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Version Post-print/accepted manuscript

Citation (published version) Kovacevic, V., Simpson, A.J., Simpson, M.J., 2016. ¹H NMR-based metabolomics of *Daphnia magna* responses after sub-lethal exposure to triclosan, carbamazepine and ibuprofen. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* 19, 199–210.
<https://doi.org/10.1016/j.cbd.2016.01.004>.

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**¹H NMR-based metabolomics of *Daphnia magna* responses after sub-lethal exposure to
triclosan, carbamazepine and ibuprofen**

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Abstract

Pharmaceuticals and personal care products are a class of emerging contaminants that are present in wastewater effluents, surface waters, and ground waters around the world. There is a need to determine rapid and reliable bioindicators of exposure and the toxic mode of action of these contaminants to aquatic organisms. ¹H nuclear magnetic resonance (NMR)-based metabolomics in combination with multivariate statistical analysis was used to determine the metabolic profile of *Daphnia magna* after exposure to a range of sub-lethal concentrations of triclosan (6.25 – 100 µg/L), carbamazepine (1.75 – 14 mg/L) and ibuprofen (1.75 – 14 mg/L) for 48 hours. Sub-lethal triclosan exposure suggested a general oxidative stress condition and the branched-chain amino acids, glutamine, glutamate, and methionine emerged as potential bioindicators. The aromatic amino acids, serine, glycine and alanine are potential bioindicators for sub-lethal carbamazepine exposure that may have altered energy metabolism. The potential bioindicators for sub-lethal ibuprofen exposure are serine, methionine, lysine, arginine and leucine, which showed concentration-dependence. The differences in the metabolic changes were related to the dissimilar modes of toxicity of triclosan, carbamazepine and ibuprofen. ¹H NMR-based metabolomics gave an improved understanding of how these emerging contaminants impact the keystone species *D. magna*.

Key words: pharmaceuticals, personal care products, metabolomics, *Daphnia magna*, aquatic toxicology, mode of action, bioindicators.

1. Introduction

Pharmaceuticals and personal care products are emerging environmental contaminants that are frequently detected in aquatic ecosystems due to their high consumption and incomplete removal by wastewater treatment technologies (Metcalf et al., 2003a; Chen et al., 2006; Kasprzyk-Hordern et al., 2009; Jeffries et al., 2010; Yu et al., 2013). Thousands of tons of these compounds are produced annually to be used for prescription drugs, veterinary drugs, cosmetics, fragrances, and diagnostic agents such as X-ray contrast media (Kot-Wasik et al., 2007). Recent studies detected pharmaceuticals and personal care products in the ng/L to µg/L range in wastewater treatment plant effluents, surface waters, groundwater, and even in drinking water (Loraine and Pettigrove, 2006; Brausch and Rand, 2011; Blair et al., 2013; Liu and Wong, 2013). Triclosan, carbamazepine, and ibuprofen are among the most frequently detected pharmaceuticals and personal care products in aquatic environments worldwide (Kolpin et al., 2002; Ferrari et al., 2004). Triclosan is an antibacterial agent that inhibits the enzyme enoyl-acyl carrier protein reductase responsible for bacterial fatty acid synthesis (Slater-Radosti et al., 2001). Carbamazepine is an anticonvulsant that stabilizes the inactivated state of sodium channels thereby making neurons less excitable (Beutler et al., 2005). Ibuprofen is a non-steroidal anti-inflammatory drug that inhibits the cyclooxygenase enzymes involved in prostaglandins synthesis (Charlier and Michaux, 2003). Triclosan, carbamazepine and ibuprofen are incompletely removed by conventional wastewater treatment technologies, with removal efficiencies of 90 – 98%, 7 – 8% and 87 – 90%, respectively (Zhang et al., 2008; Santos et al., 2009; Nakada et al., 2010). As a result, all three compounds are present in wastewater effluents and are frequently detected in surface waters worldwide especially near the discharge of wastewater treatment plants (Metcalf et al., 2003b; Gros et al., 2006; Brausch and Rand, 2011; Ramaswamy et al., 2011).

