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Metabolomics Reveals Energetic Impairments in *Daphnia magna* Exposed to Diazinon, Malathion and Bisphenol-A

Edward G. Nagato, André J. Simpson, Myrna J. Simpson*

Environmental NMR Centre and Department of Physical and Environmental Sciences,
University of Toronto Scarborough, 1265 Military Trail, Toronto, ON, M1C 1A4, Canada

*Corresponding author: Tel. (416) 287-7234, Fax: (416) 287-7279, e-mail address:

myrna.simpson@utoronto.ca

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Abstract

¹H 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 ¹H 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 at all exposure concentrations tested (0.009 µg/L – 0.135 µg/L), while for malathion the second lowest (0.08 µg/L) and two highest exposure concentrations (0.32 µg/L and 0.47 µg/L) 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 0.1 mg/L, 1.4 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 lactate. 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.

1. 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 (Clarke and Haselden, 2008; Keum et al., 2010; Lin et al., 2006; Nagato et al., 2015; 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 (Li et al., 2015; Nagato et al., 2013; Nagato et al., 2015; Poynton et al., 2011; Taylor et al., 2009; Taylor et al., 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 (Fasulo et al., 2012; Nagato et al., 2013; Nagato et al., 2015; 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 ¹H NMR metabolomics with *D. magna* (Nagato et al., 2013), 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.

2. Materials and Methods

2.1. D. 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 CaCO₃/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).

2.2. D. 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 (63.1 µM) mg/L, 0.9 µg/L (3.0 nM) and 3.2 µg/L (9.7 nM) for BPA, diazinon and malathion respectively and are consistent with values found in the literature (Table 1).

Table 1. Acute toxicity (LC₅₀) values obtained from this study and comparisons with literature values.

Contaminant	LC ₅₀ values determined in this study	LC ₅₀ values from the literature
Diazinon	0.9 µg/L (48 hours)	0.96 µg/L (48 hours) ^a
Malathion	3.2 µg/L (48 hours)	3.8 µg/L (24 hours) ^b 0.9 µg/L (48 hours) ^c
Bisphenol-A	14.4 mg/L (48 hours)	12.8 mg/L (48 hours) ^d 10.4 mg/L (Immobilization EC ₅₀ -48 hours) ^e

^afrom Sanchez et al. (2000), ^b from Ren et al. (2007), ^c from Barata et al. (2004), ^d from Hirano et al. (2004), and ^e from Jeong et al. (2013).

D. magna were exposed to 1%, 2.5%, 5%, 10% and 15% of these observed LC₅₀ values. For diazinon these corresponded to 0.009 µg/L, 0.0225 µg/L, 0.045 µg/L, 0.09 µg/L and 0.135 µg/L (0.03 nM, 0.08 nM, 0.15 nM, 0.30 nM and 0.45 nM). For malathion these corresponded to 0.03 µg/L, 0.08 µg/L, 0.16 µg/L, 0.32 µg/L and 0.47 µg/L (0.01 nM, 0.24 nM, 0.48 nM, 0.97 nM, 1.42 nM). For BPA these corresponded to 0.1 mg/L, 0.35 mg/L, 0.7 mg/L, 1.4 mg/L and 2.1 mg/L

(0.6 μ M, 1.5 μ M, 3.2 μ M, 6.3 μ M and 9.2 μ M). 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. kirchnireilla*. 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.

2.3. Metabolite extractions for ^1H NMR

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 (Nagato et al., 2015). Briefly, 1 mg of *D. magna* dry mass was weighed out with a microbalance (Sartorius ME36S, Goettingen, Germany) and inserted into a 200 μ L 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 μ L 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 deuterioxide (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.

2.4. ^1H NMR spectroscopy and data processing

Analysis was performed with a Bruker BioSpin Avance III 500 MHz NMR equipped with a microprobe (^1H - ^{13}C - ^{15}N 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 (Lankadurai et al., 2011; Nagato et al., 2013; Nagato et al., 2015). Acquired spectra were manually phased, baseline corrected and aligned to the trimethyl-silyl group of the DSS internal calibrant and set at $\delta = 0.00$ ppm. Prior to any ^1H NMR analysis, both the D_2O used and the D_2O -based phosphate buffer were analyzed to check for impurities.

2.5. 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 ¹H 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 (Lankadurai et al., 2011; Nagato et al., 2013; Nagato et al., 2015) 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). A *t*-test (two tailed, equal variance, $\alpha < 0.05$) 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.

2.6. 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 (Brown et al., 2008; Nagato et al., 2013; Nagato et al., 2015). 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; Ekman et al., 2007; Nagato et al.,

2013). Statistical significance of these changes was determined with a *t*-test (two tailed, equal variances, $p = 0.05$) that were corrected with a false discovery rate according to the Benjamini and Hochberg (1995) method (Ralston-Hooper et al., 2008; Wu et al., 2012) using an in house R-script (Supplementary Information). 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).

