMIX Bioref database, as well as existing published spectra found on the Madison Metabolomics Consortium Database (Jones et al., 2012; Vandenbrouck et al., 2010) and previously published metabolite resonances (Brown et al., 2008). An ANOVA with a Bonferroni posthoc established statistical significance of metabolic changes in response to metal exposure. NMR intensity values of the exposed group were subtracted from the bucket values of the control, then divided by the control bucket value, to produce a percent change (Ekman et al., 2007; Lankadurai et al., 2011).

2.4 Results and discussion

2.4.1 Principal component analysis (PCA)

Given the large amount of data produced in metabolomics, dimension reduction methods are often used to quickly screen for differences between groups (Macel et al., 2010; Reo, 2002), as such, PCA is commonly used in metabolomics (Macel et al., 2010). The PCA scores plot of metal exposed *Daphnia* (Figure 2-1A) shows that the metabolic profile of *D. magna* mostly differs from the control with copper exposure, followed by lithium and arsenic exposures. A mean PCA scores plot (Figure 2-1B) was also constructed, and the ANOVA shows the statistical significance of the separation between groups (PC1: $F(3,28) = 27.27$, $p = 1.85 \times 10^{-8}$, PC2: $F(3,28) = 3.01$, $p = 4.70 \times 10^{-2}$).
Figure 2-1: A) Principal component analysis (PCA) scores plot of principal component 1 versus principal component 2 for *D. magna* exposure to arsenic, copper and lithium. B) Mean PCA values (along with the associated standard error) calculated from the averages of the 8 populations used in each exposure show the separation of metal exposures from the control. Statistical significance (P < 0.05) of the separation of metal exposure from the control is indicated by an asterisk.
Distinct clusters for copper and lithium, relative to the control group (confirmed via ANOVA with Bonferroni posthoc (Appendix A, Table A2-1), p < 0.05), indicates that there is a significant metabolic shift in response to exposure to these metals, with principal component 1 explaining 73% of the variation. Although the metabolome for arsenic exposure shows a variation from the control along principal component 2 (explaining 12% of the variation), it is not statistically significant. This may be because the arsenic concentration used in the experiment was 1/10\(^{th}\) of the LC\(_{50}\) due to the high mortality rates observed in preliminary LC\(_{50}\) experiments. This is particularly true in using the trivalent form of arsenic, which is known to have a greater toxicity than the pentavalent form (Chen et al., 1999; He et al., 2009). Preliminary experiments found that there was a rapid rise in lethality when arsenic exposure concentrations were increased; the acute toxicity curve was very shallow and continued for over two orders of magnitude (data not shown). This made it difficult to gauge a proper sub-lethal concentration while minimizing \textit{D. magna} mortalities. Copper and lithium exposures were at half the LC\(_{50}\) values which may be why the metabolomic response was more distinct and statistically significant than of that observed for arsenic (Figure 2-1).

The PCA loadings plot aids in the identification of metabolites that contribute to the separation in the PCA scores plot and the spectral regions that are altered in response to an external stressor (Bundy et al., 2002). Figure 2-2 shows the loadings plots for arsenic, copper and lithium exposures in comparison to the control. The largest peaks correspond to the spectral regions that contribute most to the separation observed in the principal components (Bundy et al., 2002). A number of amino acids, as well as uracil and glycerophosphocholine were identified as contributing to the separation observed in the PCA scores plot (Figure 2-1).
Figure 2-2: Principal component loadings plot for PC1 and PC2 for *D. magna* exposed to: A) arsenic, B) copper and C) lithium. Identified metabolites are labelled.
For copper and lithium exposures, metabolites that contributed significantly to the PCA separation from the control include: alanine, leucine, isoleucine, valine, methionine, glutamate, glycine, and glycerophosphocholine. For arsenic exposure, alanine, isoleucine, glycine, methionine, glutamate, threonine and arginine contributed to the variation from the control.

2.4.2 Changes in specific metabolites

Figure 2-3 summarizes the changes in metabolite concentrations with metal exposure in comparison to the control with significance for each metabolite calculated using an ANOVA with a Bonferroni posthoc test (Appendix A, Tables A2-2 and A2-3). For arsenic exposure, though the overall metabolome does not show a statistically significant change (as seen in the PCA scores plot Figure 2-1), there is a statistically significant change in alanine. The decrease in alanine may be due to disruptions in energy metabolism (discussed further below). The increase in lysine differs from the trend seen with other amino acids, since other than glycerophosphocholine, it is the only metabolite that shows an increase. As a precursor to carnitine, it is important in fatty acid metabolism (Maity et al., 2012) and the relative increase in lysine suggests a stress induced alteration in energy production.

Both copper and lithium exposures exhibit similar decreases in amino acids such as alanine, lysine, leucine, methionine, valine, glutamine, glycine, phenylalanine, glutamate, as well as in uracil, and an increase in glycerophosphocholine (Figure 2-3).
Figure 2-3: Percent changes in the metabolites found using $^1$H NMR spectroscopy. Statistical significance ($P < 0.05$) is indicated by the asterisk.

The similarity between metabolic changes suggests that the toxic mode of action is similar for these two metals. Many of these amino acids (such as alanine, leucine, glutamate, valine and methionine) are intermediaries of the tricarboxylic acid (TCA) cycle (Kokushi et al., 2012). The observed decreases suggest that such amino acids are either being produced in lesser quantities, being depleted in mitigating the toxicity, or the organisms are suffering overall
reduced growth. Alanine in particular decreases in concentration for not just copper and lithium, but arsenic as well, and has been observed to decrease in *Mytilus galloprovicialis* exposure to nickel (Jones et al., 2012) and in *Pimephales promelas* exposure to vinclozolin (Ekman et al., 2007). As alanine can be converted to pyruvate in the TCA cycle (Ekman et al., 2007; Southam et al., 2008), this observed decrease may suggest an increase in energy metabolism as a coping mechanism with the metal exposure. It is commonly observed that organisms respond to toxins by directing a greater portion of their energy stores to maintain regular metabolism, or in the induction of defense mechanisms (Spann et al., 2011) which in turn can compromise growth and reproduction (Soetaert et al., 2007; Spann et al., 2011). Metals such as copper are well-known sources of oxidative stress (Barata et al., 2005; Lushchak, 2011), which is not only damaging, but is also energetically taxing to an organism (Booth et al., 2011; Connon et al., 2008). The overall decrease in amino acids suggests that there is an increased metabolic burden (De Schamphelaere et al., 2007) or impaired respiration (Giarratano et al., 2007; Khangarot and Rathore, 2003). Should this be the case, a lack of available amino acids can compromise downstream protein synthesis, thereby inhibiting organismal growth. The large decrease in uracil observed in both copper and lithium exposures, is indicative of alterations to nucleotide metabolism that may reduce RNA production, protein synthesis and organismal growth (Boer et al., 2010) and is consistent with the observed amino acid decrease.

Methionine and lysine also decreased as a result of copper and lithium exposure. Both are among amino acids that are stored by crustaceans as energy reserves during molting cycles (Maity et al., 2012) and the decreased availability of these amino acids may impair the molting process. This impairment is particularly important, since *D. magna* undergo ecdysis 20 hours after being released from the brood pouch, thus experiencing their first molt (Anderson, 1946;
David et al., 2011); a period that coincides with the life stage of the daphnids used in this experiment (neonates are <24 hours old). This can delay growth and prolong instar periods (De Schamphelaere et al., 2007). This inhibition of growth was observed by Fan et al. (2009) as *D. magna* exposed to cadmium directed energy towards metallothionein induction. In addition to the impairment in energy metabolism, the exposures may have also impacted the osmoregulatory status of the daphnids, as changes in several free amino acids (glycine, glutamine, proline, alanine) which are required for maintaining proper osmoregulation (Shinji et al., 2012; Giarrataro et al., 2007) was observed.

Phenylalanine is the precursor to tyrosine, which in turn is used in the production of neurotransmitters such as octopamine and dopamine (McCoole et al., 2012) and pigment compounds such as melanin (Scoville and Pfrender, 2010). Phenylalanine decreased in response to copper and lithium exposure which potentially suggests that such derivatives were being produced in greater quantities, and/or that a decreased rate of phenylalanine production had occurred. Poynton et al. (2011) found that *D. magna* exposed to cadmium responded by producing more of these products such as L-Dopa and dopamine. As such, this observation may be a general response to metal stress. In crustaceans, the amines octopamine, dopamine, histamine and serotonin likely serve as neuromodulators (Christie, 2011), are possibly important in osmoregulation (Morris and Ahern, 2003) and control aggression (Christie, 2011). Melanin is also a byproduct of L-Dopa (another tyrosine derivative), and there are ecological consequences for daphnids as they maintain a balance between being protected from UV radiation and being visible to predators (Scoville and Pfrender, 2010). Because of the importance of the phenylalanine derivatives this warrants further investigation.
2.5 Conclusions

The exposure to sub-lethal concentrations of metals, even for a short duration, was observed to manifest significant changes at the metabolic level. In addition, \(^1\)H NMR-based metabolomics provided insights into potential modes of toxicity that cannot be gleaned from mortality or fecundity tests alone. For example, Bossuyt and Janssen (2004) have found for *D. magna* an ideal copper range of 1 - 35 µg/L; a range in which reproduction rates appear to be stable. The concentration used in this experiment (12.4 µg/L) was within this range, showing that while reproduction may not be altered in *D. magna*, there are changes to the metabolome that are not detectable through reproduction tests, further bolstering the imperative to conduct experiments at sub-lethal concentrations. Our study demonstrates that exposure to arsenic, copper or lithium at sub-lethal concentrations is able to alter the metabolic composition of *D. magna* after 48 hours of exposure. Interestingly, lithium shows changes similar to copper and implies a similar mode of action for the two metals. Large decreases in metabolites such as alanine, glutamate, methionine and uracil may indicate that there are disruptions in energy production. The decreased phenylalanine concentration also suggests potential alterations in products such as dopamine and octopamine. Though arsenic exposure does not elicit a significant change in metabolome (possibly due to the low concentrations (relative to the LC\(_{50}\)) used in this experiment) we are still able to discern changes in important amino acids such as alanine. We show that \(^1\)H NMR-based metabolomics is able to detect metabolic stress after 48 hours of exposure to sub-lethal concentrations of metals. There are a number of avenues that subsequent research can explore. Reproduction tests, as well as molting time and frequency will need to be considered, particularly where chronic exposure tests are concerned. Future studies should
consider variations in exposure concentration and time, as well as study additional environmental contaminants that impair aquatic ecosystem health.

2.6 Acknowledgements

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Chapter 3

Development of an NMR microprobe procedure for high-throughput environmental metabolomics of *Daphnia magna*


3.1 Abstract

Nuclear magnetic resonance (NMR) is the primary platform used in high-throughput environmental metabolomics studies because its non-selectivity is well suited for non-targeted approaches. However, standard NMR probes may limit the use of NMR-based metabolomics for tiny organisms because of the sample volumes required for routine metabolic profiling. Because of this, keystone ecological species, such as the water flea *Daphnia magna*, are not commonly studied due to the analytical challenges associated with NMR-based approaches. Here the use of a 1.7 mm NMR microprobe in analyzing tissue extracts from *D. magna* is tested. Three different extraction procedures (D$_2$O-based buffer; Bligh and Dyer; and acetonitrile: methanol: water) were compared in terms of the yields and breadth of polar metabolites. The D$_2$O-buffer extraction yielded the most metabolites and resulted in the best reproducibility. Varying amounts of *D. magna* dry mass were extracted to optimize metabolite isolation from *D. magna* tissues. A ratio of 1-1.5 mg dry mass to 40 μL of extraction solvent provided excellent signal-to-noise and spectral resolution using $^1$H NMR. The metabolite profile of a single daphnid was also investigated (approximately 0.2 mg). However the signal-to-noise of the $^1$H NMR was considerably lower, and while feasible for select applications, would likely not be appropriate for high-throughput NMR-based metabolomics. Two-dimensional NMR experiments on *D. magna* extracts were also performed on the 1.7 mm NMR probe to confirm $^1$H NMR metabolite assignments. This study provides a NMR-based analytical framework for future metabolomics studies that use *D. magna* in ecological and ecotoxicity studies.
3.2 Introduction

Metabolomics is the analysis of metabolites in a biological sample (Aliferis and Chrysayi-Tokousbalides, 2011; Lin et al., 2007) and has seen increased development and use over the past decade in toxicological studies, particularly in the medical sciences (Lankadurai et al., 2013a). In spite of this advancement, the use of metabolomics in the environmental sciences is relatively new and the baseline metabolome (the composite overview of all small endogenous metabolites in a sample) for many keystone ecological organisms remains undefined (Simpson and Bearden, 2013). Given its potential use in aquatic toxicology and environmental monitoring regimes, the further development and improvement of metabolomics in environmental studies is necessary. In particular, species-specific methodologies need to be developed and standardized so that experiments can be replicated across laboratories (Brown et al., 2008; Liebeke and Bundy, 2012).

Nuclear magnetic resonance (NMR) is the most commonly used analytical platform in routine, high-throughput environmental metabolomics because of its reproducibility, non-selectively, and ability to identify metabolites (Dunn and Ellis, 2005; Jordan et al., 2012; Viant, 2008). However, NMR is often considered to be less sensitive relative to mass spectrometry (MS)-based methods (Grimes and O'Connell, 2011; Jones and Larive, 2012; Pan and Raftery, 2007; Schroeder and Gronquist, 2006). As such, it is difficult to perform a metabolomics experiment when there are sample size limitations, such as with small organisms, or when samples are difficult to obtain (Dossey et al., 2006). For example, the tiny water flea *Daphnia magna*, which is a keystone species commonly used to study both food web ecology and ecotoxicity in freshwater ecosystems (Altshuler et al., 2011; Olmstead and Leblanc, 2000), has been comparatively understudied using NMR-based environmental metabolomics methods. To
date, only 5 published studies report on the *D. magna* metabolome (Chapter 2; Poynton et al., 2011; Taylor et al., 2009; Taylor et al., 2010; Vandenbrouck et al., 2010). In two of these studies, hundreds of organisms needed to be pooled to produce adequate signal to noise (S/N) using a conventional 5 mm NMR probe (Chapter 2; Vandenbrouck et al., 2010). Even though it is the largest in the daphnia genus, *D. magna* only grow to 2.3-6.0 mm in length, with a mass between 0.25-0.4 mg (Gulati, 1978). With such a mass limited organism, individuals cannot be analyzed and in some cases this prohibits an understanding of the natural variation in the metabolome in a population, as has been done with earthworms (Aslund et al., 2011) and nematodes (Szeto et al., 2011).

To address this challenge, there are a number of technologies that enable the examination of small, mass-limited samples. For example, capillary NMR, often hyphenated with other analytical instrumentation such as liquid chromatography and capillary electrophoresis (Schroeder and Gronquist, 2006), has been used successfully in studies identifying novel steroids in fireflies (Gronquist et al., 2005) and metabolites in the scarlet bugler plant (Hu et al., 2005). The use of microprobe technology in tandem with high-resolution magic-angle spinning has been used in examining the nematode *Caenorhabditis elegans* (Wong et al., 2014) with even an analysis of a single *C. elegans* individual. In addition, cryoprobes can help mitigate thermal noise (Schroeder and Gronquist, 2006) and have been used in examining stick insect venom (Zhang et al., 2007). As these technologies were not available to us at the time, the focus of this study is with a commercially available room temperature 1.7 mm microprobe, which is a viable alternative to the more cost-prohibitive cryoprobes (Schroeder and Gronquist, 2006).

With a microprobe, the smaller coil and the reduced sample volume requirements (typically down to 30 μL (Martin, 2005)), allow for greater salt tolerance (Aramini et al., 2007)
which in turn results in better line shape and increased S/N (Grimes and O'Connell, 2011; Kim et al., 2010; Martin, 2005). This is aided by the closer proximity of the sample to the detector coil region which results in improvements in magnetic field homogeneity (Aramini et al., 2007; Grimes and O'Connell, 2011). The microprobe is especially useful when samples are limited or difficult to acquire. The small volume requirement is in contrast to the use of the more common 5 mm NMR probes which typically require 600 µL (Fratila and Velders, 2011; Grimes and O'Connell, 2011) of sample, potentially requiring sample dilution, which results in decreased sensitivity. As a result, there are a number of advantages that are possible with the use of microprobe, but there are much fewer instances of its use in environmental metabolomics studies compared to the more conventional 5 mm probe (Asakura et al., 2013; Dossey et al., 2006; Gronquist et al., 2005; Poynton et al., 2011; Zhang et al., 2007).

In addition to NMR hardware, the optimization of a metabolomics experiment also requires comparative testing of the metabolite extraction procedure (Brown et al., 2008; Lankadurai et al., 2013a). Each extraction procedure has its own biases and no single method can capture all the metabolites present (Lin et al., 2007; Martineau et al., 2011; Sardans et al., 2011). Ultimately, an extraction procedure will need to be investigated for each particular species while maximizing efficiency, metabolite content and minimizing variability (Kaiser et al., 2009; Le Belle et al., 2002; Martineau et al., 2011). There are several studies that have examined species-specific extraction procedures; species which include the Chinook salmon (Lin et al., 2007), marine ragworms (Alvarez et al., 2010), the plant *Arabidopsis thaliana* (Kaiser et al., 2009), the bacterium *Escherichia coli* (Maharjan and Ferenci, 2003), and earthworms (Liebeke and Bundy, 2012). Thus, the extraction protocol that is most suited for particular use in studies using *D. magna* should also be tested. As such, this study examines the efficacy of three extraction
procedures (Brown et al., 2008; Roemisch-Margl et al., 2012), the Bligh and Dyer (BD; Bligh and Dyer, 1959) extraction, the aqueous buffer (D$_2$O) extraction, as well as an acetonitrile: methanol: water (AMW) extraction method developed by Liebeke et al. (2012) which has been optimized for use with the annelid *Lumbricus rubellus*. The first objective of this study is to examine various extraction procedures to determine which consistently yields the best S/N with *D. magna*. The second objective is to optimize the amount of daphnia dry mass needed for a metabolomics experiment using *D. magna*.

3.3 Experimental

3.3.1 *Daphnia magna* culturing and extraction preparation

*D. magna* were purchased from Ward Science Canada (St. Catherines, ON, Canada) and have been maintained in a laboratory for 6 months in de-chlorinated municipal tap water (hardness approximately 120 mg/L). Stock cultures were raised on a diet of *chlorella* spp. algae and were fed three times per week. Water was also changed three times per week prior to feeding. Stock cultures were maintained at 20°C and kept under a 16:8 light/dark photoperiod. To avoid natural variation in the *D. magna* metabolite profile due to differences in age, only fully grown adults (>2 weeks old) were used in this study. Daphnids were flash frozen in liquid nitrogen to cease enzymatic activity (Lin et al., 2007), subsequently lyophilized for two days and weighed using a microbalance (Sartorius ME36S, Sartorius AG, Goettingen, Germany). They were stored in a freezer at -25 °C until extraction.
3.3.2 Extraction methods

For the testing of the extraction procedures, 3 mg of dry mass was used as preliminary experiments showed that yields varied with the three different extraction methods. 3 mg of lyophilized *D. magna* was weighed into a 2 mL centrifuge tube and homogenized into a powder with a metal spatula. Metabolites were extracted using three different methods: D$_2$O-buffer, BD, and AMW. Each extraction was repeated 8 times.

The D$_2$O-buffer comprised of 0.2 M sodium phosphate dihydrate (NaH$_2$PO$_4$ • 2H$_2$O, 99.3%, Fisher Scientific Company, Toronto, ON, Canada), 10 mg L$^{-1}$ of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS; 97% purity, Sigma Aldrich, St. Louis, MO, USA) to serve as an internal reference standard, and 0.1% w/v sodium azide (99.5% purity, Sigma Aldrich) added as a preservative (Lankadurai et al., 2011; Chapter 2) dissolved in D$_2$O (99.9% purity, Cambridge Isotope Laboratories, Andover, MA, USA). The buffer solution was adjusted to a pD = 7.4 by adding NaOD (30% w/w in 99.5% D$_2$O, Cambridge Isotope Laboratories Inc.). To extract metabolites with the D$_2$O-buffer, 40 μL of the buffer solution was added to the homogenized daphnids and subsequently vortexed for 30 seconds and sonicated for 15 minutes. The mixture was then centrifuged (Eppendorf 5804R, at 14,000 rpm for 20 minutes). The resulting supernatant was transferred into a new 2 mL centrifuge tube and centrifuged again (14,000 rpm for 20 minutes). The supernatant was then transferred with a pipette into a 1.7 mm NMR tube (Bruker BioSpin) for NMR analysis.

The BD extraction (Bligh and Dyer, 1959) was used based on Wu et al. (2008). Briefly, 320 μL of MeOH and 128 μL of H$_2$O (HPLC grade) were added to the daphnia homogenate. The mixture was vortexed for 30 seconds. 320 μL of chloroform and 160 μL of H$_2$O were subsequently added and the mixture was vortexed for 30 seconds and put on ice for 10 minutes.
The samples were then centrifuged (12,000 rpm for 10 minutes) and the polar fraction (the upper layer) was removed and inserted into a 2 mL centrifuge tube. This supernatant was evaporated to dryness under a stream of nitrogen gas. The resulting residue was stored in a freezer and then reconstituted in 50 μL of the D$_2$O-buffer solution (as previously described) and transferred into a 1.7 mm NMR tube prior to analysis.

The AMW extraction entailed (Liebeke and Bundy, 2012) using a 1:1 ratio of cold acetonitrile/methanol (200 μL of each) which was added to 3 mg of each daphnid homogenate and the solution was centrifuged for 10 minutes at 14,000 rpm. The supernatant was removed and transferred into a new 2 mL centrifuge tube. The remaining pellet was re-extracted by adding a 2:2:1 ratio of acetonitrile: methanol: water (200 μL: 200 μL: 100 μL) and the mixture was centrifuged again. The two supernatants were combined and evaporated to dryness under a stream of nitrogen gas and the residue was stored frozen and reconstituted in 50 μL of a D$_2$O-buffer and transferred into a 1.7mm NMR tube prior to analysis.

### 3.3.3 Nuclear magnetic resonance spectroscopy and data analysis

All *D. magna* extracts were analyzed using a Bruker BioSpin Avance III 500 MHz NMR equipped with a $^1$H- $^{13}$C-$^{15}$N TXI 1.7 mm microprobe fitted with an actively shielded gradient. $^1$H NMR analysis was performed with 256 scans, a relaxation delay of 3 s, 32 k time domain points, a 90° pulse calibrated on a per sample basis. Spectra were apodized through multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectra, with a zero filling factor of 2 (Chapter 2). Water suppression was accomplished using the Presaturation Using Relaxation Gradients and Echoes (Brown et al., 2008). Spectra were manually phased and calibrated to align with the trimethylsilyl group of the DSS internal standard and set to δ = 0.00 ppm. In addition to acquiring one-dimensional (1D)
spectra, 3 mg of \textit{D. magna} dry mass was extracted with the D$_2$O-buffer (as described previously) for a two-dimensional (2D) $^1$H-$^{13}$C heteronuclear single quantum coherence (HSQC) analysis, which allowed for metabolite identification and confirmation of assignments made in the 1D $^1$H NMR spectra. HSQC acquisition was performed with 256 scans, with parameters previously optimized for 2D metabolomics studies (Yuk et al., 2010). For each of the three extraction procedures, the total spectral S/N was calculated using the TopSpin program (version 3.0, Bruker BioSpin, Rheinstetten, Germany).

3.3.4 Statistical analyses and metabolite quantification

The 1D NMR spectra from the different extraction methods were further analyzed using principal component analysis (PCA) with the Analysis of MIXtures program (AMIX, version 3.9.3, Bruker BioSpin, Rheinstetten, Germany). Spectra were divided into bucket regions of 0.02 ppm, between the chemical shift region of 0.5 and 10 ppm. The region between 4.7 and 4.9 ppm (corresponding to the remaining water signal) was removed. Buckets were normalized to the total sum of intensities and were used in the PCA. PCA scores plots were used to visualize differences between spectra and identify whether there were discrete clustering patterns with the three extraction procedures. A loadings plot was also prepared using AMIX, based on the results of the PCA.