Despite the widespread occurrence of pharmaceuticals and personal care products in aquatic environments, the potential ecotoxicity of these contaminants to non-target organisms is still poorly understood (Fent et al., 2006; Brausch and Rand, 2011). Sub-lethal concentrations of pharmaceuticals and personal care products can alter physiological processes and adversely impact the health of aquatic organisms by unanticipated mechanisms (Fent et al., 2006; Jjemba, 2006). Recent studies evaluated the toxicity of triclosan, carbamazepine and ibuprofen on different aquatic organisms (Binelli et al., 2009; Contardo-Jara et al., 2011; Parolini et al., 2011; Matozzo et al., 2012). Triclosan exposure is believed to have cytotoxic and genotoxic impacts on the zebra mussel *Dreissena polymorpha* (Binelli et al., 2009) stimulates the activity of glutathione transferase and glutathione reductase in the Mediterranean mussel *Mytilus galloprovincialis* (Canesi et al., 2007) and is immunotoxic to the Manila clam *Ruditapes philippinarum* (Matozzo et al., 2012). Reports of carbamazepine exposure to aquatic organisms include oxidative stress and protein damage in the zebra mussel *D. polymorpha* (Contardo-Jara et al., 2011) growth retardation in the water flea *Daphnia magna* (Dietrich et al., 2010) and developmental abnormalities in the zebrafish *Danio rerio* (van den Brandhof and Montforts, 2010). Exposure to ibuprofen altered antioxidant and detoxifying enzyme activities in the zebra mussel *D. polymorpha* (Parolini et al., 2011) and alters the transcription of key genes involved in eicosanoid metabolism and oogenesis in *D. magna* (Heckmann et al., 2008b). Evaluations of molecular and biochemical mechanisms for the toxic responses induced by these contaminants in non-target aquatic species are less commonly undertaken (Canesi et al., 2007; Heckmann et al., 2008b; Martin-Diaz et al., 2009).

Detecting changes in metabolite concentrations in cells, tissues, or biofluid of an organism after sub-lethal contaminant exposure can give insight into the toxicological mode of action of

the contaminant (Bando et al., 2011; Liu et al., 2011). Metabolomics measures the concentration of endogenous metabolites from biological samples using methods such as NMR spectroscopy in combination with multivariate statistical analysis (Wu and Wang, 2010; Wagner et al., 2014). ^1H NMR-based metabolomics is capable of high-throughput analysis that is non-destructive, and gives highly quantitative assessments and reproducibility (Clarke and Haselden, 2008; Naz et al., 2014). The field of environmental metabolomics has been increasingly using ^1H NMR spectroscopy to examine the metabolic responses of organisms to a wide range of environmental stressors, such as temperature, disease, and contaminant exposure (Viant et al., 2005; Li et al., 2014; Shao et al., 2015). Recently, ^1H NMR-based metabolomics conducted on the water flea *D. magna* was successfully applied to evaluate the metabolic response to cadmium (Poynton et al., 2011), the polycyclic aromatic hydrocarbons pyrene and fluoranthene (Vandenbrouck et al., 2010), the heavy metals arsenic, copper and lithium (Nagato et al., 2013), flame-retardants (Scanlan et al., 2015) and silver nitrate and coated silver nanoparticles (Li et al., 2015). *D. magna* is a model organism for these studies because it has vital ecological roles in freshwater ecosystems and is sensitive to water contaminants (Lampert, 2006; Altshuler et al., 2011). Previous research has shown that metabolomics techniques are possible with *D. magna*, however there is limited knowledge of the metabolic responses to emerging contaminants such as pharmaceuticals and personal care products.

In this study, ^1H NMR-based metabolomics was applied to characterize the metabolic profile of *D. magna* after 48 hour exposures to a range of sub-lethal triclosan, carbamazepine and ibuprofen concentrations. Previous studies on the exposure of these select contaminants to aquatic invertebrates were mostly focused on the activity of antioxidant enzymes, or on reproductive and developmental toxicity, which are not adequate to propose molecular

mechanisms of toxicity. Therefore, the objectives of this study were to use ^1H NMR-based metabolomics to discern the mode of action and identify probable bioindicators of sub-lethal triclosan, carbamazepine and ibuprofen exposures to *D. magna*. This research aims to give new perspectives on the toxicological mechanisms of pharmaceutical and personal care product exposures on aquatic organisms. In addition, the results of this study can aid in the development of targeted analytical tests for biomonitoring the health of aquatic ecosystems.