3. Results

3.1. Multivariate statistical analyses of ^1H NMR spectra

Figure 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 all diazinon exposed groups along PC1 (accounting for 77% of the variation) that was statistically significant ($p < 0.05$). However, at exposure concentrations of 0.045 $\mu\text{g/L}$ and 0.09 $\mu\text{g/L}$, the PCA scores plot showed that these averaged spectra were uniquely different from the others based on their positioning within the PCA. On PC1 the lowest concentrations (0.009 $\mu\text{g/L}$ and 0.0225 $\mu\text{g/L}$) 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 (Supplementary Information, Fig. 1A) for PC1 indicated that resonances from the amino acids leucine, valine, alanine, threonine, glycine and glutamate and signals from fatty acid components (terminal $-\text{CH}_3$ and mid-chain methylene $-\text{CH}_{2(n)}$ groups) accounted for the separation seen in this PC, while threonine, alanine and $-\text{CH}_{2(n)}$ groups accounted for changes on PC2. A PCA scores plot for malathion exposures (Fig. 1.B) accounted for 85% of the variation within the first two PCs. There was also a pattern similar to that of diazinon, where along PC1

254 (accounting for 60% of variability), the lowest and the highest exposure concentrations resulted
255 in closer grouping and are distinct from the second lowest concentration (0.08 $\mu\text{g/L}$). The
256 loadings plot for both PCs indicated the cause for the separation between the groups
257 (Supplementary information, Fig. 1B) with resonances from the amino acids leucine, valine,
258 alanine, threonine and glutamate, as well as $-\text{CH}_3$ and $-\text{CH}_{2(n)}$ groups causing separation along
259 the first two PCs. A PCA scores plot for BPA exposure (Fig. 1C) indicated that there was
260 separation between the control and exposed groups, with the exception of the 0.35 mg/L and 1.4
261 mg/L exposure concentrations. There were differences in the direction of change, where the
262 lowest concentrations (0.1 mg/L) separated along PC2 (accounting for 16% of the variation) and
263 the higher concentrations were separated along PC1 (accounting for 66% of the variation) with
264 the highest concentration incurring the largest shift in metabolome. This showed a general
265 grouping between low exposures and higher exposures. The loadings plot (Supplementary
266 Information, Fig. 1C) indicates which metabolites account for these changes and include
267 resonances from the amino acids valine, leucine, alanine, threonine along PC1 and $-\text{CH}_{2(n)}$ on
268 PC2.

269

270 Figure 1. Averaged principal component analysis (PCA) scores plots for diazinon (A), malathion (B) and bisphenol-
 271 A (C). Statistically significant separation from the control ($p < 0.05$) is indicated by an asterisk (*).

3.2. 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 (Fig. 1), examining individual metabolites provides greater insight into the mechanisms of toxicity. The percent metabolite changes with diazinon exposure (Fig. 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, arginine, glycine, glutamate, phenylalanine and tyrosine. These trends reversed at 0.045 µg/L and 0.09 µg/L, where there were decreases in amino acids such as leucine, valine, arginine, glycine, threonine, lysine, glutamate, glutamine, phenylalanine and tyrosine. 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 (Fig. 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, threonine, 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, isoleucine, tyrosine and phenylalanine 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 (Fig. 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, glutamine, 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 2. Percent changes of metabolites showing statistically significant changes after 48 hours of diazinon exposure. A *t*-test, modified with a false discovery rate, was used to determine statistical significance and is indicated by an asterisk (*), n=10.

309

310 Figure 3. Percent changes of metabolites showing statistically significant changes after 48 hours of malathion
 311 exposure. A *t*-test, modified with a false discovery rate, was used to determine statistical significance and is
 312 indicated by an asterisk (*), n=10.

313

314 Figure 4. Percent changes of metabolites showing statistically significant changes after 48 hours of bisphenol-A
 315 exposure. A *t*-test, modified with a false discovery rate, was used to determine statistical significance and is
 316 indicated by an asterisk (*), n=10.

4. Discussion

4.1. Metabolomic responses with OP exposure

The PCA scores plots showed that diazinon and malathion exposures (Fig. 1A and 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.

4.2. Mechanisms of OP toxicity to *D. 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; Fingerman et al., 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 result 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, isoleucine, phenylalanine and tyrosine 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 LC₅₀ 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.

4.3. Metabolomic responses with BPA 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.

4.4. Mechanisms of BPA toxicity to D. magna

With increasing BPA exposure concentrations, the relative increase in free amino acids with increasing exposure concentrations (Fig. 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.

5. Conclusions

Using ^1H 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 ^1H 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.

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