In addition to the PCA, metabolite quantification was also performed for each extraction procedure to test for reproducibility in metabolite quantification. Alanine, glycine, phenylalanine and threonine were selected, based on their varying degrees of protonation and because at least one proton resonance could be clearly distinguished within the $^1$H NMR spectra. The metabolites were quantified with AMIX, using the DSS peak ($\delta = 0.00$) as an internal standard. Integrated values were normalized to mass quantities using the following formula: $\gamma _{mip}/m _{pm}$ (Caligiani et
where $\gamma$ is the integrated value from $^1$H NMR, $m_1$ is the molecular mass of the metabolite, $p_i$ is the number of protons on the internal standard, $m_s$ is the molecular mass of the internal standard and $p_m$ is the number of protons on the metabolite. Average quantities and relative standard errors (RSE) were calculated to gauge the variability of metabolite quantities between samples.

### 3.3.5 Optimizing extraction mass

After determining the optimal extraction procedure for metabolomics, the optimal dry mass for use with the 1.7 mm microprobe was examined. A range of masses was tested including: 0.5 mg, 1 mg, 1.5 mg and 2 mg of daphnia dry mass; all extracted 8 times to also test reproducibility. A single daphnid (approximately 0.2 mg) and 60 neonates (approximating 1 mg in mass) were also examined as well. S/N values for the methyl resonance of alanine (between 1.49 and 1.45 ppm) were calculated using TopSpin, and statistical significance between groups was determined for the four mass ranges with a $t$-test (two tailed, equal variance, 95% confidence interval) using Microsoft Excel (version 12, Microsoft Corporation, Redmond, WA, USA). Because this was an initial range finding component to the study, only a D$_2$O-buffer (preparation described in the next section) was used to extract metabolites. Spectra were examined to determine which mass to solution ratio provided optimal signal intensity, baseline quality for quantification and bucketing purposes, while minimizing the number of daphnids needed.
3.3.6 Metabolite identification

Spectra were compared to an array of compounds found in the Bruker Biofluid Reference Compound Database (v. 2.0.3). Pattern matching of both 1D and 2D spectra was performed using AMIX (Woods et al., 2011). Compounds with a greater than 80% confidence match, based on automated searches, were further selected for detailed manual inspection. Only compounds that showed near perfect matches in all spectral regions were retained and considered as accurate assignments. The chemical shifts of the identified compounds were compared with the database values and were plotted against each other to provide confirmation of the quality of the assignments.

3.4 Results and discussion

3.4.1 Extraction procedures

Three extraction procedures were evaluated for this experiment. Figure 3-1 shows example ¹H NMR spectra from the three extraction procedures tested. The DSS internal standard peak was visible in all three and the relatively low intensity of the peak (at δ = 0.00) in relation to the others revealed that the D₂O-buffer extract yielded a larger spectral intensity for the metabolites present, relative to the BD polar extract and the AMW extract. This disparity is highlighted by the S/N calculations for the entire spectra which show that there is an order of magnitude difference in S/N between the D₂O-buffer and the BD and AMW extractions, with respective overall spectra S/N values of 2159.0, 287.3 and 215.4.

There was also a difference seen in the composition of the metabolites represented in the three extracts which is apparent in ¹H NMR profiles (Figure 3-1).
Figure 3-1: $^1$H NMR spectra for three extraction procedures: A) The Bligh and Dyer (BD) extraction (polar extract is shown) B) Acetonitrile: Methanol: Water (AMW) extraction and C) The D$_2$O-buffer extraction. The 1.2-1.5 ppm region is expanded for each spectrum. Signal to noise (S/N) calculations are also listed.
In the AMW extract, there were broad resonances overlapping in the region between 1.27-1.29 (Figure 3-1 inset) from main chain methylenes \((\text{CH}_2(n))\) and in the 1.5-1.6 ppm region, which was likely from methylene \(\beta\) to double bonds and carboxylic groups in lipids (Ekman et al., 2009; Wu et al., 2008). The presence of lipids makes metabolite identification more challenging as the broad resonances obscure key peaks from amino acids and other metabolites that resonate in this region. The similar is true for additional lipid signals at around 2.23, 2.78 and 5.3 ppm (Knothe and Kenar, 2004). Their presence is likely because the AMW accounts for both polar and nonpolar phases. With the BD, the small presence of fatty acids may be the result of the added separation that occurs with biphasic mixtures, where there is difficulty separating the two phases (polar and nonpolar), especially given the small volumes that are used. This is in contrast with the D$_2$O-buffer extract, which does not require this extra step in sample preparation. Metabolite signals in the D$_2$O-buffer extract are not obscured by fatty acids, and this reduced overlap permits a wider range of metabolites to be identified.

3.4.2 Reproducibility of extraction procedures

A PCA scores plot (Figure 3-2) indicates a clear separation between the three extraction procedures with all three showing general clustering patterns. PCA is an unsupervised statistical method that reduces multivariate data into fewer dimensions (Giansante et al., 2003; Reo, 2002; Weckwerth and Morgenthal, 2005). Each data point represents an individual $^1$H NMR spectrum and the closer proximity spectra have with each other is an indication of the greater similarity between the metabolite profiles.
Figure 3-2: A principal component analysis (PCA) scores plot illustrating the variation between the Bligh and Dyer (BD), Acetonitrile: Methanol: Water (AMW) and D₂O-buffer extracts (n=8). The clustering of the BD and D₂O-buffer spectra show excellent repeatability of these extraction methods, while there is greater variation in the AMW method, particularly along PC1.

The PCA shows that the spectra for the D₂O-buffer were very similar to each other, as evinced by the close clustering in the PCA, in particular along the first principal component (PC1), which explains 83.4% of the variation. This indicates that there is minimal variation between replicates. This was also seen to a lesser extent with the BD extracts, which show similar variance in PC1 to the D₂O-buffer extract, albeit with much larger variance with respect to PC2. In contrast the AMW extract showed a great deal of variation, in particular along PC1. This suggests that there
is considerably more variability with this method. While the AMW extraction was seen to be effective in the analysis of earthworm metabolites (Liebeke and Bundy, 2012), it did not prove to be as effective in analyzing the *D. magna* metabolome, possibly owing to the differing metabolite profiles that exist between annelids and crustaceans. These results highlight the important need to develop species-specific extraction protocols for model organisms (Le Belle et al., 2002). The loadings plot (Appendix B, Figure A3-1) accounts for the variables which contribute to the variation seen in the PCA. Spectral buckets corresponding to regions where broad fatty acid resonances are present (0.85-0.95, 1.27-1.29) are contributing to the variation, and based on the statistics of each of these bucket regions, this is the result of the large variation in the AMW method (individual statistics not shown). The presence of these fatty acids is likely the result of the inherent nature of an extraction procedure that extracts both polar and nonpolar compounds.

The distribution of variance observed in the PCA for the extraction procedures is also reflected in the quantification of select metabolites. Table 3-1 lists the values of the quantified metabolites and their corresponding RSE values. The D$_2$O-buffer extraction yielded the largest quantities of the individual metabolites, compared to the BD and AMW. It also had the least variation, as seen by the low RSE values associated with each metabolite. There is slightly more variation with the BD extraction and much higher variation with the AMW extraction. The threonine spectral region ($\delta = 4.20$-4.28 ppm) could not be discerned for the AMW and the BD extraction methods, since there was too much interference from background noise. The low variation found in the quantification process corroborates the PCA results; that the D$_2$O-buffer extraction procedure yields more reproducible results.
Table 3-1. Quantification of metabolites from three different extraction procedures with associated relative standard error.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Bligh and Dyer (BD)</th>
<th>Acetonitrile: Methanol: Water (AMW)</th>
<th>D$_2$O-buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Metabolite concentration (µg/g dry mass)</td>
<td>Relative Standard Error (%)</td>
<td>Metabolite concentration (µg/g dry mass)</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.16</td>
<td>3.46</td>
<td>1.06</td>
</tr>
<tr>
<td>Threonine</td>
<td>Not discernible$^1$</td>
<td>Not discernible$^1$</td>
<td>17.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.48</td>
<td>3.58</td>
<td>0.35</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.51</td>
<td>6.31</td>
<td>0.31</td>
</tr>
</tbody>
</table>

$^1$Threonine could not be discerned for the AMW and BD extractions because of interference from background noise.

Combined with the high quantities of metabolites found and the ease of using this procedure, it is concluded that the D$_2$O-buffer extraction is the optimal extraction procedure to use in high-throughput metabolomics experiments for $D$. magna when examining polar metabolites. However, it should be noted that the AMW extract provides insight into the fatty acid profiles that occur in $D$. magna. Because the AMW extraction procedure allows both nonpolar and polar compounds to be extracted, there is valuable information that would be missed in a solely polar extraction. While it does not constitute a large portion of metabolomics studies, lipid metabolomics has provided valuable information in studies using earthworms (Brown et al., 2014; Lankadurai et al., 2013b) and fathead minnows (Ekman et al., 2009) and should be considered in future studies.
3.4.3 Dry mass optimization

Figure 3-3 shows the NMR spectra obtained for a range of dry masses, as well as for 1 mg of neonates (approximately 60 individuals) and a single adult daphnid.

**Figure 3-3**: $^1$H NMR spectra for dry mass ranges are shown for 0.5 mg, 1 mg, 1.5 mg, 2.0 mg, for a single daphnid (approximately 0.2 mg) and for daphnia neonates (1 mg). The spectral region corresponding to the methyl resonance of alanine (1.45-1.49 ppm) is highlighted and S/N calculations are shown.
Between 1 and 2 mg of dry mass, high quality spectra were obtained, with clear metabolite signals and a flat baseline (Figure 3-3). However, 0.5 mg of dry mass exhibited lower spectral quality, where the overall signal intensity was lower and baseline noise greater. This difference is reflected in the S/N calculations for the spectral regions corresponding to the methyl resonance of alanine, where there is a statistically significant increase from 0.5 mg (248) to 1 mg (702) and even higher at 1.5 mg (1043). These differences are important when considering metabolite quantification, where an S/N greater than 100 is recommended (Barding et al., 2012). Interestingly, at 2 mg of dry mass, there is a drop in S/N, possibly suggesting a saturation of metabolites that occurs in 40 μL of buffer solution. It is possible then that extraction with samples with dry masses between 1 and 1.5 mg are before a point where metabolite saturation occurs, making the extraction of higher dry masses unnecessary. For masses greater than 2 mg, extraction with 40 μL of D$_2$O-buffer resulted in increased viscosity that reduced spectral resolution (data not shown). Though some metabolites were detected, the higher viscosity and concentration led to peak broadening that in turn affected S/N and metabolite discrimination which is problematic for NMR-based metabolomics experiments. In addition, as more dry tissue is used, the presence of a macromolecular background may also increase, resulting in interference with small molecule identification and quantification. Increases in protein concentrations may also lead to further reductions in spectral line shape through binding or non-specific interactions (Daykin et al., 2002; Nowick et al., 2003). Consequently, it was difficult to discern the DSS internal calibrant from baseline noise, which is problematic because the trimethylsilane (TMS) peak of DSS (δ=0.00 ppm) is routinely used as an internal standard for quantification and referencing in metabolomics (Nowick et al., 2003). There is less macromolecular background and a more defined baseline when 1-2 mg of dry mass is used and
the TMS peak is clearly resolved (Figure 3-3). However, given the potential saturation of metabolites at 2.0 mg, it was concluded that a range between 1-1.5 mg of dry mass to 40 μL of extraction solvent provided an optimal metabolite yield while minimizing the number of organisms used, and was therefore used for our subsequent experiments.

A spectrum for a single daphnid was also acquired (Figure 3-3). While metabolites can be discerned, both the number of individual metabolites found and the spectral intensity were low, as seen by the large intensity of the TMS peak in DSS relative to the metabolites. This is also apparent in the low S/N of 76 for alanine, which does not meet the minimum requirements for quantification (Barding et al., 2012). Therefore, with the current room temperature NMR technology, routine metabolic analysis using a single daphnid would be challenging. However, it is important to note that the 1.7 mm NMR probe was sensitive enough to detect a number of metabolites. Further honing of technologies, for example microcryoprobes, may allow future metabolomics experiments to be conducted on single daphnids. Single daphnid metabolomics experiments would allow for further examination of the natural variation that exists within conspecifics and would encourage the use of a greater number of experimental replicates, since so few would be required to acquire a large number of spectra.

An extraction of 1 mg of daphnia neonates produced a spectrum with an S/N of 495 for alanine (Figure 3-3), though interestingly not as high as it is with adults even with a similar dry mass. This is attributed to the different physiological differences between neonates and adult daphnids. 1 mg of neonates was a sufficient dry mass to produce a quality spectrum that could be used for a metabolomics experiment. It is seen that neonate metabolites are largely consistent with what is found in adults. The use of the microprobe is in contrast with our previous study which required 250 neonatal daphnids (between 5-10 mg dry mass) to acquire a spectrum.
(Chapter 2) and a sample spectrum from this study indicates a relatively low S/N of 282, even with the increase in dry mass used for extraction (Appendix B, Figure A3-2). While the 5 mm QXI probe is able to provide spectra adequate for quantification in metabolomics studies, there are greater efficiencies with regards to sample requirements which makes the microprobe more amenable for high-throughput metabolomics studies.

3.4.4 Identification of metabolites

A sample 1D spectrum for the D$_2$O-buffer extract is shown in Figure 3-4 and identified metabolites are labelled.

![A 1H NMR spectrum from the D$_2$O-buffer extract with identified metabolites labeled.](image)

**Figure 3-4:** A 1H NMR spectrum from the D$_2$O-buffer extract with identified metabolites labeled.

A number of amino acids and sugars were identified: phenylalanine, tyrosine, glucose, diphosphate sugars, glycine, lysine, glutamine, glutamate, methionine, arginine, alanine,
threonine, isoleucine, valine and leucine. To confirm these assignments, a 2D $^1$H-$^{13}$C HSQC spectrum was also obtained (Figure 3-5).

**Figure 3-5:** 2D $^1$H-$^{13}$C Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum with metabolites labeled.

With HSQC NMR, the greater dispersion into the $^{13}$C chemical shift region created less overlap in signals and cross peaks between the $^1$H and $^{13}$C allowed for better identification of compounds (Kim et al., 2010; Xi et al., 2008). Here, the metabolites asparagine, aspartic acid, betaine, proline, serine and tryptophan were further identified, and added to those identified in the 1D spectrum. Identified metabolites and their associated $^1$H and $^{13}$C peaks are listed in Table 3-2.
Table 3-2. Metabolites identified with both 1D and 2D (\(^{1}H\text{-}^{13}C\) HSQC) in *D. magna* extracts and the associated chemical shifts. Peak types are indicated in parentheses: s = singlet, m = multiplet, d = doublet, dd = doublet of doublets, t = triplet, and q = quartet.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>1H NMR Chemical shift (ppm)</th>
<th>13C NMR Chemical Shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>1.48 (d), 3.78 (q)</td>
<td>18.8, 53.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.71 (m), 1.92 (m), 3.24 (t), 3.76 (t)</td>
<td>26.2, 30.4, 43.1, 57</td>
</tr>
<tr>
<td>Asparagine</td>
<td>2.84 (dd), 2.93 (dd), 4.01 (q)</td>
<td>37.4, 53.9</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>2.82 (dd), 2.7 (dd), 3.88 (q)</td>
<td>39.3, 54.8</td>
</tr>
<tr>
<td>Betaine</td>
<td>3.26 (s), 3.89 (s)</td>
<td>56.2, 69.2</td>
</tr>
<tr>
<td>Glutamate</td>
<td>2.05 (m), 2.34 (t), 3.75 (t)</td>
<td>29.5, 36, 57.2</td>
</tr>
<tr>
<td>Glutamine</td>
<td>2.13 (m), 2.45 (m), 3.77 (t)</td>
<td>29.1, 33.7, 57</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.55 (s)</td>
<td>43.9</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.25 (t), 3.40 (m), 3.48 (m), 3.54 (d), 3.72 (m), 3.80 (m), 3.89 (dd), 4.64 (d), 5.24 (d)</td>
<td>63.3, 72.3, 74.0, 75.6, 77, 78.5, 94.6, 98.6</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.95 (t), 1.02 (d), 1.26 (m), 1.48 (m), 1.98 (m), 3.68 (d)</td>
<td>13.4, 17.4, 27.3, 38.5, 62.3</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.95 (t), 1.70 (m), 3.75 (m)</td>
<td>24.1, 24.2, 26.4, 42.4, 56.9</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.48 (m), 1.74 (m), 1.91 (m), 3.03 (t), 3.75 (t)</td>
<td>24.1, 29, 32.4, 41.6, 56.7</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.13 (s), 2.63 (t), 3.84 (t)</td>
<td>16.6, 31.6, 32.5, 56.6</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.13 (dd), 3.26 (dd), 3.9 (q), 7.39 (m)</td>
<td>39.0, 57.7, 132</td>
</tr>
<tr>
<td>Proline</td>
<td>2.02 (m), 2.28 (m), 3.35 (m), 3.41 (m), 4.12 (q)</td>
<td>26.5, 31.8, 48.9, 63.9</td>
</tr>
<tr>
<td>Serine</td>
<td>3.83 (m), 3.97 (m)</td>
<td>59.1, 63.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.33 (d), 3.58 (d), 4.24 (m)</td>
<td>22.3, 63.1, 68.8</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>3.33 (q), 3.4 (dd), 4.03 (q), 7.19 (t), 7.28 (m), 7.53 (d), 7.72 (d)</td>
<td>28.9, 57.5, 115, 121.9, 124.9, 127.8</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.05 (dd), 3.2 (dd), 3.94 (q), 6.9 (d), 7.19 (d)</td>
<td>38.2, 58.5, 118.5, 133.4</td>
</tr>
<tr>
<td>Valine</td>
<td>0.98 (d), 1.03 (d), 2.25 (m), 3.60 (d)</td>
<td>19.3, 20.7, 31.9, 63.1</td>
</tr>
</tbody>
</table>

Confirmation of the HSQC assignments by plotting actual spectral values against Bruker Biofluid Reference Compound Database derived values (Appendix B, Figure A3-3) shows a high degree of consistency between obtained spectra and database values (with \(r^2 = 0.999\)).
metabolites were further cross referenced and checked against identifications from HSQC from another species (Yuk et al., 2012).

3.5 Conclusion

The 1.7 mm NMR microprobe reduced the 10 mg sample size required by 5 mm probes in our prior study (Chapter 2) to only 1-1.5 mg of dry mass. While spectra with 5 mm probes (Chapter 2) are of high quality and suitable for metabolomics studies, the microprobe allows a degree of sample reduction that makes metabolomics with this keystone species more logistically feasible and efficient, given that culturing of organisms can be both time consuming and costly. This would allow for its greater use in situations where metabolomics holds promise, such as in environmental monitoring of freshwater aquatic ecosystems. The D$_2$O-buffer extraction was the most efficient and consistent extraction procedure for polar metabolites, with metabolite yields about an order of magnitude higher than the other extraction methods tested. The development of these protocols is important for the further implementation of environmental metabolomics studies using *D. magna*, but also highlights the need for species-specific protocols for metabolomics studies. Through this study a reliable protocol for future metabolomics studies using keystone species such as *D. magna* was developed. This will directly support future studies to focus on integral species rather than species that meet NMR hardware requirements. As such, future environmental monitoring programs, based on high-throughput NMR analysis can be developed and expanded to include freshwater ecosystems.

3.6 Acknowledgements

The authors thank the Krembil Foundation for generous support of this research.
3.7 References


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Chapter 4

Metabolomics reveals energetic impairments in *Daphnia magna* exposed to diazinon, malathion and bisphenol-A

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(Modified to include an ANOVA with a Bonferroni posthoc test, to replace the multiple t-tests and false discovery rate that were used in calculating statistical significance in the PCA and metabolite percent changes. Text in the methods and results sections has been modified to reflect these changes. Figures 4-1, 4-2, 4-3, 4-4 have also been modified to reflect the changes in statistical significance after the ANOVA and Bonferroni posthoc test.)

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4.1 Abstract

$^1$H nuclear magnetic resonance (NMR)-based metabolomics was used to study the response of *Daphnia magna* to increasing sub-lethal concentrations of either an organophosphate (diazinon or malathion) or bisphenol-A (BPA). Principal component analysis (PCA) of $^1$H NMR spectra were used to screen metabolome changes after 48 hours of contaminant exposure. The PCA scores plots showed that diazinon exposures resulted in aberrant metabolomic profiles (0.0225 μg/L, 0.045 μg/L and 0.135 μg/L), while for malathion the second lowest (0.08 μg/L) exposure caused significant shifts from the control. Individual metabolite changes for both organophosphates indicated that the response to increasing exposure was non-linear and described perturbations in the metabolome that were characteristic of the severity of exposure. For example, intermediate concentrations of diazinon (0.045 μg/L and 0.09 μg/L) and malathion (0.08 μg/L) elicited a decrease in amino acids such as leucine, valine, arginine, glycine, lysine, glutamate, glutamine, phenylalanine and tyrosine, with concurrent increases in glucose and lactate, suggesting a mobilization of energy resources to combat stress. At the highest exposure concentrations for both organophosphates there was evidence of a cessation in metabolic activity, where the same amino acids increased and glucose and lactate decreased, suggesting a slowdown in protein synthesis and depletion of energy stocks. This demonstrated a similar response in the metabolome between two organophosphates but also that intermediate and severe stress levels could be differentiated by changes in the metabolome. For BPA exposures, the PCA scores plot showed a significant change in metabolome at 0.7 mg/L and 2.1 mg/L of exposure. Individual metabolite changes from 0.7 to 2.1 mg/L of BPA exposure showed increases in amino acids such as alanine, valine, isoleucine, leucine, arginine, phenylalanine and tyrosine. These metabolite changes were correlated with decreases in glucose and lactose. This pattern of response was also
seen in the highest organophosphate exposures and suggested a generalized stress response that
could be related to altered energy dynamics in *D. magna*. Through studying increasing exposure
responses, we have demonstrated the ability of metabolomics to identify discrete differences
between intermediate and severe stress, and also to characterize how systemic stress is
manifested in the metabolome.
4.2 Introduction

Organisms in aquatic environments are invariably exposed to an array of contaminants (Altshuler et al., 2011), many of which have toxicities that are poorly understood. Risk assessors are under constant duress to monitor the potential toxicities of these contaminants (Khangarot and Rathore, 2003) and there is need for a rapid procedure for this task. Currently, routine tests using the microcrustacean *Daphnia magna* in acute and chronic toxicity tests constitute a large portion of aquatic invertebrate toxicology studies (Baird et al., 1989; Martins et al., 2007). While they are simple and robust tests, the terminal endpoints of mortality and reproduction do not describe the biochemical mode of action of a toxicant (Dang et al., 2012; Lin et al., 2006) and may not accurately reflect toxicity, especially at sub-lethal concentrations. As a result, the potential toxicities may be underestimated with tests using these types of endpoints and therefore tests using sub-lethal endpoints need to be developed (Flaherty and Dodson, 2005; Lorenzon et al., 2000; Martins et al., 2007).