2. Materials and methods

2.1. Daphnia culturing

D. magna were originally purchased from Ward Science Canada (St. Catharines, ON, Canada) and have been cultured in our laboratory since 2013. Culture and maintenance of *D. magna* was performed at 20°C with a light/dark cycle of 16/8 h, and with dechlorinated tap water which has been aerating for more than 24 hours and has a hardness of approximately 120 mg $\text{CaCO}_3 \text{ L}^{-1}$ (consistent with local freshwater conditions). *D. magna* were fed with a 50:50 ratio of *Pseudokirschneriella subcapitata*: *Chlorella vulgaris* and both algae were grown in a Bristol medium. Feeding and a 50% water change were done three times a week. Selenium and cobalamin (1 $\mu\text{g/L}$ of each) were included in the food broth twice a week to ensure that the daphnids could meet Environmental Canada standards of reproduction within 12 days with a minimum clutch size of 15 neonates per individual (Environment Canada, 2000).

2.2. Daphnia exposure to sub-lethal concentrations of triclosan, carbamazepine, and ibuprofen

Triclosan ($\text{C}_{12}\text{H}_7\text{Cl}_3\text{O}_2$, 97% purity), carbamazepine ($\text{C}_{15}\text{H}_{12}\text{N}_2\text{O}$, 98% purity), and ibuprofen ($\text{C}_{13}\text{H}_{18}\text{O}_2$, 98% purity) were purchased from Sigma-Aldrich (Mississauga, ON). Previous studies have examined the acute toxicity of triclosan, carbamazepine and ibuprofen to *D. magna* and reported 48 hour LC_{50} (the concentration that causes 50% mortality) values of

330 µg/L, 111 mg/L, 132.6 mg/L, respectively (Han et al., 2006; Peng et al., 2013) After preliminary tests to confirm the 48 hour sub-lethal contaminant concentrations, *D. magna* adults were exposed over 48 hours to either sub-lethal triclosan concentrations (0, 6.25, 12.5, 25, 50, 75 and 100 µg/L), sub-lethal carbamazepine concentrations (0, 1.75, 3.5, 5.25, 7, 10.5 and 14 mg/L) or sub-lethal ibuprofen concentrations (0, 1.75, 3.5, 5.25, 7, 10.5 and 14 mg/L). For every exposure concentration, two groups of 60 individuals each were kept in separate 2 L beakers with a density of 1 daphnid per 30 mL. *D. magna* were fed halfway through the acute test with a 50:50 ratio of *P. subcapitata*: *C. fusca* at an amount of 0.1 mg of carbon per litre. Light and temperature conditions were consistent with the culturing conditions. After 48 hours the daphnids were removed from the test solution and rinsed in dechlorinated tap water. They were subsequently flash frozen in liquid nitrogen, lyophilized and stored in a freezer at -25°C until extraction.

2.3. Sample preparation and metabolite extraction

The *D. magna* metabolite extraction procedure was centred on previous work done with a Bruker 1.7 mm NMR microprobe that reduces sample volume requirements and therefore the amount of daphnia dry mass needed for metabolomics experiments (Nagato et al., 2015). *D. magna* dry mass was first pooled and then weighed out to 1 mg with a microbalance (Sartorius ME36S) and inserted into a 200 µl centrifuge tube where it was homogenized with a small metal spatula. This procedure was repeated 10 times for each exposure concentration which resulted in 10 replicates for NMR analysis. Metabolites were extracted with 40 µl of a 0.2 M monobasic sodium phosphate buffer solution (NaH₂PO₄ • 2H₂O, 99.3%, Fisher Canada) 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. (Lankadurai et al., 2011; Nagato et al.,

2015). The buffer also contained 0.1% w/v sodium azide (99.5% purity; Sigma Aldrich) as a preservative and 10 mg L⁻¹ of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS; 97%, Sigma Aldrich) as an internal calibrant (Lankadurai et al., 2011; Nagato et al., 2015). Each sample was vortexed for 45 seconds and then sonicated for 15 minutes. Samples were then centrifuged (Eppendorf centrifuge 5804 R) at 4°C for 20 minutes at 12000 rpm (~15294g) and the supernatant was transferred into 1.7 mm Highthroughput^{plus} NMR tubes (Norell Inc., NJ, USA) for ¹H NMR analysis.

2.4. ¹H NMR acquisition

¹H NMR spectra were acquired using a Bruker BioSpin Avance III 500 MHz NMR spectrometer equipped with a ¹H-¹⁵N-¹³C TXI 1.7 mm microprobe fitted with an actively shielded Z gradient. ¹H NMR experiments were performed with Presaturation Using Relaxation Gradients and Echoes (PURGE) water suppression (Simpson and Brown, 2005), 256 scans, a recycle delay of 3 s, and 32 K time domain points. Spectra were apodized through 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). All spectra were manually phased and calibrated to the trimethylsilyl group of the DSS internal reference set to a chemical shift (δ) of 0.00 ppm.

2.5. Data and statistical analysis

Data analysis was performed on phased and baseline corrected ¹H NMR spectra with AMIX software v. 3.9.7 (Bruker BioSpin, Rheinstetten, Germany). The ¹H NMR spectra were analyzed in the chemical shift range between 0.5 and 10 ppm and the spectral region between 4.70 and 4.90 ppm was excluded to eliminate the small residual H₂O/HOD signals. The ¹H NMR spectra were divided into buckets of 0.02 ppm, for a total of 475 buckets, to control for small

variations in peak positions and to facilitate bioindicator identification. The integration mode was set to the sum of intensities and the spectra were scaled to total intensity (Lankadurai et al., 2011). Average Principal Component Analysis (PCA) scores plots were assembled to compare the metabolic profile of each exposed daphnid group to the control group. The average PCA scores plots were constructed using a PCA model that described all of the data and then the scores belonging to each group were imported into Microsoft Excel (v. 12. Microsoft Corporation, Redmond, WA) and were averaged per group (exposure concentration or control) and re-plotted with their associated standard errors (Lankadurai et al., 2011; Nagato et al., 2013). One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post-hoc test was conducted on the PCA score values from each group to examine statistical significance ($p < 0.05$) of the separations. The statistically significant separations from the control ($p < 0.05$) are indicated by an asterisk (*) on the PCA scores plots. To identify the metabolites that contributed to the separation between the control and the exposed daphnids in the PCA scores plots, the corresponding PCA loadings plots were also acquired that show the relative weight of each bucket (Lankadurai et al., 2011; Nagato et al., 2013).

The metabolite signals were identified using published spectra found on the Madison Metabolomics Consortium Database (Cui et al., 2008) and previously published metabolite resonances (Brown et al., 2008; Nagato et al., 2015). Metabolite percent changes were obtained by subtracting the NMR intensity values of the exposed group from the bucket values of the control, then dividing this difference by the control bucket value (Nagato et al., 2013). A t-test (two-tailed, equal variances) was used to compare the buckets of the control with that of the exposure to identify the metabolites that were statistically different at $\alpha = 0.05$ (Lankadurai et al., 2011).

Partial least squares (PLS) regression analysis was performed in R (v. 3.1.2, The R Foundation, Vienna, Austria) using the Chemometrics package (Filzmoser and Varmuza, 2010) on the binned ^1H NMR spectra generated by the AMIX 3.9.7 statistics tool. PLS-regression was done via the NIPALS PLS algorithm using the normalized bucket intensities from the ^1H NMR spectra as the X matrix of multiple predictors and the exposure concentrations of the three tested contaminants as the Y (response) matrix (Åslund et al., 2011; Lankadurai et al., 2013). PLS models were cross validated using leave-one-out cross validation (Westerhuis et al., 2008; Varmuza and Filzmoser, 2009) and the optimal number of components for each final PLS model was determined by the single cross validation strategy (Westerhuis et al., 2008). The $R^2\text{X}$ and $R^2\text{Y}$ values were obtained for each PLS model to indicate how well the model fit the training data (Eriksson et al., 2006). The cross-validated $R^2\text{Y}$ value (represented as $Q^2\text{Y}$) was used as a preliminary measure of the predictive ability of the PLS model (Varmuza and Filzmoser, 2009; Åslund et al., 2011). Response permutation testing was conducted with 400 iterations to assess the significance of each PLS model (Eriksson et al., 2006; Åslund et al., 2011). In addition an alternative PLS model was made using 80% of the data by randomly removing two out of ten samples from each treatment and then the remaining 20% of the data were used as independent prediction sets to assess the predictive performance (Åslund et al., 2011).