Metabolomics shows promise as an efficient method for assessing and understanding sub-lethal toxic stress on an organism (Aliferis and Jabaji, 2011; Viant, 2007). Metabolomics is the holistic characterization of a suite of endogenous metabolites in an organism; termed the metabolome (Keum et al., 2010). While it is useful in the characterization of an organism’s metabolome, the particular strength of metabolomics is in discerning the biochemical mode of action in response to a particular stressor (Aliferis and Jabaji, 2011; Lin et al., 2006). While predominantly used in human health studies, it has gained traction in the environmental sciences and is being developed as a useful addition to risk assessment programs (Lin et al., 2006; Yoshida et al., 2014). Since toxic stress often manifests first in the metabolome (Clarke and Haselden, 2008; Keum et al., 2010), metabolomics is a more sensitive indicator of stress
responses than traditional toxicity tests and other omic approaches. Within metabolomics studies, nuclear magnetic resonance (NMR) has emerged as a highly reproducible, high-throughput platform for analyzing the metabolome that requires minimal sample preparation (Chapter 3; Clarke and Haselden, 2008; Keum et al., 2010; Lin et al., 2006; Yoshida et al., 2014). Because of its excellent interlaboratory comparability, it is particularly appropriate for incorporation into risk assessment programs (Viant et al., 2009; Yoshida et al., 2014).

*Daphnia* spp. are small crustaceans inhabiting lentic ecosystems, where they are keystone grazers in food webs (Heckmann et al., 2008; Martin-Creuzburg et al., 2007). They are sexually parthenogenic and their clonal reproduction makes them ideal for toxicological studies since genetic variability is largely controlled for (Altshuler et al., 2011; Heckmann et al., 2008). It is a species that serves as a useful proxy for the toxicities of compounds to mammalian systems (Guilhermino et al., 2000). They are sensitive to toxic stress and are easily cultured in a laboratory setting (Soetaert et al., 2007; Von Der Ohe and Liess, 2004) and thus are ubiquitous in toxicological studies. The species *D. magna* accounts for a large number of all toxicological studies with standardized tests for toxicity (Environment Canada, 2000; Guilhermino et al., 2000; Mansilha et al., 2013; Martins et al., 2007; OECD, 2012). While common in toxicity studies, they have only recently been used in metabolomics studies with most of these focusing on acute toxic stress (Chapters 2 and 3; Li et al., 2015; Poynton et al., 2011; Taylor et al., 2009; 2010; Vandenbrouck et al., 2010).

The organophosphate (OP) insecticides are a large group of widely used chemicals that are an alternative to the more persistent organochlorine pesticides (Barata et al., 2004; Printes and Callaghan, 2004; Zeng et al., 2014). While they degrade quickly in the environment, pulse exposures are common, and as a result many non-target organisms are subjected to consistent OP
exposure (Ren et al., 2007; Zeng et al., 2014). OPs are known to inhibit the action of acetylcholinesterase (AChE; Kretschmann et al., 2011; Ren et al., 2007) and a number of studies have focused on their action on the AChE inhibition in D. magna (Barata et al., 2004; Duquesne, 2006; Li and Tan, 2011; Printes and Callaghan, 2004; Toumi et al., 2015). There is however, evidence that OPs interfere with other processes as well (Printes and Callaghan, 2004), for example being agents causing oxidative stress (Lushchak, 2011; Wu et al., 2011) and even potentially altering endocrine function in Daphnia (Barry, 2002). Therefore they require further investigation beyond examinations of AChE inhibition and a more holistic view of the toxic mode of action. Among OPs, malathion and diazinon are commonly used in agriculture as insecticides/acaricides but also have uses in human health uses in treating lice and parasites (Maroni et al., 2000; Sanchez et al., 2000).

Bisphenol-A (BPA) is used largely in the production of polycarbonate plastic and epoxy resins (Mihaich et al., 2009) and can be found in a wide array of products, from food packaging to electronics. This large breadth of uses has resulted in its discharge into aquatic systems (Chen et al., 2002; Flint et al., 2012; Mansilha et al., 2013). Studies with D. magna are primarily focused on its role as an endocrine disruptor, with endpoints typically measured in terms of mortality, molt frequency and fecundity (Brennan et al., 2006; Caspers, 1998; Klecka et al., 2001; Mansilha et al., 2013; Mu et al., 2005), with fewer studies directly examining biochemical indicators of stress (Jemec et al., 2012; Park and Choi, 2009). Though it is a known endocrine disruptor in mammalian systems, it is not entirely known if BPA acts on invertebrate endocrine systems alone or through other mechanisms (Flint et al., 2012) and therefore warrants further examination.
The current study aims to provide a biochemical examination of the metabolomic responses of *D. magna* to sub-lethal contaminant stress. In particular, polar metabolites will be analyzed as they include a large breadth of endogenous metabolites such as amino acids, sugars, nucleotides and fatty acids (Chapters 2 and 3; Fasulo et al., 2012; Wu and Wang, 2010). Polar metabolites have also been shown to be sensitive to various types of external stressors and have been linked to many biochemical pathways (Lankadurai et al., 2011; Li et al., 2014; Viant et al., 2006). While we have previously examined the efficacy of $^{1}$H NMR metabolomics with *D. magna* (Chapter 2), the purpose of this study is to examine the response of the *D. magna* metabolome, after exposure to two OPs (malathion and diazinon), as well as BPA, in order to investigate how the metabolome responds to increasing exposure concentrations. Given that both OPs function as AChE inhibitors, we hypothesize that OPs will induce similar metabolome changes and that these changes can be distinguished from BPA exposure. Ultimately, a metabolomic analysis will be able to provide a more holistic overview of the systemic toxicity of these contaminants that can serve as a complement to the 48 hour acute toxicity test. While a 48 hour test does not provide information on the larger population level changes incurred by toxic stress, it will provide greater insight into the toxicity incurred by pulse contaminant exposures.

4.3. Materials and Methods

4.3.1 *Daphnia magna* and algae culturing

*D. magna* were from a culture reared in the laboratory since 2013. *D. magna* were reared under a 16:8 light to dark ratio at an ambient room temperature of 20°C. Water used was dechlorinated municipal tap water (hardness approximately 120 mg CaCO$_3$/L) and was aged for at least a week prior to use. *D. magna* were fed a diet of 50:50 *Chlorella vulgaris* and
*Pseudokirchneriella subcapitata*, both of which were grown in a Bristol medium (Tam and Wong, 1990). Feedings occurred three times a week, at which time a 50% water change was also performed. 1 μg/L of selenium and cobalamin were added as supplements to the food twice a week (Environment Canada, 2000).

4.3.2 *Daphnia magna* acute toxicity exposures

*D. magna* were exposed over 48 hours to either BPA (Sigma-Aldrich, >99%), diazinon (Sigma-Aldrich, PESTANAL analytical standard, 98.5%) or malathion (Sigma-Aldrich, PESTANAL, analytical Standard, 97.5%). 48 hour 50% lethality concentrations (LC50) tests were conducted, using methods outlined by the OECD and Environment Canada (Environment Canada, 2000; OECD, 2012). Briefly, *D. magna* neonates (<24 hours old), taken from isolated gravid females, were placed in clear glass jars (250mL) filled with 200mL of test solution (1 daphnid/20mL). Water used for the test is dechlorinated municipal tap water that has been aged for at least 5 days and constantly aerated prior to the start of the LC50 test. Each condition consisted of an unexposed control and 5 concentrations that were based on a geometric series, and a probit analysis was used to obtain final LC50 values. Based on this, LC50 values were 14.4 mg/L, 0.9 μg/L and 3.2 μg/L for BPA, diazinon and malathion respectively and are consistent with values found in the literature (Table 4-1).
Table 4-1. Acute toxicity (LC$_{50}$) values obtained from this study and comparisons with literature values.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>LC$_{50}$ values determined in this study</th>
<th>LC$_{50}$ values from the literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazinon</td>
<td>0.9 μg/L (48 hours)</td>
<td>0.96 μg/L (48 hours)$^a$</td>
</tr>
<tr>
<td>Malathion</td>
<td>3.2 μg/L (48 hours)</td>
<td>3.8 μg/L (24 hours)$^b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.9 μg/L (48 hours)$^c$</td>
</tr>
<tr>
<td>Bisphenol-A</td>
<td>14.4 mg/L (48 hours)</td>
<td>12.8 mg/L (48 hours)$^d$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.4 mg/L (Immobilization EC$_{50}$-48 hours)$^e$</td>
</tr>
</tbody>
</table>


*D. magna* were exposed to 1%, 2.5%, 5%, 10% and 15% of the observed LC$_{50}$ values for diazinon (0.009 μg/L, 0.0225 μg/L, 0.045 μg/L, 0.09 μg/L and 0.135 μg/L), malathion (0.03 μg/L, 0.08 μg/L, 0.16 μg/L, 0.32 μg/L and 0.47 μg/L) and BPA (0.1 mg/L, 0.35 mg/L, 0.7 mg/L, 1.4 mg/L and 2.1 mg/L). Exposures were conducted with a maximum *D. magna* density of 1 daphnid per 30 mL. Since there was little variability between individuals in preliminary tests, *D. magna* were grown in populations of 60 in 2L beakers and pooled samples were drawn from these populations. *D. magna* were fed halfway through the test a 50:50 ratio of *C. vulgaris* and *P. kirchniriella*. Food was measured so that each daphnid received the equivalent of 0.2 mg of carbon content. Light and temperature conditions were consistent with culturing conditions. After 48 hours, daphnids were removed from the test solution, flash frozen in liquid nitrogen and lyophilized to cease enzymatic activity. They were then stored at -25°C until the day of extraction for metabolomic analyses.

4.3.3 Metabolite extractions for $^1$H nuclear magnetic resonance

The metabolite extraction procedure was based on previous work developed using a Bruker 1.7 mm NMR microprobe, which minimizes the amount of sample needed for metabolomics analysis while still producing adequate signal to noise for metabolite
quantification (Chapter 3). Briefly, 1 mg of *D. magna* dry mass was weighed out with a microbalance (Sartorius ME36S, Goettingen, Germany) and inserted into a 200 μL microcentrifuge tube, where they were homogenized with a small metal spatula. This procedure was repeated ten times for each condition for a total of ten analytical replicates for each exposure condition. 40 μL of a D₂O buffer was added to each homogenized sample. The D₂O buffer was comprised of 0.2 M sodium phosphate dihydrate (NaH₂PO₄ • 2H₂O, 99.3%, Fisher Canada), 10 mg L⁻¹ of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS; 97%, Sigma Aldrich) to serve as an internal calibrant and 0.1% w/v sodium azide (99.5%, Sigma Aldrich) added as a preservative. The pH was adjusted to 7 (pD = pH + 0.4) using sodium deuteroxide (30% w/w in 95.5% D₂O, Cambridge Isotope Laboratories). The mixture was vortexed for 45 seconds and sonicated for 15 minutes. Samples were then centrifuged for 20 minutes at 12,000 rpm (15294 g; Eppendorf 5804-R, Hamburg, Germany). The resulting supernatant was then pipetted into 1.7 mm NMR tubes (Norell Inc., NC, USA) for ¹H NMR analysis.

4.3.4 ¹H nuclear magnetic resonance spectroscopy and data processing

Analysis was performed with a Bruker BioSpin Avance III 500 MHz NMR equipped with a microprobe (¹H-¹³C-¹⁵N TXI) fitted with an actively shielded Z gradient. Analysis was performed using 256 scans, a relaxation delay of 3 seconds, 32k time domain points and a 90° pulse calibrated on a per sample basis. Water suppression was accomplished by using the Presaturation Using Relaxation Gradients and Echoes program (Brown et al., 2008; Simpson and Brown, 2005). Spectra were apodized through multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectra, with a zero filling factor of 2 (Chapters 2 and 3; Lankadurai et al., 2011). Acquired spectra were manually phased, baseline corrected and aligned to the trimethyl-silyl group of the DSS internal calibrant and set at δ = 0.00
ppm. Prior to any $^1$H NMR analysis, both the D$_2$O used and the D$_2$O-based phosphate buffer were analyzed to check for impurities.

4.3.5 Multivariate statistical analysis

NMR spectra were analyzed by using an unsupervised principal component analysis (PCA; Lankadurai et al., 2011). The PCA is an unsupervised multivariate statistical tool that is commonly the first step in analyzing the large amounts of spectral data from metabolomic studies (Saccenti et al., 2014). The large number of variables (in the case of NMR, the integrated bucket values) are reduced to a few latent variables that describe as much of the original data as possible (Eriksson et al., 2006; Kemsley et al., 2007; Saccenti et al., 2014) with two to three principal components (PCs) usually being sufficient to describe most of the variation (Reo, 2002).

PCA was performed using the Analysis of MIXtures (AMIX) program (ver. 3.9.7, Bruker BioSpin). Each $^1$H NMR spectrum was divided into buckets of 0.02 ppm between 0.5 and 10 ppm, resulting in 475 buckets. Buckets were normalized to the total sum of intensities (Chapters 2 and 3; Lankadurai et al., 2011) and the spectral region corresponding to the residual water signal (between 4.7 - 4.9 ppm) was omitted from analysis. This produced a matrix where the rows were samples and columns were the integrated spectral buckets. PCA scores plots were created (with up to 5 PCs) and the data from the PCA was then exported into Microsoft Excel (version 12, Microsoft Corporation, Redmond, VA) to create averaged scores plots (n=10). An analysis of variance (ANOVA) with a Bonferroni posthoc test using an in house R script (Appendix A, A2-1) was performed to assess whether the groups showed statistically significant separation from each other. Standard error of the mean was calculated using Microsoft Excel. PCA loadings plots were also generated using AMIX.
4.3.6 Metabolite identification and percent change calculations

Metabolites were identified using the AMIX metabolite database for comparison, as well as by comparison with spectra of reference standards measured in house and using spectral assignments from previous studies (Chapters 2 and 3; Brown et al., 2008). To calculate the relative percent change of individual metabolites, the integrated bucket values of the control group were subtracted from those of the exposed group. This was divided by the control to give a percent change value (Brown et al., 2008; Chapter 2; Ekman et al., 2007). Statistical significance of these changes was determined using an ANOVA with a Bonferroni posthoc test. Identified metabolites were related to the particular metabolic pathways and this was assisted by using the Kyoto Encyclopedia of Genes and Genomics database (Kanehisa et al., 2014).

4.4 Results

4.4.1 Multivariate statistical analyses of $^1$H nuclear magnetic resonance spectra

Figure 4-1A shows the PCA scores plot for D. magna exposed to diazinon, with the first two PCs describing 85% of total variance. The PCA scores plot showed separation between the control and the 0.045 μg/L, 0.09 μg/L and 0.135 μg/L groups along PC1 (accounting for 77% of the variation) that was statistically significant (ANOVA: F(5,48) = 38.94, p = 8.94 x 10^{-16}, Bonferroni posthoc test results in Appendix C, Table A4-1). However, at exposure concentrations of 0.045 μg/L and 0.09 μg/L, the PCA scores plot showed that these averaged spectra were uniquely different from the others based on their positioning within the PCA.
Figure 4-1. Averaged principal component analysis (PCA) scores plots for diazinon (A), malathion (B) and bisphenol-A (C). Statistically significant separation from the control ($p < 0.05$), based on an analysis of variance with a Bonferroni posthoc test is indicated by an asterisk (*).
On PC1 the lowest concentrations (0.009 μg/L and 0.0225 μg/L) were clustered closer to the highest concentration, though the latter also showed separation from the control on PC2 (accounting for 8% of the variation). The loadings plot (Appendix C Figure A4-1A) for PC1 indicated that resonances from the amino acids leucine, valine, alanine, threonine, glycine and glutamate and signals from fatty acid components (terminal -CH₃ and mid-chain methylene -CH₂(n) groups) accounted for the separation seen in this PC, while threonine, alanine and -CH₂(n) groups accounted for changes on PC2. A PCA scores plot for malathion exposures (Figure 4-1B) accounted for 85% of the variation within the first two PCs. There was also a pattern similar to that of diazinon, where along PC1 (accounting for 60% of variability), the lowest and the highest exposure concentrations resulted in closer grouping and are distinct from the second lowest concentration (0.08 μg/L) with significance seen at 0.08 μg/L (ANOVA: F(5,50) = 12.29, p = 8.62 x 10⁻⁸, Bonferroni posthoc test results in Appendix C, Table A4-2). The loadings plot for both PCs indicated the cause for the separation between the groups (Appendix C, Figure A4-1B) with resonances from the amino acids leucine, valine, alanine, threonine and glutamate, as well as -CH₃ and -CH₂(n) groups causing separation along the first two PCs. A PCA scores plot for BPA exposure (Figure 4-1C) indicated that there was separation between the control and exposed groups, at 0.7 mg/L and 2.1 mg/L exposure concentrations, based on the PC1 ANOVA (F(5,53) = 7.99, p = 1.15 x 10⁻⁵, Bonferroni posthoc test results in Appendix C, Table A4-3). There were differences in the direction of change, where the lowest concentrations (0.1 mg/L) separated along PC2 (accounting for 16% of the variation) and the higher concentrations were separated along PC1 (accounting for 66% of the variation) with the highest concentration incurring the largest shift in metabolome. This showed a general grouping between low
exposures and higher exposures. The loadings plot (Appendix C, Figure A4-1C) indicates which metabolites account for these changes and include resonances from the amino acids valine, leucine, alanine, threonine along PC1 and -CH$_2$(n) on PC2.

4.4.2 Metabolite changes with contaminant exposure

While the PCA scores plots for all three contaminant exposures showed that there were changes in metabolome incurred in response to either OP or BPA exposure (Figure 4-1), examining individual metabolites provides greater insight into the mechanisms of toxicity. Statistically significant changes in metabolite content was calculated using an ANOVA with a Bonferroni posthoc test (results for each exposure condition are in Appendix C, Figures A4-4 to A4-9). The percent metabolite changes with diazinon exposure (Figure 4-2) indicate that at the lowest concentrations (0.009 μg/L and 0.0225 μg/L) there were increases in a number of amino acids that include leucine, valine, isoleucine, glycine, glutamate and phenylalanine. These trends reversed at 0.045 μg/L and 0.09 μg/L, where there were decreases in amino acids such as leucine, valine, glycine, threonine, glutamate and phenylalanine. The highest concentrations resembled the changes that observed at the lowest concentrations of diazinon with increases in many of the amino acids. The pattern of amino acid responses contrasted with the changes in glucose and lactate, both of which were negatively correlated with amino acid changes throughout all the exposure concentrations. The exception to this was the amino acid alanine, which increased throughout all the exposure concentrations.

Malathion exposures showed a trend similar to those seen with diazinon exposure (Figure 4-3). While there were no significant changes in metabolites at the lowest concentration (0.03 μg/L), the second lowest concentration (0.08 μg/L) resulted in a decrease in many amino acids
including alanine, leucine, isoleucine, valine, arginine, glycine, lysine, glutamate, glutamine, phenylalanine and tyrosine. Both glucose and lactate increased at this concentration, though only lactate was statistically significant. Though the changes were not as pronounced as those seen in diazinon exposures, at the highest concentration (0.47 μg/L) these trends were also reversed, though only alanine, valine and isoleucine increased significantly and threonine did not increase. Most striking about both OP exposures is the non-linear response of the metabolites as exposure increases, especially with diazinon exposures.

With BPA exposures (Figure 4-4), there were decreases initially in a number of amino acids at the lowest concentrations, with steady rises as the exposure concentrations increased, though the metabolite changes at 1.4 mg/L were not as marked as those in 0.7 and 2.1 mg/L. This was a pattern seen in the metabolites alanine, leucine, isoleucine, valine, arginine, glycine, lysine, glutamate, phenylalanine and tyrosine. Glucose and lactate decreased significantly at the highest BPA concentrations. Though 1.4 mg/L of BPA exposure incurred a muted response of metabolites (compared to 0.7 mg/L and 2.1 mg/L), there were still increases in amino acids and decreases in the energy molecules glucose and lactate.
Figure 4-2. Percent changes of metabolites showing statistically significant changes after 48 hours of diazinon exposure. An analysis of variance with a Bonferroni posthoc test, was used to determine statistical significance and is indicated by an asterisk (*), n=10.
Figure 4.3. Percent changes of metabolites showing statistically significant changes after 48 hours of malathion exposure. An analysis of variance with a Bonferroni posthoc test was used to determine statistical significance and is indicated by an asterisk (*), n=10.
Figure 4-4. Percent changes of metabolites showing statistically significant changes after 48 hours of bisphenol-A exposure. An analysis of variance with a Bonferroni posthoc test was used to determine statistical significance and is indicated by an asterisk (*), n=10.
4.5 Discussion

4.5.1 Metabolomic responses with organophosphate exposure

The PCA scores plots showed that diazinon and malathion exposures (Figures 4-1A and 4-1B) incurred significant changes in \( D. \) magna metabolite profiles. When the individual metabolite changes were examined, the inverse correlation between energetic molecules (glucose and lactate) and amino acids, as well as their pattern of change, indicates an analogous exposure response. This confirms the hypothesis that two OPs of similar structure and function would induce comparable changes in the metabolome. However, these individual metabolite changes were non-linear, with diazinon in particular showing initial increases in many metabolites, then decreases that were followed with increases again. Both OP exposures indicated that the intermediate concentrations used in this study elicited the most pronounced changes in metabolite profiles and contrasted with what was seen at the lowest and highest concentrations in terms of the direction of the changes. While unexpected and inconsistent with the assumption that toxic responses work in a linear fashion, non-linear exposure responses to toxic stress have been reported elsewhere (Bundy et al., 2008; Graney and Giesy, 1986; Guler and Ford, 2010; Printes and Callaghan, 2004; Sanchez-Arguello et al., 2009; Uno et al., 2012; Villeneuve et al., 2012; Zhang et al., 2011) and suggest different modes of dealing with toxicity that are difficult to predict. Observed responses are not always linearly concentration dependent since the multiple biochemical mechanisms involved are complex (Graney and Giesy, 1986). This is relevant in the context of risk assessment, as many measures of toxicity using \( D. \) magna are based on acute toxicity tests, with mortality as an endpoint, and do not provide information about the changes that are occurring at sub-lethal levels. It is then difficult to make assessments on toxicity when
aberrant changes can occur well below a threshold value dictated by acute toxicity tests which assume linear toxicity.