3. Results and Discussion

3.1. NMR-based metabolomics of triclosan exposure

^1H NMR spectra of *D. magna* metabolite extracts were used to assemble average PCA scores plots (Figure 1) to compare the metabolic profiles of the triclosan exposed daphnids to the control daphnids (Nagato et al., 2013). The average PC1 and PC2 scores plot for triclosan exposure (explains 91.2% of the metabolic variation) showed separation between the control and

215 triclosan exposed daphnids but the extent of separation varied with exposure concentration
216 (Figure 1A). This suggests that triclosan exposure caused a response in *D. magna* that varied
217 with triclosan concentration and that influenced the metabolic deviation from the control. The
218 PC3 vs PC4 scores plot also showed separation of triclosan exposed daphnids from the control
219 that was not related to exposure concentration, with a statistically significant ($p < 0.05$)
220 separation of the 100 $\mu\text{g/L}$ exposure along PC3 (Figure S1A in Supplementary Material). PLS-
221 regression models (Figure 2) were developed to determine the strength and significance of the
222 correlation between the metabolic profile and the triclosan exposure concentration (Lankadurai
223 et al., 2013). Triclosan exposure produced a PLS-regression model that suggested a weak but
224 significant linear relationship between the *D. magna* metabolic profile and the triclosan exposure
225 concentration (cross-validated PLS-regression with 9 components, $R^2X=0.98$, $R^2Y=0.78$,
226 $Q^2Y=0.34$, $P=9 \times 10^{-4}$; Figure 2A). The PLS-regression model is not robust and cannot
227 discriminate well between the treatments, suggesting that the toxic mode of action of triclosan is
228 not related to concentration over this range of tested exposure concentrations (Eriksson et al.,
229 2006; Lankadurai et al., 2013). Similar results were noted for the alternative PLS model that was
230 constructed with 80% of the total data and validated with an independent prediction set (Figure
231 S2A in Supplementary Material). To explore this relationship further, PCA loadings plots
232 (Figure 3) were used to identify the NMR resonances of metabolites that contribute most to the
233 separation between the control and the triclosan exposed daphnids in the corresponding PCA
234 scores plots (Nagato et al., 2015). The loadings plot for PC1 and PC2 (Figure 3A) for triclosan
235 exposure showed that leucine ($\delta= 0.95$ ppm), valine ($\delta= 1.03$ ppm), glycine ($\delta= 3.55$ ppm) and
236 glutamate ($\delta= 2.33$ ppm) had the greatest influence on the separation from the control in the PCA
237 scores plot. The PC3 and PC4 loadings plot for triclosan exposure identified that alanine ($\delta= 1.47$

ppm) also contributed to the metabolic variation from the control (Figure S3A in Supplementary Material).

The percent changes in metabolite concentrations of the triclosan exposed daphnids relative to the control are shown in Figure 4. Triclosan impedes bacterial growth by inhibiting the enzyme enoyl-acyl carrier protein reductase that is needed for bacterial fatty acid synthesis, and the mode of action of triclosan in eukaryotic cells is not precisely known (Slater-Radosti et al., 2001). Triclosan exposure to *D. magna* showed an increase in amino acid concentrations for a majority of the amino acids, particularly valine, isoleucine, glutamate, glutamine and methionine (Figure 4). Increases in amino acid levels may be a result of protein breakdown which releases free amino acids for energy metabolism during times of stress (Gillis and Ballantyne, 1996). Triclosan has been reported to induce oxidative stress in *D. magna* (Peng et al., 2013) and other freshwater invertebrates (Binelli et al., 2009; 2011; Riva et al., 2012). Triclosan exposure in *D. magna* may have induced the oxidation of proteins, altering their three-dimensional structure and leading to complete catabolism to their amino acids constituents (Lushchak, 2011). The branched-chain amino acids valine, leucine and isoleucine all increased significantly ($p < 0.05$) after triclosan exposure (Figure 4). In addition, valine and leucine strongly influenced the PC1 and PC2 loadings plot for triclosan exposure (Figure 3A) and therefore contributed the most to the separation between the controls and the exposed daphnids (Figure 1A). The abundance of the branched-chain amino acids may indicate that protein degradation is elevated or that there is a decrease in the synthesis of proteins as valine, leucine and isoleucine are essential amino acids and are important for protein synthesis (Kimball and Jefferson, 2006). Branched-chain amino acids provide energy and are used by the immune system for the synthesis of new protective molecules, therefore increased valine, leucine and isoleucine in response to triclosan exposure

may suggest immune-related stress in *D. magna* (Calder, 2006). Triclosan exposure suppressed immune functions both *in vitro* and *in vivo* of the Mediterranean mussel *Mytilus galloprovincialis* (Canesi et al., 2007) and was observed to be immunotoxic to the Manila clam *Ruditapes philippinarum* (Matozzo et al., 2012). The amino acids proline, glutamate and glutamine also revealed significant ($p < 0.05$) increases after triclosan exposure (Figure 4). Proline is known to protect cells from oxidative stress and is an antioxidant in *D. magna*, therefore elevated proline levels may suggest an increase in its synthesis for antioxidant defense (Smirnov, 2013). Glutamine is a major source of glutamate, and glutamate is in turn is used for glutathione synthesis to defend cells from oxidative stress (Wu et al., 2004). The increases in glutamine and glutamate levels in *D. magna* can be the result of their up-regulation for antioxidant defense in response to triclosan exposure (Figure 4).