4.5.2 Mechanisms of organophosphate toxicity to *Daphnia magna*

Sokolova et al. (2012) have proposed a general model of how stress is manifested in aquatic invertebrates in relation to the severity of the stressor (i.e. exposure dependant toxic stress), and this model may provide insight into the non-linear changes observed in this study. They have observed that aquatic invertebrates, responding to stress, will have distinctly different responses between low/intermediate and high stress exposures. At low/intermediate exposures, compensatory metabolic adjustments can be made to deal with the added expenditures of cellular damage, homeostatic maintenance and physiological activity (Sokolova et al., 2012). At more extreme stress exposures, there is a systemic decrease in metabolic activity, possibly as a temporary condition until the return of more favourable conditions (Sokolova et al., 2012). As such, non-linear changes in metabolite content may be observed and this model appears to be consistent with the metabolite changes observed in this study. For example, at the lowest concentrations of diazinon (0.009 μg/L and 0.0225 μg/L) a number of amino acids increased, and though these responses are also seen at the highest concentration, they likely have a different underlying mechanism. While increased feeding in response to stress may account for the increased amino acid content (Sokolova et al., 2012), it is also possible that a general cessation in protein synthesis may be occurring as an energy conservation strategy, though depletions in lactate at 0.0225 μg/L of diazinon exposure suggest that lactate is being used as a gluconeogenesis source (Jones et al., 2008). However, the commensurate increases in glucose that would be expected were not observed.
At intermediate concentrations, glucose increased in both diazinon and malathion exposures, though only significantly for diazinon. There are two possible reasons for this. First is the release of crustacean hyperglycemic hormone (CHH), initiating a hyperglycemic response that is known to occur in crustaceans responding to stress (Chang et al., 2006; Fingerman et al., 1981; 1998; Lorenzon et al., 2000; Reddy and Rao, 1986; Rodriguez et al., 2007). The function of CHH is believed to be analogous to vertebrate cortisol/corticosterone, which elevate blood glucose and lactate levels to meet increased energy requirements (Elwood et al., 2009; Fanjul-Moles, 2006). However, it is also possible that the elevated levels of circulating glucose are the results of amino acids being used in the process of gluconeogenesis. Responding to toxicity is an energetically demanding process, and puts an added strain on top of maintaining basal metabolism (Soetaert et al., 2007). Under stressful conditions, amino acids can be used as keto acids to produce energy through the tricarboxylic acid cycle (Naveed et al., 2010; Sancho et al., 2009; Villarroel et al., 2009). This appeared to be the case in this study, where at intermediate exposure concentrations, increases in glucose were correlated with decreases with glucogenic amino acids such as glycine, glutamine, glutamate, phenylalanine and tyrosine. This has also been observed in other aquatic species responding to toxicity (Roznere et al., 2014; Schock et al., 2012; Uno et al., 2012). However, it is possible that the amino acids used in this process are not only drawn from free amino acids, but that they are being supplied from the breakdown of proteins. Often, protein catabolism occurs as a stress response and serves as a source for these amino acids (Barber et al., 1990; Naveed et al., 2010; Sancho et al., 2009). OPs in particular, acting on AChE and resulting in an increased intracellular Ca\(^{2+}\), are known to trigger proteolysis (Lushchak, 2011). Decreases in overall amino acids then may be a sign of their excess use as an energy source, resulting in a net decrease in their concentrations.
Under stressful conditions, aquatic invertebrates are also known to switch to anaerobic respiration to produce energy (Li et al., 2014; Sokolova et al., 2012). Evidence of this switch is indicated in our observations by the increase in lactate, which is the main by-product of anaerobic metabolism (Paul et al., 1998; Xuan et al., 2011). Anaerobic metabolism is likely being induced to meet the heightened energy demands incurred by OP metabolic disruption, even though it is less efficient than aerobic respiration (Li et al., 2014). The surplus of lactate is thought to cause metabolic acidosis and is mitigated by its conversion to glucose/glycogen (Xuan et al., 2011), though this is a minor component of lactate removal (Stentiford et al., 2001). The surplus of lactate seen in intermediate doses of OPs may indicate that a reduced ability to convert lactate and may actually be a consequence of toxicity (Xuan et al., 2011). While the induction of anaerobic metabolism was generally seen at higher stress responses (Sokolova et al., 2012), the induction of a hyperglycemic response and energetic compensation via the catabolism of amino acids is characteristic of a low to moderate stress response (Sokolova et al., 2012) and suggests that at 0.045 μg/L and 0.09 μg/L of diazinon exposure, as well as 0.08 μg/L of malathion exposure, D. magna were transitioning from a state of intermediate to severe toxic stress.

These changes are in contrast with the highest concentration of OP exposure, where the relationship between energetic molecules and amino acids was reversed. At the highest diazinon exposures, there were large increases in free amino acids and significant decreases in both glucose and lactate. This suggests that amino acids are not being used in protein synthesis, which is thought to be an energy conservation strategy when adenosine triphosphate production is reduced (Soetaert et al., 2007), and that there was overall less energy available in the organism. This is consistent with what occurs at more extreme exposure concentrations, where there is a general suppression of metabolism that serves as a general survival strategy until the return of
more favourable conditions (Sokolova et al., 2012). While this was clear in the diazinon exposures, with malathion these responses were less evident with only decreases in lactate and small increases in alanine, valine and isoleucine being statistically significant. This is likely the result of the greater toxicity of diazinon over the 48 hour test duration, as diazinon undergoes greater transformation into its more toxic oxon analogue than malathion does (Bavcon et al., 2003) and while exposure concentrations are based on LC$_{50}$ values, diazinon may have a steeper concentration exposure response and therefore show more toxicity relative to malathion. The higher toxicity of diazinon may be why there are increases in alanine across all concentrations, even at the lowest concentrations, for diazinon but not for malathion. Alanine serves as an important biomarker of stress as it plays a central role in energy production and as an osmolyte (Tuffnail et al., 2009; Yoshida et al., 2014).

Among the amino acids with altered concentrations were the aromatic amino acids phenylalanine and tyrosine, which are important precursors to biogenic amines such as dopamine and octopamine (McCoole et al., 2012). Perturbations in these particular amino acids are important because of the varied and pleiotropic roles their downstream products play in invertebrate systems (Christie, 2011; McCoole et al., 2012). They are important multifunctional neurotransmitters and circulating hormones in crustaceans (McCoole et al., 2012). Their release in response to acute stress precedes the release of hormones that initiate energy responses (Adamo, 2014). Serotonin initiates ecdysteroid and juvenile hormone responses which are primary drivers of molting, reproduction and growth in crustaceans (Flaherty and Dodson, 2005; Martin-Creuzburg et al., 2007) and dopamine and octopamine have been observed to play a similar role in Drosophila (Rauschenbach et al., 2007). If these precursor amino acids are being
used as keto acids in the tricarboxylic acid cycle, there are possible consequences at the hormonal and neurotransmitter levels in *D. magna*.

What was seen in the metabolome changes in response to OP exposure was a systemic response to stress that involved both increased energy production through aerobic respiration and the induction of anaerobic glycolysis. Altered energy dynamics are important, as responding to toxic stimuli means that less energy is used in somatic growth and reproduction (De Coen and Janssen, 1998; Soetaert et al., 2007; Villarroel et al., 2009) and this has consequences for not just individual *D. magna*, but at higher levels of biological organization as well. For example, *D. magna* size has direct relation to reproductive output (Villarroel et al., 2009), so any metabolic adjustments to combat stress that reduce growth can alter population dynamics. The differences seen in intermediate OP concentrations and the highest concentrations were consistent with what would be predicted from the model proposed by Sokolova et al. (2012), though the increased amino acids at the lowest levels remain difficult to account for. The highest concentrations of both OPs showed what would likely be a general slowdown in metabolic activity, with decreased protein synthesis and decreases in glucose and lactate, while intermediate concentrations indicate the increased energetic expenditures in maintaining homeostatic conditions.

4.5.3 Metabolomic responses with bisphenol-A exposure

The individual metabolite changes for BPA exposures indicated that at low concentrations, there were overall decreases in amino acid concentrations. These decreases did not correspond to significant changes in glucose levels, as seen with OP exposure. However, at the higher concentrations, the correlations between amino acids and energy molecules were similar to that seen with the OPs, where increases in amino acids were negatively correlated with glucose/lactate levels and suggest a generalized stress response. While not statistically significant,
the 1.4 mg/L exposure concentrations still showed trends consistent with 0.7 mg/L and 2.1 mg/L concentrations.

4.5.4 Mechanisms of bisphenol-A toxicity to *Daphnia magna*

With increasing BPA exposure concentrations, the relative increase in free amino acids with increasing exposure concentrations (Figure 4-4) suggests that protein synthesis is reduced. This suggests a method of dealing with toxicity that is consistent with what is seen at the highest levels of OP exposure in this study; that after a certain threshold it is better for the organism to conserve energy by halting protein synthesis even if it is at the cost of somatic growth and reproduction. A reduction in protein synthesis in response to toxic stress has been observed in other *D. magna* toxicity studies as well (Barber et al., 1990; Mckee and Knowles, 1986; Soetaert et al., 2007). This reflects the extreme stress that is incurred by BPA exposure at these concentrations, where a general reduction of metabolism is observed. However, it is also plausible that degradation of proteins is occurring as a result of toxicant induced protein damage (Barber et al., 1990) and thus producing an increase in amino acids. The decreased lactate levels seen in the higher concentrations suggest that is being used in gluconeogenesis, though given the depressed glucose levels, the contribution from this source may be insufficient. The overall metabolite changes indicate that *D. magna* have a more severe response to BPA and at these sub-lethal levels there is no moderate toxicity phase of energetic adjustments, as suggested by Sokolova et al. (2012), though it is possible that this may occur at lower levels of exposure not used in this study. While the BPA concentrations used in this study are above environmentally relevant concentrations, we have demonstrated that BPA induces a sub-lethal response that can help explain how a generalized change in energetic parameters is manifested in the *D. magna* metabolome.
4.6 Conclusions

Using $^1$H NMR-based metabolomics, responses to OPs and BPA exposure at sub-lethal levels were elucidated. The non-linear exposure responses to increasing exposures between the two OPs differed from what was observed with the BPA exposures. There were significant changes in response to all three contaminants, though for OPs, changes were not linearly correlated to exposure concentrations. Interestingly, because of this lack of linearity, there is greater information gleaned about the toxic modes of action of OPs, in particular with regards to being able to distinguish moderate from severe stress. Although OPs are known to disrupt AChE activity, the ability of metabolomics to see greater systemic responses of the metabolome provides additional insight into the mode of action of OPs and may serve as a useful tool in the analysis of other aquatic contaminants. BPA exposures also show that energy is impaired at sub-lethal values, though the inverted response seen with moderate OP exposures does not occur with BPA. While the concentration response patterns between the OPs and BPA differ, at the highest concentrations both show profiles indicating a consistent stress response that acts on altering the energy dynamics of D. magna. We have demonstrated that pulse contaminant exposures can incur aberrant metabolome profiles over 48 hours. However, chronic exposure studies that are linked to reproductive parameters may also reveal responses that have consequences at the population level. This could provide greater specificity in metabolite response and provide insight into the adaptive capacity of D. magna and the associated costs of these adaptations. Though we have demonstrated that $^1$H NMR is a powerful analytical modality in metabolomics research, the obscuration of individual variation when pooling samples is a particular limitation of NMR-based metabolomics.
4.7 Acknowledgements

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Chapter 5

Chronic bisphenol-A exposures induce metabolomic changes in *Daphnia magna* that are age dependent
5.1 Abstract

$^1$H nuclear magnetic resonance (NMR)-based metabolomics was used to investigate the changes in *Daphnia magna* biochemistry after chronic exposure to sub-lethal bisphenol-A (BPA) concentrations, with tests conducted over 4, 7, 14 and 21 days of exposure. The metabolomic examination was accompanied by a 21 day reproduction test, as well as a liquid chromatography - tandem mass spectrometry examination of the molting hormone 20-hydroxyecdysone (20HE). While the reproduction test did not show a change in total neonate output or moulting frequency, NMR-based metabolomics identified differences at 7 and 14 days of exposure. These differences included changes in metabolites such as threonine, glutamate, glutamine, methionine, asparagine, isoleucine and glucose. These changes in aggregate suggest a compensatory energy strategy, where amino acids are being used to create energy to mitigate the damage incurred by BPA exposure. By 21 days, there were no changes in metabolite content between the exposed and control groups, indicating that *D. magna* may have an adaptive capacity to respond to sub-lethal BPA exposure. These results suggest that the life stage of *D. magna* may be an important consideration in aquatic toxicology studies, as in the case of BPA exposures, the *D. magna* adults may be more susceptible to toxicity than neonates, although the latter is generally considered to be more sensitive to toxicity. As 20HE levels did not change throughout the course of the experiment, toxicity is not linked to changes in circulating ecdysone levels and so there is no evidence that BPA is acting directly on the hormonal axis.
5.2 Introduction

Bisphenol-A (BPA) is a plasticizer with a multitude of industrial uses and is one of the most used chemicals in the world (Richardson et al., 2014). Its widespread use has led to it becoming a common aquatic ecosystem contaminant (Chen et al., 2015; Crain et al., 2007). Its use is controversial as there is strong evidence that BPA is an endocrine disruptor and obesogen in mammalian systems (Flint et al., 2012; Jordão et al., 2016; Mu et al., 2005; Vandenberg, 2014), and implicated in altered neurobehaviour in children (Mustieles et al., 2015). While much focus has been on mammalian responses to BPA exposures, in spite of its presence as an aquatic ecosystem contaminant, there is a limited understanding of the biochemical responses of aquatic invertebrates (Brennan et al., 2006; Clubbs and Brooks, 2007; Plahuta et al., 2015) as well as the potential hormonal influence of BPA on these organisms.

Within aquatic toxicology studies, *Daphnia magna* is a routinely used invertebrate species and serves as a proxy for the health of global freshwater ecosystems (Altshuler et al., 2011; David et al., 2011; Jordão et al., 2016). However, studies using *D. magna* are predominantly tests of fecundity or lethality, yielding little information about the mechanisms of toxicity (David et al., 2011). For example, some research on the toxicity and endocrine impairment of BPA exposure in *D. magna* has been performed and suggests possible anti-ecdysteroidal activity, but has relied on reproductive parameters as proxies (i.e. reproductive output, intermoult duration, male offspring production) to estimate endocrine disruption (Brennan et al., 2006; Caspers, 1998; Clubbs and Brooks, 2007; Mansilha et al., 2013; Mu et al., 2005; Wang et al., 2005). Direct analyses of the biochemical responses of *D. magna* have been constrained to studies of oxidative stress biomarkers such as glutathione S-transferases and catalase and indicate that BPA can induce oxidative stress (Jemec et al., 2012; Park and Choi,
While useful, specific bioassays such as enzyme assays can be time consuming, suffer from variability and may only be useful for a specific biomarker (Robert et al., 2007; Tufi et al., 2016). There is a necessity then, for methods that can understand the holistic biochemical changes in response to long term BPA exposure.

Metabolomics has become an increasingly popular technique that can serve this function, with the potential to be an integral component of risk assessment regimes (Lin et al., 2006; Van Aggelen et al., 2010). Metabolomics is the analysis of an organism’s collective metabolites, referred to as the metabolome, and often responds to stress earlier than the other “omics”, such as proteomics or transcriptomics (Clarke and Haselden, 2008; Lin et al., 2006). In particular, 

1H nuclear magnetic resonance (NMR) has also been shown to be a high-throughput and reproducible platform in metabolomics analyses (Dunn and Ellis, 2005; Viant et al., 2009; Yoshida et al., 2014). D. magna has seen increasing use in metabolomics studies (Chapters 2 and 4; Kovacevic et al., 2016; Li et al., 2015; Poynton et al., 2011; Taylor et al., 2009; 2010; Vandenbrouck et al., 2010). While most of these have observed short term exposures (between 24 and 96 hours), metabolomic changes over longer periods are limited (Taylor et al., 2016a; 2016b). One of these studies demonstrated that dissolved organic matter is able to ameliorate the toxicity of copper (Taylor et al., 2016b) while another demonstrated an effective use of metabolomics data in creating predictive models in response to copper and nickel toxicity (Taylor et al., 2016a). Yet, there remains much that is not understood about the D. magna metabolome responses, when subjected to chronic toxic exposure and therefore this warrants further investigation.

Like all crustaceans, the endocrine axis in D. magna is regulated by ecdysteroids (Martin-Creuzburg et al., 2007; Mu et al., 2005), specifically 20-hydroxyecdysone (20HE), and
alterations in the circulating levels of this steroid can impair embryo growth (Mu et al., 2005). In a mysid species, BPA has shown possibility as an ecdysteroid antagonist (Plahuta et al., 2015), but its role in *D. magna* is unknown. In addition to 20HE, methyl farnesoate (MF), the unepoxidated form of the juvenile hormones found in insects, is also believed to be a regulator of endocrine activity in crustaceans (Mu et al., 2005; Toyota et al., 2015). Little work has been done on directly examining 20HE and MF levels in *D. magna* (Martin-Creuzburg et al., 2007), in spite of their importance in the life history and endocrine function in *D. magna*. Given that BPA is suspected to have hormonal properties, in addition to examining the metabolome, the endocrine axis should also be directly examined, to assess whether changes seen in reproduction parameters involve direct hormonal perturbation or are the result of a generalized stress response of *D. magna*.

Our previous study demonstrated that acute BPA exposure (over 48 hours) on adult *D. magna* resulted in energetic changes suggesting that metabolic activity had slowed down at BPA concentrations greater than 0.7 mg/L (Chapter 4). Little is known about the biochemical changes of *D. magna* in response to chronic BPA exposure from the neonatal stage where they are generally thought to be more susceptible to toxicity (Hanazato, 1998; Sarma et al., 2007) and if *D. magna* have an adaptive capacity to endure BPA stress over this duration. Therefore, the objective of this study is to determine whether sub-lethal chronic toxicity, over 21 days, will result in a change in metabolome profiles. Another objective will be to monitor the changes in 20HE over the 21 day period, to discern if changes can be related to the hormonal axis of *D. magna*. Using the Organization for Economic Co-operation and Development (OECD) guidelines, a 21 day reproduction test will also be performed (OECD, 2012), to determine whether metabolomics is a more sensitive test than the standard reproduction test used in
assaying chronic toxicity. Given that a brief 48 hour exposure incurred metabolome changes suggesting a cessation in metabolic activity (Chapter 4), and the greater sensitivity of neonates to toxicity, it is hypothesized that early exposures will result in toxic stress early on, that carries into adulthood and into the reproductive stages of *D. magna*.

5.3 Experimental Methods

5.3.1. *Daphnia magna* culturing conditions

*D. magna* were initially purchased from Ward Science and have been reared in the laboratory since 2013 (Chapters 3 and 4). Cultures were housed in 2 L jars with a maximum of 30 daphnids in each, kept under a 16:8 light to dark regime and at a constant temperature of 20°C. The medium used was aerated dechlorinated municipal tap water (hardness approximately 120 mg CaCO₃/L) and half the medium was changed every two days. *D. magna* were fed *Raphidocelis subcapitata* (formerly *Pseudokirchneriella subcapitata*) daily, which was grown using a Bristol medium (Tam and Wong, 1990). 1 μg/L of selenium (as NaSeO₃) and cobalamin were added to the food twice a week (Environment Canada, 2000) in order to ensure optimal reproduction and carapace health.

5.3.2. *Daphnia magna* toxicity testing

Using the toxicity data from our previous study as a guide (Chapter 4), preliminary experiments were performed and indicated total survivability at 1 mg/L and that higher values incurred mortalities over 21 days, and so this concentration was selected for sub-lethal chronic toxicity testing. *D. magna* neonates were grown in 600 mL beakers filled with 500 mL of test solution, with 10 daphnids in each. *D. magna* were exposed to BPA over 4, 7, 14 and 21 days, with each condition having its own unexposed control. Each condition had 10 biological
replicates \(n = 10\). *D. magna* were fed *R. subcapitata* every two days, at which point the media was renewed. At each time point, water samples were taken to monitor BPA levels using high performance liquid chromatography (LC) coupled to a diode array detector (Agilent 1100) equipped with a Prevail C18, 250 mm x 4.6 mm column. Samples were monitored using an isocratic method with a water and acetonitrile mobile phase (at a 40:60 ratio) at 30°C and an external calibration curve was developed for quantification purposes. Percent recoveries of BPA in water samples were 75%, 65%, 75% and 78% at 4, 7, 14 and 21 days respectively. For each time point, *D. magna* were flash frozen in liquid nitrogen, lyophilized and stored at -25°C until metabolites were extracted on the day of NMR analysis.

5.3.3. 21 day reproduction test

A 21 day reproduction test was performed, with neonates taken from the same population of *D. magna* used in the metabolomics test and following the guidelines set by the OECD (OECD, 2012). The reproduction test was a static renewal test, with medium changes every two days, at which point the *D. magna* were fed 2 mg carbon L\(^{-1}\) of *R. subcapitata*. Individual *D. magna* were kept in 100 mL beakers, which were filled with 50 mL of test medium, or uncontaminated control. Each condition had 10 replicates \(n = 10\). Endpoints that were examined were total neonate production, time to first moult and number of clutches over the 21 day test period. Statistical significance for differences between clutch size and total reproductive output was calculated using a \(t\)-test \((\alpha = 0.05)\).

5.3.4. Metabolite extractions for \(^1\)H nuclear magnetic resonance analysis

The metabolite extraction procedure is based on a previous study that optimized the use of a microprobe (Bruker BioSpin 1.7 mm) for \(^1\)H NMR analysis of *D. magna* tissue extracts.
(Chapter 3). For each sample, 1 mg of *D. magna* dry mass was weighed out using a microbalance (Sartorius ME36S, Goettingen, Germany) and homogenized inside a 200 μL centrifuge tube with a metal spatula. This was repeated for each sample, resulting in 10 biological replicates for each condition (BPA exposed and control). 40 μL of D$_2$O buffer was added to each sample. The buffer was comprised of 0.2 M sodium phosphate dihydrate (NaH$_2$PO$_4$ • 2H$_2$O, 99.3% Fisher Scientific), 10 mg L$^{-1}$ of 4,4 dimethyl-4-silapentane-1-sulfonic acid (DSS; 97%, Sigma Aldrich) as an internal calibrant and 0.1% w/v sodium azide (99.5%, Sigma-Aldrich) as a preservative to deter microbial degradation of the sample. The pH was adjusted to 7 (pD = 7.4) using NaOD (30% w/w in 95.5% D$_2$O, Cambridge Isotope Laboratories). The mixture was vortexed for 45 seconds and sonicated for 15 minutes before being centrifuged for 20 minutes at 12,000 rpm (15,294 g; Eppendorf 5804-R, Hamburg Germany). The supernatant was removed and inserted into a 1.7 mm NMR tube (Norell Inc., NC, USA) for NMR analysis.

5.3.5. $^1$H nuclear magnetic resonance spectroscopy and post-acquisition processing

NMR analysis was performed with a Bruker BioSpin Ascend 500 NMR equipped with a cryoprobe Prodigy TCI probe and SampleJet autosampler. Spectra were acquired using 256 scans, a relaxation delay of 3 seconds, 32k time domain points and a 90° pulse calibrated on a per sample basis. Suppression of the water signal was performed using the Presaturation Using Relaxation Gradients and Echoes program (Simpson and Brown, 2005). Spectra were apodized by multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectra and had a zero filling factor of 2 (Chapters 3 and 4). Neat D$_2$O and D$_2$O-based phosphate buffer were also analyzed to ensure that reagents were contaminant-free. Spectra were phased manually, baseline corrected and the trimethyl-silyl group of the DSS internal calibrant was set to $\delta = 0.00$ ppm.
5.3.6. Multivariate analysis of $^1$H nuclear magnetic resonance spectra

Spectra were analyzed using an unsupervised principal component analysis (PCA) and this was performed using the Analysis of MIXtures (AMIX; ver. 3.9.7, Bruker BioSpin) program. NMR spectra were divided into buckets of 0.02 ppm, between 0.5 and 10 ppm, with the region corresponding to the residual water signal (4.7 - 4.9 ppm) excluded. The integrated area of each bucket was normalized to the total sum of intensities. The buckets produced a matrix where the integrated areas were represented in the columns and the samples were represented in the rows. This matrix was used to create PCA scores plots (up to 5 PCs), as well as their associated scores plots, using AMIX. Averaged scores plots were created with this data using Microsoft Excel (version 12, Microsoft Corporation, Redmond, VA). A $t$-test (two tailed, equal variance, $\alpha = 0.05$) was used to assess whether changes between conditions were statistically significant. Standard error of the mean was calculated using Microsoft Excel.

5.3.7. Individual metabolite percent changes

Metabolites were identified by ascribing characteristic proton resonances with reference spectra from the AMIX database, as well as by comparing spectra obtained from previous experiments which included the analysis of various metabolite standards (Chapters 3 and 4). The percent change for each metabolite was calculated by taking the integrated bucket value of the control group and subtracting these values from the BPA exposed bucket. This value was then divided by the control value and multiplied by a hundred to provide a percent change. With 10 replicates each, the individual metabolite changes were averaged and statistical significance was determined by using a $t$-test ($\alpha = 0.05$) and p-values were corrected using a false discovery rate, as per Benjamini and Hochberg (1995), using an in house R script (Appendix D, A5-1).
5.3.8. Targeted liquid chromatography-tandem mass spectrometry analyses

Several extraction procedures for analyzing 20HE and MF were tested for liquid chromatography - tandem mass spectrometry (LC-MS/MS) analysis and modified to assess their applicability to *D. magna* samples (Hikiba et al., 2013; Hirano et al., 2009; Westerlund and Hoffmann, 2004; Zhou et al., 2011). Preliminary work with standards of 20HE (99.25%, Selleckchem) and MF (>95%, Echelon) were analyzed and MS conditions were optimized for multiple reaction monitoring (MRM) of 20HE and MF (Table 5-1).

**Table 5-1.** Optimized liquid chromatography-tandem mass spectrometry conditions and fragmentation patterns for 20-hydroxyecdysone and methyl farnesoate analyzed under multiple reaction monitoring and positive ion mode. Results are consistent with what is found in Zhou et al. (2011).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transition ions (m/z)</th>
<th>Collision energy (eV)</th>
<th>Declustering potential (V)</th>
<th>Dwell time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-hydroxyecdysone</td>
<td>481.2 → 445.3</td>
<td>25</td>
<td>86</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>481.2 → 371.2</td>
<td>21</td>
<td>86</td>
<td>200</td>
</tr>
<tr>
<td>Methyl farnesoate</td>
<td>251.2 → 219.3</td>
<td>13</td>
<td>46</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>251.2 → 191.3</td>
<td>13</td>
<td>46</td>
<td>200</td>
</tr>
</tbody>
</table>

From these preliminary tests, it was concluded that only 20HE could be reliably monitored and an extraction procedure developed for the analysis of mysid samples (Hirano et al., 2009) was followed to specifically examine 20HE levels. Information on the LC-MS/MS analysis of MF can be found in Appendix D, A5-1. Briefly, for each sample, 5 mg of freeze dried *D. magna* was placed in a 1.5 mL centrifuge tube and homogenized with a metal spatula. A 4:1 ratio of ice cold methanol and water was added to the homogenate and vortexed for 45 seconds and subsequently sonicated for 15 minutes. Samples were then centrifuged for 20 minutes at 12,000 rpm (15,294 g) and the supernatant was removed and placed into a separate centrifuge tube. The remaining pellet was re-extracted with 200 μL of cold methanol centrifuged for 10 minutes and was added to the other supernatant. This step was performed twice. Samples were blown down to dryness in
a stream of N\textsubscript{2} gas and were reconstituted in 200 \(\mu\)L 10:90 H\textsubscript{2}O (0.1% formic acid): acetonitrile (0.1% formic acid) and filtered through a 0.2 \(\mu\)m filter before LC-MS/MS analysis.

The LC-MS/MS system consisted of an Agilent 1200 LC and an AB Sciex 4000 Q Trap mass spectrometer. Chromatographic separation was carried out on a Phenomenex Synergi Hydro-RP column (150 mm x 4.6 mm, 4 \(\mu\)m; Phenomenex, Torrance, California). Mobile phase A consisted of Millipore water and mobile phase B consisted of acetonitrile with both solvents containing 0.1% formic acid. Separation was carried out with an isocratic flow at 10:90 ratio of mobile phases A and B. Flow rate was 1 mL/min, injection volume was 40 \(\mu\)L and the column temperature was set at 45°C. Detection was performed through MRM in the positive ion mode with a Turbo Spray electrospray ionization source. Chromatograms were processed and analyzed using the Analyst software (AB Sciex, Framingham, MA, USA).

5.4 Results and discussion

5.4.1. 21 day reproduction test

Results of the 21 day reproduction test are shown in Figure 5-1.
The number of broods produced (6) and the time to first brood (7 days) did not differ between the BPA exposed and control groups. The number of neonates produced in the second broods, occurring at nine days, is significantly different, with the BPA exposed *D. magna* producing fewer offspring. However, the total neonate output between the BPA exposed and unexposed control was not statistically different (α = 0.05). Although there is one point where clutch sizes differ, because the gross output was not changed, it cannot be concluded that BPA exposure at this concentration alters the reproductive rate of *D. magna*. Mansilha et al (2013) also found no difference in reproductive output either at 1 mg/L but observed that the offspring from the test
had conspicuously high birth rates and a low survivorship of the fifth brood, indicating that the exposed parent suffered toxicity that resulted in weaker offspring. Similarly, at the same BPA exposure concentrations (1 mg/L), Brennen et al (2006) reported that over 21 days, moulting frequency of *D. magna* did not change. However, the offspring from these tests did not survive over 21 days, indicating that there was a weakening of the parental generation that the 21 day reproduction test did not detect and was subsequently manifested in the form of poor offspring production. That offspring, in other studies conducted at the same concentration, suffered abnormalities (Brennan et al., 2006; Mansilha et al., 2013) strongly suggests that the parental generation undergoes toxic stress that the reproduction test cannot detect.

5.4.2. Non-targeted $^1$H nuclear magnetic resonance-based metabolomics

While the reproduction test yielded little information on toxicity, the metabolomic analysis revealed that there was indeed a sub-lethal toxic response incurred by the *D. magna*. The PCA scores plots for each time point (Figure 5-2) indicate that at 4 and 21 days, there is no divergence in the metabolome between BPA exposed and control *D. magna* groups.
Figure 5-2. Averaged principal component analysis scores plots (n = 10) for the four time points tested in this study. Statistical separation between the control and bisphenol-A exposure is indicated by an asterisk (*) and is calculated using a t-test (α = 0.05).

However, at 7 and 14 days, there are statistically significant changes in metabolome that suggest that exposures are inducing a response at the biochemical level. At 7 days, the loadings plots (Figure 5-3) show that the major contributors to change are terminal methyl group (-CH$_3$) and midchain methylene groups (-CH$_2(n)$) that are part of lipids (Chapter 3)
However, the loadings plot for the 14 day exposure shows that other signals, such as the resonances corresponding to threonine and the broad region corresponding to sugar resonances are contributing to the difference in metabolome (Figure 5-3). It is at 14 days then that a metabolomic analysis is able to detect the largest change in metabolome profile and challenges our hypothesis that exposure would manifest initially in the early juvenile stages. Interestingly, the change seen at 14 days does not continue into day 21, where there are no changes in the individual metabolites relative to the control group. While the PCA and the corresponding loadings plots provide an overview of overall spectral differences, looking at individual
metabolite changes reveals greater detail about the biochemical perturbations occurring at all time points.

The shifts in metabolite concentrations, relative to the control group (Figure 5-4), provide more specific information on the biochemical changes that occur at 14 days (a full table of FDR transformed p values can be found in Appendix D Table A5-1).

![Graph showing metabolite changes over time](image)

**Figure 5-4.** Metabolite changes expressed as percent differences for identified metabolites. Statistical significance based on a t-test ($\alpha = 0.05$) is indicated with an asterisk (*).

These changes include a decrease in the amino acids threonine, glutamate, glutamine, methionine, asparagine and isoleucine, as well as an increase in glucose. These results are in
contrast with what was seen in our previous acute toxicity study, where adults responded to increasing BPA exposures by inducing a general slowdown of metabolic activity, while increasing anaerobic respiration to produce energy (Chapter 4). This manifested as a general increase in amino acids and a decrease in energetic molecules such as glucose and lactate. However, our previous study also found that at certain concentrations of organophosphate exposure, there was a general decrease in amino acids, with a concomitant increase in energetic molecules (specifically glucose and lactate) at levels before severe stress is observed; suggesting a mobilization of energy stores in order to maintain a homeostatic balance (Chapter 4). The distinction between the two patterns is likely a reflection of the differing degrees of stress in the organism, where moderate stress results in energy mobilization and higher levels of stress cause a slowdown in metabolic activity (Chapter 4). In the current study it may be that with chronic exposures to BPA, the compensatory energy strategy seen with the organophosphate exposures is now being seen, as evinced by the decreases in amino acids and increase in glucose stores, though the branched chain amino acids valine and leucine, as well as alanine, did not show significant changes, as was seen in the previous study (Chapter 4). As such, this response pattern may be characteristic of a generalized response to contaminant stress in *D. magna* (Chapter 4). There is however, no change in metabolites at 4 and 21 days, and only a significant increase in threonine stores at 7 days suggesting little toxic stress at these time points.

The life stages for *D. magna* are very different for the 4 and 7 day olds, compared to the mature *D. magna* seen after 14 days and may explain why toxicity is manifested differently at this point. It is largely believed that juvenile stage daphnids are more sensitive to toxicity, given that they have fewer energy resources to draw from (Hanazato, 1998; Sarma et al., 2007) and so any change in metabolome would have been expected by 4 days. However, Lyu et al. (2014),
examining hypoxic stress in *D. magna*, observed that 14 day old daphnids were particularly susceptible to reduced oxygen levels, experiencing higher mortalities and reduced gene expression of catalases, α-esterase and heat shock proteins. In accounting for the reduced α-esterase expression, the authors note the possibility that the ability to express α-esterase was reduced with age, causing the older *D. magna* to be more susceptible (Lyu et al., 2015).

Similarly, David et al. (2011) note the possibility that the toxicity induced increase in genes encoding responses to DNA damage and oxidative stress may actually be the result of adults experiencing greater DNA damage and oxidative stress. It is then possible, that adult *D. magna* may be more sensitive to particular stressors and may explain why toxicity is only manifested after 14 days of exposure. While neonates are growing rapidly as juveniles, in addition to growth, they are also expending resources in reproduction, which appears to plateau after 14 days (the third clutch). The increased metabolic rate at this stage may account for the differences and also account for the why the only changes seen in the 21 day reproduction test was with the second clutch, which occurred between the 7 and 14 day time points used in this experiment. Thus the different life stages of the *D. magna* need to be considered in aquatic toxicity testing, as ecosystems are comprised of individuals of varying age and therefore, of varying susceptibility to stress (Lyu et al., 2015).

At 21 days, there is no difference in metabolome shown in the PCA and the individual metabolite changes indicate no changes either, even though at 14 days there were changes in a number of metabolites. This may be due to the induction of cytochrome-P450 (CYP) defenses in an effort to detoxify the BPA and indicates an adaptive capacity of *D. magna*. In a study of other known endocrine disruptors, *D. magna* may have been responding to long term exposure through the induction of CYP enzymes and thus acclimating to toxicity (Clubbs and Brooks, 2007). For
this reason, a chronic test might show at the end of 21 days an adaptive capacity of *D. magna* to BPA stress.

5.4.3. Monitoring of 20-hydroxyecdysone levels

20HE was detected and showed that it likely exists as a conjugate, though the free form could be detected and monitored. If there are indeed conjugates, based on our study, there are far more than reported by Martin-Creuzberg et al. (2007), who observed that the majority of 20HE exists in the free form. Circulating levels were found in the pg range per individual and were shown to increase with age (Figure 5-5).

![Graph showing 20-hydroxyecdysone levels in *D. magna*](image)

**Figure 5-5.** 20-hydroxyecdysone levels in *D. magna* throughout the 21 day test period. There was no statistically significant change between the two conditions (α = 0.05) at any of the time points.

There were no statistically significant changes at any time points between the BPA exposed and control groups. This suggests that the changes seen in the metabolome at days 7 and 14 are not a result of perturbations of the ecdysone system of *D. magna*. However, this does not discount the
possibility that MF is not affected and future work will need to examine methods for determining whether MF is bound to other macromolecular components in order to properly monitor this hormone.

5.5 Conclusion

In this study, the response of *D. magna* to BPA exposure over 21 days was studied and found that there were changes in the metabolome at 7 and 14 days, though not at 4 and 21 days. Changes were particularly pronounced at 14 days in a number of amino acids and glucose and this closely follows in time the only change seen in the 21 day reproduction test, where the second clutch was significantly lower. However, since the total neonatal output did not change, it is difficult to conclude that the reproduction test could provide evidence of toxic stress. This is in contrast with the metabolomics data, which was able to detect the perturbations in energy partition at 14 days of exposure. This is inconsistent with our initial hypothesis, where it was thought that neonatal exposure would manifest as a change in metabolome at an early juvenile stage that would carry on into adulthood. These changes raise the issue of age dependant susceptibility to stress. While it is generally assumed that neonates are more susceptible to toxicity than adults, our study shows with BPA, this may not be the case and so time dependant studies will help provide more insight into how *D. magna* stress responses differ with age. Furthermore, the changes observed did not appear to be the result of perturbations of circulating ecdysone levels and suggest that sub-lethal BPA exposure does not act on the ecdysteroid axis.
5.6. References


Chapter 6

$^{1}$H NMR-based metabolomics shows *Daphnia magna* responds to different carbon resources through a change in energy partitioning
6.1 Abstract

The responses of *Daphnia magna* to a change in available carbon sources were characterized using $^1$H nuclear magnetic resonance (NMR)-based metabolomics. *D. magna* were initially raised on a mixture of two chlorophyta (*Chlorella vulgaris* and *Raphidocelis subcapitata*) and then raised under conditions representing different autochthonous and allochthonous carbon forms which included: cyanobacterium (*Synechocystis* spp.), dissolved organic carbon (DOC), a mixture of DOC mixed with the two chlorophyta and only one of the chlorophyta. NMR-based metabolomics was accompanied by an analysis of the fatty acid (FA) components of the food sources using gas chromatography-mass spectrometry (GC-MS). A principal component analysis indicated that there were distinct changes in the *D. magna* metabolome profiles for all treatments with *Synechocystis* and DOC resulting in the greatest differences. More specifically, *Synechocystis* induced a decrease in a number of amino acids and an increase in glucose that suggested an increased mobilization of energy resources to deal with the less nutritious, potentially toxic food source. In response to DOC exposure there was also a large change in the metabolome, but metabolite changes were opposite of what was seen in the *Synechocystis* treatment. Instead there were large increases in amino acids and a decrease in glucose, indicating a starvation response. The changes seen in the metabolome are reflected in the GC-MS analysis of the various food sources. Compared to the chlorophyta, *Synechocystis* and DOC are lacking in FAs, particularly the poly-unsaturated FAs that are a physiological necessity for *D. magna*. This lack is manifested in the metabolome, which changed even after just 48 hours to a change in carbon quality of potential food sources. This study demonstrates that the biochemical response in a model organism responds rapidly to changes in their immediate environment.
6.2 Introduction

Zooplankton acquire the nutrients they need from the phytoplankton and bacteria they consume (Mariash et al., 2011). These food sources can be from autochthonous primary production (i.e. chlorophyta) or from secondary assimilation of allochthonous carbon sources, such as dissolved organic carbon (DOC; Karlsson et al., 2012; Taipale et al., 2014). These primary and secondary producers serve as important entry points for the transfer of carbon through to higher trophic tiers (Jansson et al., 2007), but they also reflect the quality of the water around them and they respond quickly to a changing environment (Thakur et al., 2013). In general, the abundance of nutrients leads to eutrophic conditions (Smith et al., 2006), and the excess grazing on phytoplankton can drive their reduction in number, allowing less digestible cyanobacteria to proliferate (Arnold, 1971; Schwarzenberger et al., 2013). By contrast oligotrophic conditions may have greater DOC than carbon contained in biota and so zooplankton may rely more on this as a carbon source, specifically through heterotrophic bacterial assimilation (Bouchnak and Steinberg, 2013; Karlsson et al., 2012). These are however dynamic systems that are subject to seasonal flux. For example, in one lake, autochthonous sources of carbon comprised 30-40% of the carbon for Daphnia spp., dropping to 1-5% in the autumn, meaning that there was a greater input from other carbon sources in order for zooplankton to maintain their mass; namely terrestrially derived allochthonous sources (Taipale et al., 2007). These allochthonous carbon inputs, though considered low quality substrates, by sheer bulk become important for bacterial growth and the transfer of carbon through the food chain (Jansson et al., 2000; Taipale et al., 2007). Zooplankton must then sustain themselves on a mixture of both autochthonous and allochthonous forms of carbon (Brett et al., 2009; Van den Meersche et al., 2009). However, these changes in phytoplankton
and bacterial composition can have large consequences given the nutritional differences that exist within them and this poses as a potential stressor to zooplankton. For example, fatty acids (FAs) are important nutrients, and their levels in food sources vary, with diatoms being considered the best foods, followed by chlorophyta and cyanobacteria/bacteria being the poorest (Brett and Muller-Navarra, 1997; Taipale et al., 2014). It is from these sources that zooplankton need to accrue, amongst other resources, the FAs such as the polyunsaturated FAs (PUFAs), that are required for survival and reproduction (Burns et al., 2011; Goulden and Place, 1993; Taipale et al., 2014; Wacker and Martin-Creuzburg, 2007).

Some of these zooplankton are cladocerans such as *Daphnia magna*, which play an important role in ecosystems as linkages between trophic levels (Altshuler et al., 2011). However, as generalized feeders with little capacity to selectively graze (Arnold, 1971; Tessier et al., 2001; Vandonk and Hessen, 1993), their survival is dictated by the nutritional quality of the food around them, which in turn is a function of water quality. Like other zooplankton, *D. magna* derive their biomass from a mixture of autochthonous and allochthonous sources, each with variable quality (Taipale et al., 2014). Examinations of food quality changes on zooplankton have typically been assessments of survival, reproduction, growth or specific examinations of lipid profiles (Bouchnak and Steinberg, 2010; Brett et al., 2009; Burns et al., 2011; Wacker and Martin-Creuzburg, 2007), and while providing valuable information, do not provide much biochemical insight into the changes wrought by a changing environment.

An emerging approach to obtain biochemical insight into an organism’s health is metabolomics. Metabolomics is the holistic characterization of the small molecular weight metabolites in a tissue or organism (Lin et al., 2006) and provides mechanistic information that is absent in traditional assays (Lin et al., 2006). While many of the metabolomics studies using *D.*
magna have been toxicity tests (Chapters 2, 4 and 5; Kovacevic et al., 2016; Li et al., 2015; Poynton et al., 2011; Taylor et al., 2009; 2010; 2016a; 2016b; Vandenbrouck et al., 2010), few have specifically examined parameters that simulate changes in aquatic ecosystems related to carbon resources. While there are a number of analytical modalities that are used in metabolomics studies, nuclear magnetic resonance (NMR) provides a rapid, high-throughput platform (Dunn and Ellis, 2005; Yoshida et al., 2014) and is particularly useful in the analysis of polar compounds (Lankadurai et al., 2013; Li et al., 2015). However, it is less capable in distinguishing non-polar molecules such as FAs. Therefore, in examining the FA quality of carbon sources, gas chromatography-mass spectrometry (GC-MS) is an appropriate analytical modality (Bigelow et al., 2011; Volkman et al., 1989).

In this experiment we subjected D. magna to a ¹H-NMR-based metabolomics analysis as they undergo a short-term change in the carbon sources available to them. This will provide a molecular context for how D. magna will respond to differing degrees of carbon quality, whether autochthonous or allochthonous in origin. FA profiles of different carbon sources will be characterized using GC-MS and will complement the NMR analysis. This will provide insight into the biochemical function of D. magna because cladocerans depend on dietary sources for their FAs (Goulden and Place, 1993). For this experiment, we reared D. magna on a mixture of two chlorophyta species (Raphidocelis subcapitata and Chlorella vulgaris) that are characteristic of healthy food sources for D. magna and are ecologically relevant species (Espinosa-Rodriguez et al., 2014; Tessier et al., 2001). This was compared to lower quality carbon sources including: cyanobacteria (Synechocystis spp.), terrestrially derived DOC, a mixture of chlorophyta (the two they were reared on) and DOC, or only one of the chlorophyta. We hypothesize that the metabolome for the D. magna raised under chlorophyta will be similar, but that the metabolome
of *D. magna* reared under cyanobacteria will differ considerably, as cyanobacteria lack essential FAs and sterols (Wacker and Martin-Creuzburg, 2007). We will also investigate whether there is evidence of any assimilation of DOC but we hypothesize that DOC will be a poor food source, owing to the lack of FAs in this allochthonous source. The addition of better quality carbon (as chlorophyta) may offset the potential poor quality of DOC which will also be studied in this experiment. This research will provide a biochemical context for the environmental changes that occur, whether seasonal or anthropogenic, in lakes and the way zooplankton respond to these changes in the short-term.

6.3 Methods

6.3.1 Chlorophyta and *Daphnia magna* culturing

*C. vulgaris* and *R. subcapitata* were grown in batch culture under continuous light in Bristol medium (Tam and Wong, 1990). Algal cells were concentrated by centrifugation at 4500 rpm for 20 min and an approximate 1:1 ratio of the two chlorophyta were fed ad libitum to the brood mother *D. magna*. The *D. magna* were initially purchased from Ward Science Canada and have been maintained in the laboratory for over two years in de-chlorinated municipal tap water (hardness approximately 120 mg CaCO$_3$/L). Three times a week 50% of the water was changed to keep ammonia levels low and prevent the decomposition of excess food. *D. magna* were maintained at 20°C and kept under a 16:8 light/dark photoperiod. Only *D. magna* that could meet the minimum Environment Canada standards of reproduction within 12 days and a minimum clutch size of 15 neonates per individual were used for experiments (Environment Canada, 2000). The *Synechocystis* was generously provided by Professor John Coleman from the University of Toronto.
6.3.2 Dissolved organic carbon preparation from Pahokee Peat

International Humic Substances Society (IHSS) Pahokee peat was used to isolate the DOC. 20 g of the IHSS Pahokee peat was ground using a mortar and pestle and transferred into a 250 mL Nalgene Centrifuge bottle to which 200 mL of Milli-Q water (Millipore Corporation) was added and shaken for 2 hours. The solution was then centrifuged at 4500 rpm for 20 minutes using a Sorval RC-5B centrifuge. The supernatant was decanted into a 1 L Nalgene bottle. The remaining Pahokee peat pellet was repeatedly extracted using the same procedure and the supernatants were combined together. The extracts were then filtered using glass fiber filters (Whatman GF/F 55 mm diameter, 70 µm pore size) to remove large particles. The DOC fraction was subsequently isolated by filtering the water through a Whatman 0.2 µm Durapore membrane. The final extract was freeze-dried to produce the DOC that was used as a carbon source.

6.3.3 *Daphnia magna* responses to different carbon sources

*D. magna* aged 14 days were removed from stock cultures and placed in replicate jars with a density of 1 individual per 35ml of water. *D. magna* were then randomly assigned to one of six treatments that were standardized to the amount of total carbon (6 mg carbon L⁻¹) in each replicate jar as per Organization for Economic Co-operation and Development (OECD) guidelines (OECD, 2012). The six treatments were: 1. Chlorophyta mixture (3 mg carbon L⁻¹ each of *C. vulgaris* and *R. subcapitata*), 2. *C. vulgaris*, 3. *R. subcapitata*, 4. *Synechocystis*, 5. DOC, and 6. A 3 mg carbon L⁻¹ mixture of the chlorophyta mixture diet with 3 mg carbon L⁻¹ of DOC (referred to herein as C+DOC). Carbon content was determined by freeze drying a subsample of all the treatments for elemental analysis (Thermo Scientific Flash 2000, TRACES centre, University of Toronto, Scarborough) by combusting at 950 °C using oxygen gas. Both *C. vulgaris* and *R. subcapitata* had a carbon content which was 50% of dry mass, whereas the
Synechocystis was 45% carbon and the DOC was 33% carbon. The 48 hour experiments were maintained at the same room temperature and photoperiod as the D. magna stock culture.

6.3.4 Sample preparation and extraction for 1H nuclear magnetic resonance

After the 48 hours, D. magna were removed from the test medium and rinsed in dechlorinated municipal tap water, quickly flash-frozen in liquid nitrogen and then lyophilized to restrict enzyme activity (Dunn and Ellis, 2005). They were stored at -20 °C until the day of extraction. D. magna were measured to yield approximately 1 mg of dry mass per sample. The extraction procedure is based on the method outlined in Chapter 3. Briefly, 1 mg dry mass of D. magna was placed in a 200 µL centrifuge tube and homogenized using a stainless steel spatula. 45 µL of a 0.2 M monobasic sodium phosphate buffer solution (NaH₂PO₄·2H₂O; 99.3%; Fisher Chemicals) containing 0.1% (w/v) sodium azide (99.5% purity; Sigma Aldrich) as a preservative was then added. The buffer solution was made with D₂O (99.9% purity, Cambridge Isotope Laboratories) and adjusted to a pD of 7.4 using NaOD (30% w/w in 99.5% D₂O; Cambridge Isotope Laboratories Inc). The buffer solution also contained 10 mg/L of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS; 97%, Sigma Aldrich) as an internal calibrant. The samples were then vortexed for 45 seconds and sonicated for 15 minutes. This was followed by centrifugation for 20 minutes at 12 000 rpm (~15 000 g at 4 °C) using an Eppendorf 5804-R centrifuge. The supernatant was then pipetted into 1.7 mm High Throughput plus NMR tubes (Norell Inc., NJ, USA) for 1H NMR analysis. Freeze dried C. vulgaris, R. subcapitata, and Synechocystis (1.1 to 1.5 mg) were also extracted following the same procedure as the D. magna tissue and the samples were transferred into 1.7 mm High Throughput plus NMR tubes (Norell Inc., NJ, USA) for 1H NMR analysis.
6.3.5 $^1$H nuclear magnetic resonance spectroscopy and data analysis

*D. magna* extracts were analyzed with a Bruker BioSpin Avance III 500 MHz NMR equipped with a $^1$H- $^{13}$C- $^{15}$N TXI 1.7 mm microprobe fitted with an actively shielded Z gradient. $^1$H NMR analysis was performed with 256 scans, a relaxation delay of 3 seconds, 32k time domain points and a 90° pulse calibrated on a per sample basis (Chapter 3). Spectra were apodized through multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectra, with a zero filling factor of 2 (Chapter 3). Water suppression was accomplished using the Presaturation Using Relaxation Gradients and Echoes program (PURGE; Simpson and Brown, 2005). Spectra were manually phased and calibrated to align with the trimethyl silyl group of the DSS internal calibrant and set to $\delta = 0.00$ ppm.

The chemical range between 0.5 and 10 ppm represented all $^1$H NMR resonances in extracts and were divided into buckets that were 0.02 ppm in width using the Analysis of MIXtures (AMIX, 3.9.7, Bruker BioSpin, Rheinstetten, Germany) statistics tool for a total of 475 buckets. The area between 4.7–4.9 ppm was excluded to eliminate the residual H$_2$O/HOD signals. The integration mode was set to the sum of intensities and the spectra were scaled to total intensity. This created a matrix in which each row represented a *D. magna* sample and each column contained the integrated area of the bucket region. A principal component analysis (PCA) scores plot was constructed to compare the metabolic responses of *D. magna* that were subjected to the six different carbon sources. The values from the PCA scores plots were then imported into Microsoft Excel (version 12), Microsoft Corporation, Redmond, WA) and were averaged for each condition and re-plotted with their associated standard errors. An analysis of variance (ANOVA) with a Bonferroni posthoc test (using an in house R script, Appendix A2-1) was performed on the PCA scores plot values to determine statistically different differences ($\alpha =$
0.05). Corresponding PCA loadings plots were also generated with AMIX to determine which metabolites contributed to the separation observed in the scores plots.

The buckets generated by AMIX were then imported into Microsoft Excel. The percent changes in the metabolite bucket intensities of *D. magna* exposed to alternative carbon sources relative to the chlorophyta mixture were calculated by dividing the buckets related to the metabolites in carbon sources (either DOC, Synechocystis, C+DOC or one of the two chlorophyta) by the corresponding buckets in the chlorophyta mixture. An ANOVA with a Bonferroni posthoc test was then performed comparing the buckets of the chlorophyta mixture with that of the *D. magna* exposed to various carbon sources to identify the buckets that were statistically different at α = 0.05. The metabolite peaks in the 1H NMR spectra of the *D. magna* tissue extracts were identified by comparing to 1H NMR spectra of metabolite standards that were previously reported (Chapters 2 and 3).

6.3.6 Fatty acid analysis using gas chromatography-mass spectrometry

Freeze dried carbon sources were individually ground to a fine powder in liquid nitrogen using a mortar and pestle, and then weighed to the nearest microgram. Lipids were extracted using a modified method from Folch et al. (Folch et al., 1957). In brief, each sample was extracted three times, using 2 mL of chloroform/methanol (2:1; v/v) and then pooled (for a total of 6 mL). Polar compounds were removed by adding 1.6 mL NaCl solution (0.9% w/v); this layer was discarded following centrifugation. The resulting lipid-containing solvent was concentrated to 2 mL and 2 aliquots (100 µL each) were removed and evaporated to dryness to determine total lipid content through gravimetric analysis. The lipid extract was then prepared for GC-MS by derivatizing FAs, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to FA methyl esters (FAMEs) using sulfuric acid as the catalyst. FAMEs were
extracted twice using hexane: diethyl ether (1:1; v/v), then dried under a gentle stream of nitrogen. The dried FAMEs extract was re-dissolved in hexane and individual FAMEs were separated using a GC (Shimadzu-2010 Plus, Nakagyo-ku, Kyoto, Japan) equipped with an SP-2560 column (Sigma-Aldrich, St. Louis, Missouri). All solvents used in the extraction and FAME derivatization procedures were of high purity (>99%, HPLC grade). FAMEs in samples were identified by comparison of their retention times with a known standard (GLC-463 reference standard; Nu-chek Prep, Inc., Waterville, Minnesota) and quantified with a 5-point calibration curve using this same standard. A known concentration of 5 alpha-cholestane (C8003, Sigma-Aldrich, St. Louis, Missouri) was added to each sample prior to extraction to act as an internal standard to estimate extraction and instrument recovery efficiency. For each quantified value, standard error was calculated.

6.4 Results and discussion

6.4.1 Multivariate statistical analysis of $^1$H nuclear magnetic resonance spectra

The averaged PCA scores plot (Figure 6-1) includes the first two PCs and shows a statistically significant separation between the DOC, *Synechocystis* and *R. subcapitata* conditions on PC1, as determined by the ANOVA ($F(5,54) = 220.62$, $p = 1.22 \times 10^{-34}$, Bonferroni posthoc results are in Appendix E, Table A6-1).
Figure 6-1. An averaged principal component analysis scores plot shows that each of the conditions tested induced a separation in the metabolome after 48 hours that was statistically significant. The *Synechocystis* and dissolved organic carbon conditions induced the greatest separation on PC1. An analysis of variance with a Bonferroni posthoc test was used to determine statistically significant separation (α = 0.05) and is indicated by an asterisk (*).

This shows that *D. magna* exposed to a changing environment respond with distinctly different metabolite profiles from the chlorophyta mixture, even after only 48 hours. Changes are particularly pronounced on PC1, which accounts for 80% of the variation observed in the data, with *Synechocystis* and DOC showing separation on PC2 as well (accounting for 6% of the variation). Overall, *D. magna* receiving one of the chlorophyta (*C. vulgaris* or *R. subcapitata*) or the C+DOC were more similar to the chlorophyta mixture than with the *Synechocystis* and DOC. This relatively close clustering of the chlorophyta mixture with *D. magna* fed one of the two chlorophyta in the PCA scores plots is expected, given that it was a mixture of these two and
therefore similar metabolic responses were to be expected. To confirm whether the contents of the *D. magna* digestive tract may influence these spectra and overall results, an overlay of the *D. magna* sample with chlorophyta and *Synechocystis* extracts (which were at masses higher than the *D. magna* mass used in the extracts) was created and indicates that the contribution from these carbon sources to the *D. magna* spectra is minimal (Appendix E, Figure A6-1). However, between the two chlorophyta, the *R. subcapitata* showed the greater divergence from the chlorophyta mixture. Both *C. vulgaris* and *R. subcapitata* have similar dietary and carbon qualities (Espinosa-Rodriguez et al., 2014) and this is confirmed with the NMR profiles, which bear similarity with each other and *Synechocystis* does not (Appendix E, Figure A6-2). That the *C. vulgaris* source induced a response more similar to the chlorophyta mixture than *R. subcapitata* may be an indication of the greater assimilation efficiency of *C. vulgaris* over *R. subcapitata* when fed a mixture of the two, as it may be that it provides the greater share of nutrition to *D. magna*.

The C+DOC group clusters closely with the chlorophyta group and this suggests that there is little change in the metabolome, even with DOC present. However, the metabolome of *D. magna* in the presence of only DOC resulted in significant separation along PC1. The loadings plot indicates that this is accounted for by changes in the amino acids alanine, leucine and valine (Figure 6-2).
Figure 6-2. A loadings plot from the principal component analysis (PCA) scores plot shows which bucket regions are inducing separation in the PCA scores plot.

This is in contrast with what was seen when *D. magna* switched to an environment with only *Synechocystis*, which also showed a large and clear separation on the first PC, but is also distinctly different from the DOC group (Figure 6-1). The loadings plot indicates that this is due to changes in resonances corresponding to glucose (Figure 6-2). Based on the PCA scores plot, after 48 hours of exposure, the *D. magna* metabolome undergoes change and this vouches for the sensitivity of the metabolome as an indicator of a shift in environmental conditions. While the PCA scores plot is able to discern overall differences in spectra between various carbon sources,
the individual metabolite changes provide more specific insight into the mechanisms that underpin these differences.

6.4.2 Individual metabolite responses to different carbon sources

Individual metabolite changes are shown in Figure 6-3, with 15 metabolites identified as having changed in at least one condition, based on the ANOVA and Bonferroni posthoc results (found in Appendix E, Tables A6-2 and A6-3). The identified metabolites are amino acids, with the exception of glucose. Overall, the changes seen here reflect what was observed in the PCA scores plot (Figure 6-1), where the relatively close clustering of the chlorophyta conditions (including the C+DOC condition) are reflected in the percent metabolite changes, since few metabolites are changing in concentration. As expected, the two conditions that were most divergent in the PCA scores plot and therefore showed the most altered metabolomes, showed large changes in individual metabolites.

The different chlorophyta, whether C. vulgaris or R. subcapitata, resulted in some minor changes in the metabolome of D. magna. In particular, R. subcapitata induced a significant decrease in alanine, leucine, tryptophan and glucose. Given that the chlorophyta mixture diet was a 50:50 split between C. vulgaris and R. subcapitata, the difference seen on the PCA scores plot provides some insight into the differing quality of these chlorophyta for D. magna. While they generally have similar nutritional composition (Espinosa-Rodriguez et al., 2014), there are differences in the FA composition (Table 6-1).
Figure 6-3. Individual metabolite percent changes are shown for each carbon source, relative to the chlorophyta mixture. An analysis of variance with a Bonferroni posthoc test was used to determine statistically significant changes (α = 0.05) and is indicated by an asterisk (*).
Table 6-1. Fatty acid (FA) composition for each of the diets used in the study was measured using gas chromatography-mass spectrometry (GC-MS) and the values shown are in μg/mg of dry weight. FAs that were not detected are denoted as ND.

<table>
<thead>
<tr>
<th></th>
<th>Chlorella vulgaris</th>
<th>Raphidocelis subcapitata</th>
<th>Chlorophyta mixture</th>
<th>Synechocystis</th>
<th>C+DOC</th>
<th>DOC</th>
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<tr>
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<tr>
<td>14:0</td>
<td>ND</td>
<td>0.9±0.6</td>
<td>1.5±0.9</td>
<td>3.3±0.2</td>
<td>1.9±1.0</td>
<td>1.0±1.0</td>
</tr>
<tr>
<td>i-15:0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.6±0.6</td>
</tr>
<tr>
<td>ai-15:0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.3±0.3</td>
</tr>
<tr>
<td>15:0</td>
<td>0.2±0.0</td>
<td>0.1±0.1</td>
<td>0.2±0.1</td>
<td>0.1±0.1</td>
<td>ND</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>i-16:0</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.7±0.7</td>
</tr>
<tr>
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<td>1.8±0.9</td>
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<tr>
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<td>ND</td>
<td>ND</td>
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<td></td>
</tr>
<tr>
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<tr>
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<td>4.7±0.3</td>
<td>4.3±1.0</td>
<td>4.2±0.1</td>
<td>3.0±0.4</td>
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<td><strong>17.6</strong></td>
<td><strong>12.9</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>2.9±0.2</td>
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<td>4.5±0.3</td>
<td>2.9±0.3</td>
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<td>2.1±0.1</td>
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<td>1.9±0.1</td>
<td>2.1±0.2</td>
<td>2.3±0.2</td>
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<td>1.6±0.2</td>
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<td>0.6±0.3</td>
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<td>3.2±0.2</td>
<td>3.5±0.1</td>
<td>2.9±0.3</td>
<td>3.1±0.2</td>
<td>3.2±0.2</td>
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<tr>
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<td>2.9±0.2</td>
<td>3.7±0.3</td>
<td>3.3±0.2</td>
<td>1.6±0.8</td>
<td>3.3±0.0</td>
<td>ND</td>
</tr>
<tr>
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<td>ND</td>
<td>ND</td>
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<td>ND</td>
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<td><strong>32.5</strong></td>
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<td><strong>28.1</strong></td>
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<tr>
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<td></td>
<td></td>
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<td></td>
<td></td>
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<td>12.9±1.5</td>
<td>11.9±0.9</td>
<td>5.9±0.1</td>
<td>10.5±0.3</td>
<td>6.0±1.1</td>
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<tr>
<td>18:3n-6</td>
<td>0.7±0.4</td>
<td>1.2±0.1</td>
<td>1.3±0.1</td>
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<td>1.1±0.0</td>
<td>0.7±0.1</td>
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<td>18:3n-3</td>
<td>6.0±0.3</td>
<td>8.5±0.9</td>
<td>7.6±0.5</td>
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<td>7.0±0.3</td>
<td>4.2±0.6</td>
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<td>20:2n-6</td>
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<td>2.3±0.2</td>
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<td>1.1±0.0</td>
<td>1.9±0.1</td>
<td>1.1±0.1</td>
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<td>20:3n-3</td>
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<td>0.3±0.3</td>
<td>ND</td>
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<td>20:4n-6</td>
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<td>0.6±0.1</td>
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<td>0.5±0.0</td>
<td>0.5±0.0</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.7±0.1</td>
<td>0.6±0.1</td>
<td>0.8±0.0</td>
<td>0.7±0.0</td>
<td>0.7±0.0</td>
<td>0.8±0.0</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>ND</td>
<td>0.1±0.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>ND</td>
<td>0.1±0.1</td>
<td>0.2±0.2</td>
<td>0.7±0.4</td>
<td>ND</td>
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</tr>
<tr>
<td>Total PUFAs</td>
<td><strong>18.7</strong></td>
<td><strong>26.3</strong></td>
<td><strong>24.5</strong></td>
<td><strong>14.5</strong></td>
<td><strong>21.7</strong></td>
<td><strong>13.6</strong></td>
</tr>
<tr>
<td>Total FAs</td>
<td>62.2</td>
<td>78.3</td>
<td>74.2</td>
<td>62.2</td>
<td>67.4</td>
<td>42.4</td>
</tr>
</tbody>
</table>
For example, while the total FA content of *R. subcapitata* was higher than *C. vulgaris*, the greater quantities of mono-unsaturated FAs (MUFA) and poly-unsaturated FAs (PUFA) are important because they are physiological necessities for *Daphnia* (Taipale et al., 2014; Wenzel et al., 2012). However, both chlorophyta are lacking specifically in EPA (20:5n-3) and DHA (22:6n-3), which are essential for *Daphnia* (Taipale et al., 2014). In spite of the paucity of these FAs, there are still higher levels of linoleic acid and α-linoleic acid in *R. subcapitata*, which are also necessary to *D. magna*, and so *R. subcapitata* is likely a better quality carbon source. While this would then suggest that there is greater benefit derived from consuming *R. subcapitata*, decreases in amino acids seen in the metabolomic profile suggest *D. magna* are deriving less nutrition (Figure 6-3). To account for this, it is possible that there are differing assimilation efficiencies that manifest in the metabolome that are not derived from conventional analyses. For example, it may be more energetically favorable and more efficient to consume the larger *C. vulgaris* that is spherical shaped and 5-10 µm in diameter (Scragg et al., 2003) compared to the smaller *R. subcapitata* which is crescent-shaped and 5-6 µm in width and 10-12 µm in length (Blaise, 1986), making *C. vulgaris* slightly better for *D. magna* somatic growth. However, to the best of our knowledge, there are no studies that have directly compared this. A study using related rotifer species found that there were differences between rotifer species when fed either *C. vulgaris* or *R. subcapitata* (Espinosa-Rodriguez et al., 2014), and so in our study, the preference for *C. vulgaris* may be specific to *D. magna*. There is even the possibility that differing growth conditions of chlorophyta can have a wide variability in nutritional value and morphology and that this can alter the assimilation efficiency of *D. magna* (Ahlgren et al., 1990; Samek et al., 2013; Sterner et al., 1993; Vandonk and Hessen, 1993). This is however beyond the scope of the current study but these differences warrant further investigation.
While the changes incurred from a monoculture of chlorophyta were minute, when fed *Synechocystis*, the changes in metabolite content were more pronounced, showing a marked decrease in a number of amino acids, including leucine, isoleucine, alanine, glutamate, glutamine, lysine, phenylalanine and tryptophan, as well as an increase in glucose (Figure 6-3). The decreases in amino acids is a particular concern, as they form the building blocks required for growth (Fink et al., 2011) and are important precursors to other metabolites. This pattern of response is characteristic of a compensatory energy strategy, where *D. magna* respond to stressful events by using more energy, in an effort to maintain a homeostatic balance. This is shown in the individual metabolite changes, where amino acids change in concentrations opposite to that of an energy molecule like glucose. The decrease in amino acids and concomitant increase in glucose suggests a heightened energetic demand in response to stress where amino acids are being used in gluconeogenesis (Chapter 4). The one exception is the amino acid serine, which shows a marked increase. Serine is important in the biosynthesis of lipids and in reproduction (Ventura and Catalan, 2010), though it is unclear what the increase in this, as well as glycine, signifies in our study. Compared to chlorophyta, a cyanobacteria such as *Synechocystis* is known to be a poor food source for *D. magna* and is lacking especially in PUFAs (Burns et al., 2011). While the FA analysis indicates that the total FA concentrations are not different from *C. vulgaris*, the individual components differ (Table 6-1). *Synechocystis*, while having greater saturated FAs (SFAs) components (Table 6-1), have fewer of the important MUFAs and PUFAs in particular linoleic acid (18:2n-6) and α-linoleic acid (18:3n-3), though DHA was found in the cyanobacteria samples. As a poor food source, it is also possible that a cyanobacteria like *Synechocystis* is being rejected by the *D. magna*, thus reducing the intake and assimilation of carbon (Arnold, 1971; Porter and Mcdonough, 1984). This has been
demonstrated by Porter and McDonough (1984) who found a higher rate of food rejection in *D. magna* when a filamentous cyanobacteria were present. The higher energy expenditure of this process reduced the energy available to *D. magna* (Porter and McDonough, 1984) and the metabolome pattern seen in our study may be a reflection of the increased energetic demand required for this process.

The metabolome for the DOC treatment also showed a large divergence from the chlorophyta mixture (Figure 6-1), and the C+DOC group. This is reflected in the individual metabolite changes, where there were what appear to be indications of a starvation response or a cessation in metabolism. In this case, there were increases in a number of amino acids and a decrease in glucose and is a response opposite to that seen with the *D. magna* response to *Synechocystis*. Sweeping changes in amino acids, whether as an increase or decrease and opposite to that of glucose, appear to be common in metabolomics studies (Chapter 4; Kovacevic et al., 2016; Wagner et al., 2015) and are likely the expression of an overall change in energetic status.

The role of DOC in the life history of *Daphnia* is contradictory and complex. It is considered a ‘natural xenobiotic’ (Steinberg et al., 2003), having multiple functional groups that can interact with organisms at the biochemical level (Bouchnak and Steinberg, 2010). Specifically, it has been implicated as an oxidative stressor, which is energetically demanding on an organism (Steinberg et al., 2010), as well as having a capacity as an endocrine disruptor (Hofmann et al., 2012). However, the direct uptake of DOC as a food source has also been investigated (Hofmann et al., 2012; Mcmeans et al., 2015; Speas and Duffy, 1998) and there is evidence that this is possible, providing zooplankton with an energy source, even if it is simultaneously a xenobiotic toxin (Hofmann et al., 2012). It has been noted that even DOC can
include colloids or particulate organic carbon that pass through the filtration procedure (Hofmann et al., 2012) and these have been shown to be a food source for *Daphnia* (Brett et al., 2009). However, even if *D. magna* were able to directly assimilate DOC, it is a poor quality carbon source, lacking in FAs and sterols (Martin-Creuzburg et al., 2011; Mcmeans et al., 2015), and containing recalcitrant phenolic elements such as lignin that are resistant to enzyme digestion (Hofmann et al., 2012; Kiikkila et al., 2011). The poor nutritional quality is corroborated by our own FA analysis (Table 6-1), which showed a relative paucity of FAs, relative to the other treatments, though the arachidonic acid and EPA levels are high and comparable to the other carbon sources. Interestingly, the only iso/anteiso SFAs found in our study are in the DOC, and these are generally suggestive of microbial inputs (Kaneda, 1991). The DOC may then have provided as an indirect food source, as bacteria are able to assimilate DOC, and serve as a food source for zooplankton (Brett et al., 2012; Karlsson et al., 2012; Wenzel et al., 2012), though one study did find that mesozooplankton appear to receive little carbon input from bacterial uptake (Van den Meersche et al., 2009). McMeans et al. (2015) found that bacterial growth occurred at 24 hours, at temperatures similar to the ones in our current study and so it is possible that microbial growth may have occurred during the test. Regardless of whether the DOC is able to serve as a food source, either directly or indirectly, the evidence from this study shows either a net reduction in available energy, or a reduced metabolism and stunted growth that is the result of protein catabolism, thus explaining the increase in amino acids, while glucose levels drop off significantly. A similar metabolite response was also seen in Wagner et al. (2015) under low food concentrations, where metabolite changes reflected what was likely to be protein degradation producing amino acids for catabolism. While it is possible that a toxic response is being exerted by DOC, the response is
likely one of starvation that is either the result of the DOC being a poor quality food, whether directly or indirectly. Therefore in a system where autochthonous carbon sources are being replaced by allochthonous carbon sources, *D. magna* are likely deriving little to no sustenance from DOC.

When fed a mixture with the C+DOC, there were no changes seen in the metabolome, except for a minor decrease in tryptophan levels (Figure 6-3). One study observed that antioxidant markers, such as H$_2$O$_2$, free proline and ascorbic acid increased in response to humic substances and this suggests that even in the presence of *R. subcapitata*, there were still signs of antioxidant stress (Steinberg et al., 2010). While our results with only DOC may support the idea that the *D. magna* are stressed, the C+DOC does not suggest this. This may be because the *D. magna* in this experiment were also fed *C. vulgaris* (in addition to *R. subcapitata*) and may have had the energetic capacity to deal with the stress. Therefore, the starvation response seen with only DOC is negated by the presence of the two chlorophyta.

6.5 Conclusions

In this study we demonstrate the use of metabolomics for analyzing the response of *D. magna* to changing carbon quality and composition. The alterations in metabolome incurred by changing a diet to *Synechocystis* or only DOC are indicative of a generalized stress response. This manifested as a change in energy distribution and reflects the lower FA composition of these sources. Specifically, *Synechocystis* appears to result in an increase in energy requirements, as amino acids are decreased and glucose increases, likely as a strategy for producing more energy. In contrast, DOC induces a response that is consistent with starvation and indicates that this allochthonous source is a potentially significant stressor in aquatic environments. Also, though *R. subcapitata* contained more FAs, feeding *D. magna* only this food resulted in
decreases in amino acids and may be less efficiently assimilated than *C. vulgaris*. Even after 48 hours, there are differences in the *D. magna* metabolome as carbon quality changes, which can reflect the environmental fluctuations that can occur in natural systems.
6.6 References


Chapter 7

Conclusions, limitations and future research
7.1 Summary of research

Aquatic organisms face stressors from numerous fronts, whether anthropogenic or natural in origin. In monitoring these aquatic toxicology has been largely based on apical endpoint assays, especially the 48 hour acute toxicity test and 21 day reproduction test (Dang et al., 2012; Stark, 2005). While they are easy to conduct and are the most direct assessment of an organism’s fitness (Dang et al., 2012), they provide no information on biochemical mechanisms especially at sub-lethal levels of stress. As a result, the toxic threat of sub-lethal stress may be underestimated and this necessitates the development of more sensitive assays that examine more than just apical endpoints like mortality and fecundity. The progression from these apical endpoint tests, to singular biomarker assays, to holistic analyses of interconnected biochemical systems is now in progress and metabolomics is part of this modernization of aquatic toxicology.

In this thesis, one aspect of this change was explored and laid a basic framework for using $^1$H nuclear magnetic resonance (NMR)-based metabolomics to provide holistic analyses of *Daphnia magna* responses to stressors including contamination exposure and differing carbon sources. The first study was exploratory and tested the *D. magna* response to one of three metal contaminants; arsenic, copper or lithium at a fraction of their acute toxicity values (Chapter 2). This study found a similar response between copper and lithium that was suggestive of impairments in energy production and ionoregulation. This may have been due to the potential for both to interfere with sodium-potassium adenosine triphosphate pumps. Through this study we confirmed that $^1$H NMR-based metabolomics was sensitive enough in detecting perturbations at sub-lethal levels and the method warranted further investigation.

While the experiment in Chapter 2 was successful, *D. magna* culturing was done at the Ministry of Environment and Climate Change, where the resources for mass culturing were
available. In order for *D. magna* metabolomics to be feasible within our own lab, the number of *D. magna* used for study would need to be reduced. For this purpose, the use of a microprobe, which would allow lower mass requirements, was examined and an optimization procedure using a 1.7 mm NMR microprobe was developed (Chapter 3). By using a microprobe, a mass limited sample such as *D. magna* could be used with greater parsimony. Extraction procedures were tested for metabolite yield and also the best signal to noise ratio. It was concluded that a deuterated buffer extract yielded the best and most consistent metabolite profiles and could use a minimal mass of 1 mg; this latter point being important given the sample limitations in using an organism as small as *D. magna* (around 0.2 mg per individual). After developing an optimized method, the toxicity of two presumably similarly functioning organophosphates (OPs) as well as the suspected endocrine disruptor, bisphenol-A (BPA) were studied over 48 hours to a gradient of sub-lethal exposures (Chapter 4). It was important to test dose dependant responses, as they are normally assumed to be linear with exposure concentration. However, what was found in this study was that this was not necessarily so. Instead it was found that there were two major ways in which stress could be manifested, corresponding to the degree of stress. Moderate stress was identified as a compensatory energy strategy, where amino acids were decreased and likely used in gluconeogenesis, as glucose was seen to increase. This was in an effort to deal with the stress incurred by the toxic threat. As concentrations increased further, there was evidence of a general cessation in metabolism, as a number of amino acids increased and glucose decreased. This appeared to characterize a more severe stress. Through this study we were able to confirm the hypothesis that two similarly acting compounds (the two OPs) would induce a similar response, when compared to BPA. However, the greater importance of these findings is that the general stress response seen here could be delineated by the severity of the stressor. These were patterns
also observed in subsequent studies (Chapter 5). For example, in the chronic toxicity test examining BPA toxicity over 21 days (Chapter 5), at 14 days of exposure a response indicating a moderate stress response is seen, as amino acids decreased and glucose increased. However, the hypothesis in this study, that there would be increasing toxicity as exposure duration increased, could not be confirmed, with evidence instead of an adaptation response by day 21, as at this point there was no difference between exposed and control conditions. Also, the hypothesis that the ecdysone axis would be altered could not be proven as 20-hydroxyecdysone levels remained at the same levels as the controls, suggesting that BPA does not act on the endocrine axis of *D. magna*.

Finally, the response of *D. magna* to differing carbon sources, whether they are autochthonous or allochthonous in origin, was examined (Chapter 6). The response of *D. magna* to a cyanobacterium (*Synechocystis* spp.) and dissolved organic carbon (DOC) could be clearly distinguished from the chlorophyta fed conditions (the autochthonous carbon sources) and showed the most dramatic changes. However, the specific responses between the two were very different. The DOC condition (representing an allochthonous carbon source) showed a starvation response, where protein catabolism resulted in relative increases in amino acids and glucose depletion. *D. magna* switched to a cyanobacteria carbon source showed signs of toxic stress and an energetic compensation that manifested as the opposite response, where amino acids were depleted and likely used as a gluconeogenesis substrate. This is similar to the response seen in moderate OP exposures in Chapter 4. While it was hypothesized that *D. magna* would respond to DOC and cyanobacteria by showing these compensatory energy strategies, this could only be seen in the cyanobacteria condition.
When the metabolome responses are looked at as a whole, what is seen is a consistent response through the studies in Chapters 4 to 6; that is, there appear to be two major modes in which stress manifests in the *D. magna* metabolome, and as suggested in Chapter 4, this reflects the severity of the stress response. It is these overall changes in metabolite profile that are able to provide insight into the mode of action, and would be missed when looking at the metabolome as discrete components, rather than on a whole. Overall, the work undertaken in this thesis has been able to demonstrate that there are biochemical changes that can be detected at sub-lethal levels using $^1$H NMR-based metabolomics, and that these are largely related to alterations in energy production. These show changes that would otherwise not be evident in an apical endpoint test such as the 48 hour acute toxicity and 21 day reproduction tests and demonstrate the potential of metabolomics as a more sensitive and information rich test.

### 7.2 Limitations of the current work and future directions in metabolomics

While the main focus of this thesis work was to develop the use of $^1$H NMR-based metabolomics to study aquatic environment stressors, there remain a number of limitations to the studies performed. As will be discussed, the limitations in metabolomics studies also implicitly indicate how future metabolomics studies will change in order to provide the best ecotoxicity information.

#### 7.2.1 Studies were restricted to one analytical platform

The analytical platform used in these studies was NMR and this was because NMR confers a number of advantages over mass spectrometry (MS) based methods (as outlined in Chapter 1). However, in spite of these advantages, it remains that the full scope of the
metabolome cannot be captured with just one analytical modality. For this reason, future studies will need to include other platforms in order to capture the larger metabolome picture. While NMR is able to provide a broad overview of metabolites such as amino acids and sugars (Viant, 2008), there are other biochemical pathways in *D. magna* that need to be examined and understood. For example, neurotransmitters such as acetylcholine, gamma-aminobutyric acid and various other aminergic signaling metabolites are crucial to the function of crustaceans (McCoole et al., 2012a; 2012b), yet are not evident in an NMR spectrum. A similar case is presented when considering important elements in the crustacean endocrine system, such as ecdysteroids and methyl farnesoate (Mu et al., 2005). For this, more sensitive targeted technology would need to be used to complement what is seen using NMR. This would include MS based methods coupled to liquid chromatography or gas chromatography (GC), in spite of their inherent disadvantages such as the extra time needed in sample preparation (discussed in Chapter 1). The work to include an analysis of an important ecdysteroid in Chapter 5 was the first step in this direction, but there will need to be more targeted components added in order to provide a more comprehensive characterization of the changing metabolome. This will also require further efforts in developing extraction and optimization procedures on MS-based instrumentation. There are studies that use NMR combined with MS methods, though they are few in number compared to the rest of the NMR based studies (Booth et al., 2011; Dove et al., 2012; Jones et al., 2012; Southam et al., 2011; Spann et al., 2011). For example, the metabolome of the nematode *Caenorhabditis elegans* to nickel and chloropyrifos was analyzed with NMR and GC-MS, with GC-MS being able to analyze an organic extract to detect various fatty acids (Jones et al., 2012) and thus being able to provide a more comprehensive metabolite profile.
Though this adds more work to an experiment and detracts from the high-throughput ambitions of metabolomics, it provides the most comprehensive metabolomics data.

7.2.2 Focusing specifically on metabolomics inherently excludes other omics

Another limitation in the studies was in the specific use of metabolomics, where experiments that employ singular ‘omics’, be it genomics, proteomics or metabolomics, will not capture the entire picture of organismal change. Therefore the techniques will need to be combined to provide an even more holistic analysis (Keum et al., 2010). This can serve two roles: to provide a better overview of the changes incurred by stress but also to validate the response of the biomarkers seen in metabolomics studies. The latter is especially important, as metabolomics is able to generate many hypotheses but require further investigation for validation purposes. In *D. magna* studies the combination of metabolomics and transcriptomics has been done by Vandenbrouck et al. (2011) who examined the response of *D. magna* to sub-lethal levels of pyrene, fluoranthene or a combination of both. In this study, while metabolomics could distinguish changes that were suggestive of alterations in aminosugar metabolism, transcriptomics also provided information on cuticula protein fragments, which could be related to changes in molting and ecdysone regulation (Vandenbrouck et al., 2010). Thus, the two omics were able to complement each other to provide additional information that would otherwise be missed with a singular omics. This use of multiple omics contributes to what is referred to as systems toxicology, where instead of looking at a few select biochemical parameters an organism is viewed as being comprised of interacting networks (Garcia-Reyero and Perkins, 2011). However, the use of other omics and other analytical platforms does pose inherent problems, namely that it will require a multidisciplinary team of scientists drawing from a wide breadth of backgrounds and resources and is likely not feasible for a single laboratory to do. Nevertheless, it
will become incumbent upon aquatic toxicologists to have a greater incorporation of holistic techniques in order to produce the most accurate overview of organism responses.

7.2.3 Much of the biochemistry of *Daphnia magna* remains unknown

One of the challenges in metabolomics lies within the limited knowledge of biochemical pathways (Biales et al., 2015). While this lack of knowledge is a limitation of the studies in this thesis, it is a limitation of the field of aquatic toxicology in general. This biochemistry becomes the requisite knowledge base that makes metabolomics a stronger tool. While resources such as the Kyoto Encyclopedia of Genes and Genomes database (Kanehisa et al., 2014) allow for a consolidated knowledge pool of molecular pathways, there remains much that is still not understood specifically about crustacean biochemistry. This is compounded by the fact that many studies and databases are still based on mammalian (viz. human) metabolites (Bundy et al., 2009). The situation is being helped by the genome mining occurring with the closely related *D. pulex* (McCoole et al., 2012a; 2012b) and the homologies are useful in *D. magna* studies as they provide some important biochemical background to help better understand the responses seen in *D. magna* studies. There are for example, invertebrate specific pathways such as the octopaminergic and ecdysone pathways that would be missed if only mammalian metabolites were looked at. Further metabolomics studies will help in being able to link these metabolites to various pathways and still requires much work.

7.2.4 Studies need to be within the context of environmental conditions

In an effort to use concentrations that would be able to examine sub-lethal toxicity, *D. magna* exposures were not always at levels seen in the environment and present another potential limitation of the studies performed. This is particularly the case with BPA, for which the
concentration causing 50% mortality to a population (LC\(_{50}\)) was high, and therefore the concentration BPA was tested at was in the low mg/L range (Chapters 4 and 5). This was in order to remain at a fraction of the LC\(_{50}\) that was consistent with those used with the OPs, which were tested at concentrations more in line with levels found in the environment (Scholz et al., 2000; Sparling and Fellers, 2007). Though one study has measured BPA levels up to 17.2 mg/L (Yamamoto et al., 2001), this was in landfill leachate and is an exception. More realistic BPA levels are instead in the ng/L range (Crain et al., 2007). Future experiments will then need to be mindful of also examining concentrations reflective of what is found in the environment.

An extension to this is that in examining environmentally relevant concentrations that other environmental conditions will also need to be accounted for as well. One of these is the mixture of contaminants, as contaminants exist with others and it remains unknown whether contamination is additive, which is what is generally assumed under the toxic unit concept (Segner, 2011). In addition to this, the actual contaminants used in tests need to be broadened to examine the myriad of emerging contaminants that have little toxicological knowledge. For example, for all the attention given to BPA, the other bisphenol compounds that have replaced BPA have received much less attention. These include bisphenol-S and bisphenol-F, which have been shown to be unsafe alternatives in mammalian studies (Eladak et al., 2015). However, the toxicity of these to aquatic species is largely unexplored and presents an opportunity for examination using metabolomics.
7.3 Concluding remarks on the current state of metabolomics using

*Daphnia magna*

In addressing these future directions of metabolomics, it is clear then that it will serve two purposes. The first is from the point of view of applied science, where it can be used in environmental monitoring and risk assessment programs. This still requires extensive study and validation before it can be accepted as part of these programs (Van Aggelen et al., 2010) but is the major role that metabolomics will play. However, there is still much that remains unclear about crustacean biochemistry, and thus raises the second role that metabolomics will serve; that is in the study of the biochemistry of crustaceans and to be able to link pathways with each other. This will allow for a greater understanding of the basic mechanisms by which organisms respond to stress, but also give greater insight into the natural history of an aquatic invertebrate.

In aggregate, the use of *D. magna* in metabolomics studies can no longer be described as nascent, and much progress has been made since even the beginning of this thesis. At the beginning of this work, there were only four studies using *D. magna* in metabolomics studies (Poynton et al., 2011; Taylor et al., 2009; 2010; Vandenbrouck et al., 2010), but in the past five years a number of studies from other researchers have emerged and added to the body of *D. magna* metabolomics (Kovacevic et al., 2016; Li et al., 2015; Taylor et al., 2016a; 2016b; Wagner et al., 2015). What is increasingly being seen are the changes suggested within this thesis; that metabolomics will be incorporating other analytical modalities and other omics sciences to offset the deficiencies that they may have alone. Also, there will be greater incorporation of environmental factors as has been explored by Taylor et al. (2016b) who explored the amelioration of copper toxicity by dissolved organic matter. With this further work it is possible then that metabolomics, as well as the other omics in spite of the inherent costs
associated with this, can gain greater acceptance into environmental risk assessment and monitoring regimes.
7.4 References


Appendix A

Supplementary material for chapter 2


(modified to include an analysis of variance (ANOVA) with a Bonferroni posthoc test, which replaces the multiple student $t$-tests used in calculating statistical significance for both the principal component analysis (PCA) and the metabolite percent changes. Text in the method and results sections has been modified to account for these changes. The in house R script used in the ANOVA has been included in Appendix A, as well as the results of the Bonferroni posthoc test).
A2-1: R script used for performing the analysis of variance with a Bonferroni posthoc test

```r
#read datafile into R
metab.df <- read.csv('ANOVA.csv')

#create a list of metabolite names from the columns of the dataframe
metabolites <- names(metab.df)[2:length(names(metab.df))]

#empty variables that all results will be appended to, to create a collated table of results
anova.results <- data.frame()
mPtt <- matrix()

#create a loop through the list of metabolites to produce ANOVA, Bonferroni results
for(i in 1:length(metabolites)){
  ##ANOVA Results:
  #On first run through loop: fit a linear model of metabolite[1] as a function of Treatment
  fit <- lm(metab.df[[metabolites[i]]] ~ metab.df$Treatment)

  #Produce ANOVA on the fit
  anova.res <- anova(fit)

  #Create a temporary dataframe with the relevant outputs from the ANOVA
  temp.results <- data.frame(Metabolite = metabolites[i], Df.1 = anova.res[[1]][1],
                             Df.2 = anova.res[[1]][2], F.value = anova.res[[4]][1],
                             p.value = anova.res[[5]][1])

  #Append the results to the final dataframe
  anova.results <- rbind(anova.results, temp.results)

  ##Post-Hoc Pairwise T-test with Bonferroni Correction
  #create a temporary dataframe of the results from the pairwise t-test
  ptt <- data.frame(pairwise.t.test(metab.df[[metabolites[i]]], metab.df$Treatment, p.adj = "bonf")[[3]])

  #the output of p-values from the pairwise t-test is a lower triangular matrix
  #to make it comparable to the output of the TukeyHSD the output is manipulated.
  #each column of values is appended and stored in v
  ptt.temp.pval <- vector()
  for (j in 1:length(ptt)){
    ptt.temp.pval <- c(ptt.temp.pval, ptt[[j]])
  }

  #append those results to the collated results
  mPtt[metabolites[i]] <- data.frame(ptt.temp.pval)
}
```
# changed to dataframes and give each row a name corresponding to each pairwise comparison
ptt.rownames <- vector()
for (i in 1:length(names(ptt))) {
    rn <- paste(row.names(ptt), names(ptt[i]), sep = '\-')
    ptt.rownames <- c(ptt.rownames, rn)
}

Ptt.bonferroni.results <- data.frame(mPtt[2:length(mPtt)], row.names = ptt.rownames)
Ptt.bonferroni.fin <- subset(Ptt.bonferroni.results, complete.cases(Ptt.bonferroni.results))

# export the results as .csv files
write.csv(anova.results, 'ANOVA Results.csv')
write.csv(Ptt.bonferroni.fin, 'ANOVA Bonferroni Results.csv')
Table A2-1. A list of p values from the analysis of variance with Bonferroni posthoc tests of the principal component analysis scores values. Only conditions that are tested against the control are shown.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>PC1</th>
<th>PC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>control-arsenic</td>
<td>$4.12 \times 10^{-1}$</td>
<td>1</td>
</tr>
<tr>
<td>control-copper</td>
<td>$3.75 \times 10^{-8}$</td>
<td>$6.88 \times 10^{-2}$</td>
</tr>
<tr>
<td>control-lithium</td>
<td>$2.65 \times 10^{-5}$</td>
<td>1</td>
</tr>
</tbody>
</table>

Table A2-2. Analysis of variance results for each metabolite that was identified from the $^1$H NMR spectra for differing metal exposures against the control.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Df = 1</th>
<th>Df = 2</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>3</td>
<td>28</td>
<td>7.25</td>
<td>$9.54 \times 10^{-4}$</td>
</tr>
<tr>
<td>Uracil</td>
<td>3</td>
<td>28</td>
<td>27.35</td>
<td>$1.80 \times 10^{-8}$</td>
</tr>
<tr>
<td>Glycine</td>
<td>3</td>
<td>28</td>
<td>6.52</td>
<td>$1.75 \times 10^{-3}$</td>
</tr>
<tr>
<td>GPC</td>
<td>3</td>
<td>28</td>
<td>11.94</td>
<td>$3.26 \times 10^{-5}$</td>
</tr>
<tr>
<td>Lysine</td>
<td>3</td>
<td>28</td>
<td>7.69</td>
<td>$6.73 \times 10^{-4}$</td>
</tr>
<tr>
<td>Glutamine</td>
<td>3</td>
<td>28</td>
<td>12.17</td>
<td>$2.82 \times 10^{-5}$</td>
</tr>
<tr>
<td>Glutamate</td>
<td>3</td>
<td>28</td>
<td>21.22</td>
<td>$2.26 \times 10^{-7}$</td>
</tr>
<tr>
<td>Methionine</td>
<td>3</td>
<td>28</td>
<td>17.19</td>
<td>$1.59 \times 10^{-6}$</td>
</tr>
<tr>
<td>Alanine</td>
<td>3</td>
<td>28</td>
<td>116.92</td>
<td>$6.09 \times 10^{-16}$</td>
</tr>
<tr>
<td>Threonine</td>
<td>3</td>
<td>28</td>
<td>6.68</td>
<td>$1.52 \times 10^{-3}$</td>
</tr>
<tr>
<td>Valine</td>
<td>3</td>
<td>28</td>
<td>7.18</td>
<td>$1.01 \times 10^{-3}$</td>
</tr>
<tr>
<td>Leucine</td>
<td>3</td>
<td>28</td>
<td>6.85</td>
<td>$1.33 \times 10^{-3}$</td>
</tr>
</tbody>
</table>
Table A2-3. P values from the Bonferroni posthoc tests conducted on the analysis of variance from Table A2-2 for differing metal exposures against the control.

<table>
<thead>
<tr>
<th></th>
<th>Phenylalanine</th>
<th>Uracil</th>
<th>Glycine</th>
<th>GPC</th>
<th>Lysine</th>
<th>Glutamine</th>
<th>Glutamate</th>
<th>Methionine</th>
<th>Alanine</th>
<th>Threonine</th>
<th>Valine</th>
<th>Leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>control-arsenic</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>7.08 x 10^{-1}</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3.94 x 10^{-3}</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>control-copper</td>
<td>2.07 x 10^{-2}</td>
<td>7.58 x 10^{-7}</td>
<td>1.07 x 10^{-2}</td>
<td>1.15 x 10^{-3}</td>
<td>4.22 x 10^{-2}</td>
<td>3.50 x 10^{-4}</td>
<td>6.89 x 10^{-6}</td>
<td>6.26 x 10^{-6}</td>
<td>5.73 x 10^{-16}</td>
<td>2.13 x 10^{-2}</td>
<td>1.21 x 10^{-2}</td>
<td>1.18 x 10^{-2}</td>
</tr>
<tr>
<td>control-lithium</td>
<td>5.98 x 10^{-2}</td>
<td>4.14 x 10^{-6}</td>
<td>6.13 x 10^{-2}</td>
<td>8.05 x 10^{-4}</td>
<td>7.22 x 10^{-1}</td>
<td>1.03 x 10^{-1}</td>
<td>2.69 x 10^{-5}</td>
<td>4.59 x 10^{-3}</td>
<td>1.20 x 10^{-8}</td>
<td>1</td>
<td>3.13 x 10^{-1}</td>
<td>3.24 x 10^{-1}</td>
</tr>
</tbody>
</table>
Appendix B

Supplementary material for chapter 3


Figure A3-1: Loadings plot for Principal Component Analysis (PCA) scores (PCA scores plot based on spectra from the three extraction procedures) indicating which chemical shifts are indicative of the variation between samples.
Figure A3-2: $^1$H NMR spectrum for a D$_2$O-buffer extracted single daphnid (dry mass = approximately 0.2 mg).
Figure A3-3: $^1$H NMR spectrum of D$_2$O-buffer extracted daphnia neonates (less than 48 hours old). 60 neonates account for approximately 1 mg, the equivalent of 8-10 adults (over 2 weeks old).
**Figure A3-4:** Comparison of $^1$H-$^{13}$C HSQC NMR assignments observed versus those obtained from the Bruker Biofluid Reference Compound Database.
Appendix C

Supplementary material for chapter 4

Published as: Nagato, E.G., Simpson, A.J., Simpson, M.J. 2016. Metabolomics reveals energetic impairments in *Daphnia magna* exposed to diazinon, malathion and bisphenol-A. Aquatic Toxicology. 170: 175-186

(Modified to include an ANOVA with a Bonferroni posthoc test, to replace the multiple *t*-tests and false discovery rate that were used in calculating statistical significance in the PCA and metabolite percent changes. Text in the methods and results sections has been modified to reflect these changes. Figures 4-1, 4-2, 4-3, 4-4 have also been modified to reflect the changes in statistical significance after the ANOVA and Bonferroni posthoc test. Results of the ANOVA and Bonferroni posthoc test have also been included in Appendix C).

Figure A4-1. Loadings plots from PCAs for Diazinon (A), Malathion (B) and Bisphenol-A (C). Percentage of explained variance are in parentheses. Numbers indicate the spectral region corresponding to a particular metabolite, identified by the labels.
Table A4-1. A list of p values from the analysis of variance with a Bonferroni posthoc test of the principal component analysis scores values. Only diazinon exposure concentrations tested against the control are shown.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>PC1</th>
<th>PC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-0.009µg/L</td>
<td>3.76 x 10^{-2}</td>
<td>1</td>
</tr>
<tr>
<td>Control-0.0225µg/L</td>
<td>1.02 x 10^{-4}</td>
<td>1</td>
</tr>
<tr>
<td>Control-0.045µg/L</td>
<td>3.22 x 10^{-3}</td>
<td>5.73 x 10^{-1}</td>
</tr>
<tr>
<td>Control-0.09µg/L</td>
<td>1.75 x 10^{-1}</td>
<td>8.70 x 10^{-3}</td>
</tr>
<tr>
<td>Control-0.135µg/L</td>
<td>5.68 x 10^{-8}</td>
<td>2.80 x 10^{-5}</td>
</tr>
</tbody>
</table>

Table A4-2. A list of p values from the analysis of variance with a Bonferroni posthoc test of the principal component analysis scores values. Only malathion exposure concentrations tested against the control are shown.

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<tr>
<th>Treatments</th>
<th>PC1</th>
<th>PC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-0.03µg/L</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Control-0.08µg/L</td>
<td>7.35 x 10^{-4}</td>
<td>7.83 x 10^{-1}</td>
</tr>
<tr>
<td>Control-0.16µg/L</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Control-0.32µg/L</td>
<td>7.92 x 10^{-1}</td>
<td>8.35 x 10^{-1}</td>
</tr>
<tr>
<td>Control-0.47µg/L</td>
<td>1.27 x 10^{-1}</td>
<td>1</td>
</tr>
</tbody>
</table>

Table A4-3. A list of p values from the analysis of variance with a Bonferroni posthoc test of the principal component analysis scores values. Only bisphenol-A exposure concentrations tested against the control are shown.

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<tr>
<th>Treatments</th>
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<th>PC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-0.1 mg/L</td>
<td>1</td>
<td>2.18 x 10^{-1}</td>
</tr>
<tr>
<td>Control-0.35mg/L</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Control-0.7 mg/L</td>
<td>4.99 x 10^{-2}</td>
<td>1</td>
</tr>
<tr>
<td>Control-1.4 mg/L</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Control-2.1 mg/L</td>
<td>8.16 x 10^{-4}</td>
<td>1</td>
</tr>
</tbody>
</table>
Table A4-4. Analysis of variance results for each metabolite that was identified from the $^1$H NMR spectra for diazinon exposures.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Df = 1</th>
<th>Df = 2</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>5</td>
<td>54</td>
<td>17.33</td>
<td>3.36 x 10^{-10}</td>
</tr>
<tr>
<td>Leucine</td>
<td>5</td>
<td>54</td>
<td>35.85</td>
<td>5.39 x 10^{-16}</td>
</tr>
<tr>
<td>Valine</td>
<td>5</td>
<td>54</td>
<td>7.89</td>
<td>1.23 x 10^{-5}</td>
</tr>
<tr>
<td>Threonine</td>
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<td>54</td>
<td>7.44</td>
<td>2.28 x 10^{-5}</td>
</tr>
<tr>
<td>Alanine</td>
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<td>54</td>
<td>98.37</td>
<td>7.26 x 10^{-26}</td>
</tr>
<tr>
<td>Arginine</td>
<td>5</td>
<td>54</td>
<td>68.16</td>
<td>4.30 x 10^{-22}</td>
</tr>
<tr>
<td>Methionine</td>
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<td>54</td>
<td>20.05</td>
<td>2.99 x 10^{-11}</td>
</tr>
<tr>
<td>Glutamate</td>
<td>5</td>
<td>54</td>
<td>7.18</td>
<td>3.24 x 10^{-5}</td>
</tr>
<tr>
<td>Glutamine</td>
<td>5</td>
<td>54</td>
<td>38.76</td>
<td>1.08 x 10^{-16}</td>
</tr>
<tr>
<td>Asparagine</td>
<td>5</td>
<td>54</td>
<td>34.99</td>
<td>8.82 x 10^{-16}</td>
</tr>
<tr>
<td>Lysine</td>
<td>5</td>
<td>54</td>
<td>29.48</td>
<td>2.65 x 10^{-14}</td>
</tr>
<tr>
<td>Glycine</td>
<td>5</td>
<td>54</td>
<td>5.65</td>
<td>2.91 x 10^{-4}</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
<td>54</td>
<td>202.56</td>
<td>1.09 x 10^{-33}</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5</td>
<td>54</td>
<td>18.14</td>
<td>1.60 x 10^{-10}</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5</td>
<td>54</td>
<td>58.56</td>
<td>1.38 x 10^{-20}</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>5</td>
<td>54</td>
<td>10.30</td>
<td>5.70 x 10^{-07}</td>
</tr>
</tbody>
</table>
Table A4-5. P values from the Bonferroni posthoc tests conducted on the analysis of variance from Table A4-4 for diazinon exposures. Only values for exposure concentrations against the control are shown.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control-0.009µg/L</th>
<th>Control-0.0225µg/L</th>
<th>Control-0.045µg/L</th>
<th>Control-0.09µg/L</th>
<th>Control-0.135µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>4.68 x 10^{-1}</td>
<td>3.84 x 10^{-2}</td>
<td>1.82 x 10^{-2}</td>
<td>8.31 x 10^{-2}</td>
<td>2.54 x 10^{-2}</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1</td>
<td>1.50 x 10^{-1}</td>
<td>3.33 x 10^{-3}</td>
<td>5.98 x 10^{-2}</td>
<td>2.49 x 10^{-1}</td>
</tr>
<tr>
<td>Glucose</td>
<td>1</td>
<td>1.88 x 10^{-8}</td>
<td>1</td>
<td>4.68 x 10^{-5}</td>
<td>2.13 x 10^{-2}</td>
</tr>
<tr>
<td>Lactate</td>
<td>5.20 x 10^{-2}</td>
<td>2.59 x 10^{-5}</td>
<td>5.60 x 10^{-3}</td>
<td>2.45 x 10^{-1}</td>
<td>4.25 x 10^{-7}</td>
</tr>
<tr>
<td>Glycine</td>
<td>1</td>
<td>1</td>
<td>1.91 x 10^{-4}</td>
<td>2.37 x 10^{-4}</td>
<td>7.27 x 10^{-10}</td>
</tr>
<tr>
<td>Lysine</td>
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<td>1</td>
<td>2.38 x 10^{-3}</td>
<td>4.81 x 10^{-3}</td>
<td>1</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1.53 x 10^{-3}</td>
<td>8.01 x 10^{-4}</td>
<td>7.73 x 10^{-5}</td>
<td>5.0 x 10^{-3}</td>
<td>4.87 x 10^{-4}</td>
</tr>
<tr>
<td>Glutamate</td>
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<td>1.03 x 10^{-1}</td>
<td>5.65 x 10^{-4}</td>
<td>1.06 x 10^{-1}</td>
<td>1</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.73 x 10^{-3}</td>
<td>1.02 x 10^{-6}</td>
<td>6.48 x 10^{-3}</td>
<td>1.02 x 10^{-4}</td>
<td>4.34 x 10^{-10}</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.85 x 10^{-1}</td>
<td>1</td>
<td>9.21 x 10^{-3}</td>
<td>2.69 x 10^{-4}</td>
<td>1.84 x 10^{-3}</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.35 x 10^{-2}</td>
<td>5.00 x 10^{-3}</td>
<td>2.89 x 10^{-3}</td>
<td>1.19 x 10^{-5}</td>
<td>4.28 x 10^{-7}</td>
</tr>
<tr>
<td>Valine</td>
<td>6.79 x 10^{-2}</td>
<td>2.54 x 10^{-4}</td>
<td>3.08 x 10^{-4}</td>
<td>1.07 x 10^{-2}</td>
<td>1.12 x 10^{-9}</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.59 x 10^{-3}</td>
<td>1.68 x 10^{-4}</td>
<td>1.68 x 10^{-4}</td>
<td>1.68 x 10^{-4}</td>
<td>1.68 x 10^{-4}</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.43 x 10^{-3}</td>
<td>1.43 x 10^{-3}</td>
<td>1.43 x 10^{-3}</td>
<td>1.43 x 10^{-3}</td>
<td>1.43 x 10^{-3}</td>
</tr>
</tbody>
</table>
Table A4-6. Analysis of variance results for each metabolite that was identified from the $^1$H NMR spectra for malathion exposures.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Df = 1</th>
<th>Df = 2</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>5</td>
<td>50</td>
<td>17.87</td>
<td>3.99 x 10^{-10}</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5</td>
<td>50</td>
<td>12.43</td>
<td>7.40 x 10^{-8}</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
<td>50</td>
<td>3.77</td>
<td>5.69 x 10^{-3}</td>
</tr>
<tr>
<td>Lactate</td>
<td>5</td>
<td>50</td>
<td>8.82</td>
<td>4.75 x 10^{-6}</td>
</tr>
<tr>
<td>Glycine</td>
<td>5</td>
<td>50</td>
<td>11.00</td>
<td>3.57 x 10^{-7}</td>
</tr>
<tr>
<td>Lysine</td>
<td>5</td>
<td>50</td>
<td>5.48</td>
<td>4.27 x 10^{-4}</td>
</tr>
<tr>
<td>Glutamine</td>
<td>5</td>
<td>50</td>
<td>8.04</td>
<td>1.27 x 10^{-5}</td>
</tr>
<tr>
<td>Glutamate</td>
<td>5</td>
<td>50</td>
<td>10.24</td>
<td>8.47 x 10^{-7}</td>
</tr>
<tr>
<td>Arginine</td>
<td>5</td>
<td>50</td>
<td>11.09</td>
<td>3.22 x 10^{-7}</td>
</tr>
<tr>
<td>Alanine</td>
<td>5</td>
<td>50</td>
<td>25.94</td>
<td>8.19 x 10^{-13}</td>
</tr>
<tr>
<td>Threonine</td>
<td>5</td>
<td>50</td>
<td>1.94</td>
<td>1.04 x 10^{-1}</td>
</tr>
<tr>
<td>Valine</td>
<td>5</td>
<td>50</td>
<td>20.18</td>
<td>5.81 x 10^{-11}</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5</td>
<td>50</td>
<td>21.08</td>
<td>2.83 x 10^{-11}</td>
</tr>
<tr>
<td>Leucine</td>
<td>5</td>
<td>50</td>
<td>14.41</td>
<td>9.77 x 10^{-9}</td>
</tr>
</tbody>
</table>
Table A4-7. P values from the Bonferroni posthoc tests conducted on the analysis of variance from Table A4-6 for malathion exposures. Only values for exposure concentrations against the control are shown.

<table>
<thead>
<tr>
<th></th>
<th>Phenylnalanine</th>
<th>Tyrosine</th>
<th>Glucose</th>
<th>Lactate</th>
<th>Glycine</th>
<th>Lysine</th>
<th>Glutamine</th>
<th>Glutamate</th>
<th>Arginine</th>
<th>Alanine</th>
<th>Threonine</th>
<th>Valine</th>
<th>Isoleucine</th>
<th>Leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-0.03µg/L</td>
<td>9.12x 10^4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2.73 x 10^-1</td>
<td>3.85 x 10^-2</td>
<td>1</td>
<td>5.59 x 10^-1</td>
<td>1.34 x 10^-1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Control-0.08µg/L</td>
<td>5.92 x 10^-6</td>
<td>1.19 x 10^-4</td>
<td>3.24 x 10^-1</td>
<td>3.24 x 10^-1</td>
<td>1.55 x 10^-3</td>
<td>9.70 x 10^-6</td>
<td>2.45 x 10^-3</td>
<td>2.42 x 10^-4</td>
<td>9.97 x 10^-4</td>
<td>1.86 x 10^-7</td>
<td>6.53 x 10^-1</td>
<td>6.73 x 10^-5</td>
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<td>1.52 x 10^-5</td>
</tr>
<tr>
<td>Control-0.16µg/L</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Control-0.32µg/L</td>
<td>1</td>
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<td>1</td>
<td>9.17 x 10^-1</td>
<td>7.75 x 10^-1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2.60 x 10^-1</td>
<td>1.45 x 10^-1</td>
</tr>
<tr>
<td>Control-0.47µg/L</td>
<td>1.16 x 10^-4</td>
<td>3.41x 10^-4</td>
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<td>5.17 x 10^-2</td>
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<td>1</td>
<td>1</td>
<td>9.56 x 10^-1</td>
<td>1</td>
<td>1.34 x 10^-2</td>
<td>1</td>
<td>1.83 x 10^-5</td>
<td>7.04 x 10^-5</td>
<td>7.08 x 10^-1</td>
</tr>
</tbody>
</table>
Table A4-8. Analysis of variance results for each metabolite that was identified from the $^1$H NMR spectra for bisphenol-A exposures.

<table>
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<tr>
<th>Metabolite</th>
<th>Df = 1</th>
<th>Df = 2</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>5</td>
<td>53</td>
<td>7.49</td>
<td>2.23 x 10^{-5}</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5</td>
<td>53</td>
<td>6.48</td>
<td>9.05 x 10^{-5}</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
<td>53</td>
<td>7.94</td>
<td>1.21 x 10^{-5}</td>
</tr>
<tr>
<td>Lactate</td>
<td>5</td>
<td>53</td>
<td>7.31</td>
<td>2.86 x 10^{-5}</td>
</tr>
<tr>
<td>Glycine</td>
<td>5</td>
<td>53</td>
<td>6.71</td>
<td>6.53 x 10^{-5}</td>
</tr>
<tr>
<td>Lysine</td>
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<td>53</td>
<td>7.00</td>
<td>4.38 x 10^{-5}</td>
</tr>
<tr>
<td>Glutamine</td>
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<td>53</td>
<td>7.07</td>
<td>3.96 x 10^{-5}</td>
</tr>
<tr>
<td>Glutamate</td>
<td>5</td>
<td>53</td>
<td>8.81</td>
<td>3.91 x 10^{-6}</td>
</tr>
<tr>
<td>Arginine</td>
<td>5</td>
<td>53</td>
<td>7.93</td>
<td>1.24 x 10^{-5}</td>
</tr>
<tr>
<td>Alanine</td>
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<td>53</td>
<td>6.31</td>
<td>1.16 x 10^{-4}</td>
</tr>
<tr>
<td>Threonine</td>
<td>5</td>
<td>53</td>
<td>4.60</td>
<td>1.47 x 10^{-3}</td>
</tr>
<tr>
<td>Valine</td>
<td>5</td>
<td>53</td>
<td>7.47</td>
<td>2.28 x 10^{-5}</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5</td>
<td>53</td>
<td>9.50</td>
<td>1.64 x 10^{-6}</td>
</tr>
<tr>
<td>Leucine</td>
<td>5</td>
<td>53</td>
<td>9.27</td>
<td>2.18 x 10^{-6}</td>
</tr>
</tbody>
</table>
Table A4-9. P values from the Bonferroni posthoc tests conducted on the analysis of variance from Table A4-8. Only values for exposure concentrations against the control are shown.

<table>
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<th></th>
<th>Phenylalanine</th>
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<th>Glucose</th>
<th>Lactate</th>
<th>Glycine</th>
<th>Lysine</th>
<th>Glutamine</th>
<th>Glutamate</th>
<th>Arginine</th>
<th>Alanine</th>
<th>Threonine</th>
<th>Valine</th>
<th>Isoleucine</th>
<th>Leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>8.69 x 10^{-1}</td>
<td>1</td>
<td>9.45 x 10^{-2}</td>
<td>3.72 x 10^{-2}</td>
<td>1</td>
<td>1</td>
<td>3.04 x 10^{-2}</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.1mg/L</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>7.92 x 10^{-1}</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.35mg/L</td>
<td>1.19 x 10^{-1}</td>
<td>3.35 x 10^{-1}</td>
<td>1.89 x 10^{-1}</td>
<td>8.98 x 10^{-1}</td>
<td>1</td>
<td>2.63 x 10^{-1}</td>
<td>1</td>
<td>6.67 x 10^{-2}</td>
<td>2.06 x 10^{-2}</td>
<td>1</td>
<td>4.66 x 10^{-1}</td>
<td>3.44 x 10^{-1}</td>
<td>1.30 x 10^{-3}</td>
<td></td>
</tr>
<tr>
<td>0.7mg/L</td>
<td>1.12 x 10^{-2}</td>
<td>1.23 x 10^{-3}</td>
<td>1.12 x 10^{-3}</td>
<td>1.09 x 10^{-2}</td>
<td>1</td>
<td>3.08 x 10^{-2}</td>
<td>1</td>
<td>1.23 x 10^{-1}</td>
<td>3.28 x 10^{-2}</td>
<td>1.01 x 10^{-2}</td>
<td>3.03 x 10^{-2}</td>
<td>1</td>
<td>2.41 x 10^{-3}</td>
<td>1</td>
</tr>
<tr>
<td>1.4mg/L</td>
<td>2.35 x 10^{-3}</td>
<td>1.12 x 10^{-2}</td>
<td>2.23 x 10^{-3}</td>
<td>3.54 x 10^{-3}</td>
<td>1.09 x 10^{-2}</td>
<td>3.08 x 10^{-2}</td>
<td>1</td>
<td>1.23 x 10^{-1}</td>
<td>3.28 x 10^{-2}</td>
<td>1.01 x 10^{-2}</td>
<td>3.03 x 10^{-2}</td>
<td>1</td>
<td>2.41 x 10^{-3}</td>
<td>1</td>
</tr>
<tr>
<td>2.1mg/L</td>
<td>2.35 x 10^{-3}</td>
<td>1.12 x 10^{-2}</td>
<td>2.23 x 10^{-3}</td>
<td>3.54 x 10^{-3}</td>
<td>1.09 x 10^{-2}</td>
<td>3.08 x 10^{-2}</td>
<td>1</td>
<td>1.23 x 10^{-1}</td>
<td>3.28 x 10^{-2}</td>
<td>1.01 x 10^{-2}</td>
<td>3.03 x 10^{-2}</td>
<td>1</td>
<td>2.41 x 10^{-3}</td>
<td>1</td>
</tr>
</tbody>
</table>
Appendix D

Supplementary material for chapter 5
Figure A5.1. A. Representative chromatogram of a methyl farnesoate (MF) standard (of the 251.2 → 191.3 transition ion) that emerges from the column at 3.58 minutes. B. Representative chromatogram of an extract of *Daphnia magna* based on the method of Hirano et al. (2009), with the emergence of possibly an MF conjugate at 1.54 minutes.
A5-1 Methyl farnesoate monitoring using liquid chromatography tandem mass spectrometry

An attempt at monitoring methyl farnesoate (MF) was made with liquid chromatography-tandem mass spectrometry, based on several extraction methods (Hikiba et al., 2013; Westerlund and Hoffmann, 2004; Zhou et al., 2011) and could not definitively identify MF in *Daphnia magna* extracts. While it is possible that MF exists in levels below detection limits (Toyota et al., 2015), the presence of the compound was difficult to discern. Preliminary work with MF standards showed emergence from the column at 3.5 minutes, multiple reaction monitoring of a *D. magna* extract would consistently find a molecule emerging at 1.58 minutes; a retention time shorter than the standard (Figure A5-1). This raises the possibility that MF is forming a conjugate that is causing a quicker emergence from the column. In any case, as there was no ability to definitively identify this as MF.
A5-1 R-script used in processing p-values for a false discovery rate correction based on Benjamini and Hochberg (1995).

```r
## set working directory (wd)<- go to session-> set working directory-> click set working directory-> choose working directory
setwd("~/Desktop")

## add in data set of raw p values. These should be listed as columns with an appropriate heading at the top
rawpvalues<- read.csv("unmodifiedpvalues.csv" )

## call in method type( Benjamini and Hochberg [ "BH"]); there are others as listed below
## other options include:
#
# c("holm", "hochberg", "hommel", "bonferroni", "BH", "BY",
#   "fdr", "none"
#
#method= "BH"

## define length of raw pvalues

## call in column of p values from csv values: i.e. ‘pvalues’
(rawpvalues$pvalues)

## use length command ( length )
## set length count of p value column to arbitrary letter " n"
lengthpval<- length ((rawpvalues$pvalues))

## calculate adjusted p values from [ " BH "] methods
finalpadjusted<-p.adjust(rawpvalues$pvalues, method = "BH", lengthpval)

## rename p value
p<- (rawpvalues$pvalues)

## create plot of just ["BH"] method
plot(p,finalpadjusted, ylab="finalpadjusted", lty = 1,
     main = "P-value adjustments")

## add legend for BH type
legend(0.9,0.8, c("BH"), pch = c(1,2), lty = c(1,2))
```
## create a data frame for p values
## denote by pdf
pdf<-data.frame(p)

## print pdf to console
print (pdf)

## copy and paste pdf into Excel
## create a data frame for finalpadjusted values
## denote by finalp
finalp<-data.frame(finaladjusted)

## print pdf to console
print (finalp)

## copy and paste pdf into excel (bind by column entries)
filemerge <- cbind(p, finaladjusted)

## write over merge file as a data frame
mastermerge<-data.frame(filemerge)

## export mastermerge onto desktop from R studio.
## For this make sure that the xlsx is run beforehand. It won’t always work.
saveexcel<-write.xlsx(mastermerge, "Adjusted P values.xlsx")
Table A5-1. Adjusted p values (based on a false discovery rate transformation) for each of the metabolites detected in the $^1$H NMR spectra.

<table>
<thead>
<tr>
<th>Day</th>
<th>Glucose</th>
<th>Alanine</th>
<th>Threonine</th>
<th>Glutamate</th>
<th>Glutamine</th>
<th>Methionine</th>
<th>Asparagine</th>
<th>Valine</th>
<th>Isoleucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>$2.85 \times 10^{-1}$</td>
<td>$1.43 \times 10^{-1}$</td>
<td>$3.48 \times 10^{-1}$</td>
<td>$2.85 \times 10^{-1}$</td>
<td>$2.85 \times 10^{-1}$</td>
<td>$1.43 \times 10^{-1}$</td>
<td>$2.85 \times 10^{-1}$</td>
<td>$3.90 \times 10^{-1}$</td>
<td>$3.48 \times 10^{-1}$</td>
</tr>
<tr>
<td>7</td>
<td>$6.25 \times 10^{-1}$</td>
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<td>$8.77 \times 10^{-1}$</td>
<td>$8.77 \times 10^{-1}$</td>
<td>$8.77 \times 10^{-1}$</td>
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<td>$5.32 \times 10^{-2}$</td>
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<td>$1.18 \times 10^{-2}$</td>
<td>$2.32 \times 10^{-2}$</td>
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<td>$7.29 \times 10^{-1}$</td>
<td>$7.16 \times 10^{-1}$</td>
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</tbody>
</table>
A5-2 References


Appendix E

Supplementary material for chapter 6
Figure A6-1. An overlay of a sample *Daphnia magna* extract (in black) with the two chlorophyta *Chlorella vulgaris* (in green), *Raphidocelis subcapitata* (in red) and the cyanobacteria *Synechocystis* spp. (in blue). The shown region is between 0.7 and 2.5 ppm of the spectrum and shows that the foods contribute little to the *D. magna* spectra.
Figure A6-2. $^1$H nuclear magnetic resonance spectra for the two chlorophyta and the cyanobacteria indicate that the two chlorophyta bear greater similarity than they do to the cyanobacteria.
Table A6-1. A list of p values from the analysis of variance with Bonferroni posthoc tests of the principal component analysis scores values. Only conditions that are tested against the chlorophyta mixture are shown.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>PC1</th>
<th>PC2</th>
</tr>
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<tr>
<td>Chlorophyta-C+DOC</td>
<td>$6.12 \times 10^{-2}$</td>
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<td>Chlorophyta-C. vulgaris</td>
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</tr>
<tr>
<td>Chlorophyta-Synechocystis</td>
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<td>Chlorophyta-DOC</td>
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<tr>
<td>Chlorophyta-R. subcapitata</td>
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Table A6-2. Analysis of variance results for each metabolite that was identified from the $^1$H NMR spectra for differing carbon sources against the chlorophyta mixture.

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<th>Metabolite</th>
<th>Df = 1</th>
<th>Df = 2</th>
<th>F value</th>
<th>p value</th>
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<tbody>
<tr>
<td>Isoleucine</td>
<td>5</td>
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<td>17.33</td>
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<tr>
<td>Leucine</td>
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<td>54</td>
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<td>Valine</td>
<td>5</td>
<td>54</td>
<td>7.89</td>
<td>$1.23 \times 10^{-5}$</td>
</tr>
<tr>
<td>Threonine</td>
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<td>54</td>
<td>7.44</td>
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<tr>
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<td>54</td>
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<td>7.18</td>
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<tr>
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<tr>
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Table A6-3. P values from the Bonferroni posthoc tests conducted on the analysis of variance from Table A6-2 for differing carbon sources against the chlorophyta mixture.

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<th></th>
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<th>Valine</th>
<th>Threonine</th>
<th>Alanine</th>
<th>Serine</th>
<th>Glutamate</th>
<th>Glutamine</th>
<th>Asparagine</th>
<th>Lysine</th>
<th>Glycine</th>
<th>Glucose</th>
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<th>Phenylalanine</th>
<th>Tryptophan</th>
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