The sugar glucose generally decreased relative to the controls, with a significant ($p < 0.05$) decrease at the 75 $\mu\text{g/L}$ triclosan exposure concentration (Figure 4). The decrease in glucose levels may be linked to increased glycolysis as the organisms have an enhanced energy requirement to counteract the toxicity of triclosan. Therefore there may be rapid utilization of carbohydrate reserves in the form of glucose (Canesi et al., 2007). The amino acids phenylalanine, asparagine and methionine also revealed significant ($p < 0.05$) increases after triclosan exposure (Figure 4). Methionine is stored by crustaceans to be used as a metabolic reserve during molting (Maity et al., 2012). Elevated methionine levels may suggest impairments in the molting process, which was observed with chronic exposure of *D. magna* to triclosan, where the total number of molting per adult was decreased at 1 – 128 $\mu\text{g/L}$ triclosan concentrations (Peng et al., 2013). Tyrosine, arginine, threonine and tryptophan generally increased relative to the controls (Figure 4). A significant ($p < 0.05$) increase in tryptophan was

only observed at the 75 µg/L triclosan exposure concentration, while significant ($p < 0.05$) increases in tyrosine, arginine and threonine were only observed at the lowest triclosan exposure concentration of 6.25 µg/L (Figure 4). The significant ($p < 0.05$) increase in the levels of ten metabolites at the 6.25 µg/L triclosan treatment raises questions to the potential sub-lethal toxicity at environmentally relevant triclosan concentrations, which were detected to be from 0.035 – 1.023 µg/L in rivers in China (Peng et al., 2008; Zhao et al., 2013) and reaching 2.3 µg/L in natural streams and rivers in the USA (Kolpin et al., 2002). Therefore, exposure to very low triclosan concentrations present in the environment may cause adverse biochemical responses in *D. magna*.

3.2. NMR-based metabolomics of carbamazepine exposure

Average PCA scores plots (Figure 1) compare the metabolic profiles of the carbamazepine exposed daphnids to the control daphnids (Nagato et al., 2013). The average PC1 vs PC2 scores plot of carbamazepine exposure showed clear separation of carbamazepine exposure concentrations ≤ 10.5 mg/L from the control along PC1 (63.6% explained variation) with exposure concentrations 1.75 and 7 mg/L showing a statistically significant ($p < 0.05$) separation (Figure 1B). The highest carbamazepine exposure treatment (14 mg/L) did not separate well from the control along PC1 (Figure 1B). This implies that the metabolic profiles of daphnids exposed to ≤ 10.5 mg/L of carbamazepine were more similar to each other than that of the 14 mg/L carbamazepine exposure which was more similar to the control. The PC3 vs PC4 scores plot for carbamazepine exposure showed that along PC3 exposure concentrations of 7 and 10.5 mg/L and along PC4 exposure concentrations of 7, 10.5 and 14 mg/L showed statistically significant ($p < 0.05$) separation from the control (Figure S1B in Supplementary Material). However this does not dominate the observed variation in the metabolic profile as PC3 and PC4

only explained approximately 4% and 2% of the metabolic variation, respectively (Figure S1B in Supplementary Material). Carbamazepine exposure produced a PLS-regression model with the highest predictive power and the strongest linear correlation between the metabolic profile and the carbamazepine exposure concentration (cross-validated PLS-regression with 9 components, $R^2X=0.97$, $R^2Y=0.96$, $Q^2Y=0.87$, $P=2 \times 10^{-6}$; Figure 2B). This suggests that carbamazepine exposure may have a concentration-dependent mode of action in *D. magna* as there is little variation between treatment groups that can be discriminated by PLS regression. A reconstructed PLS model using 80% of the total data that was then validated with an independent prediction set showed similar results (Figure S2B in Supplementary Material). The PC1 and PC2 loadings plot for carbamazepine exposure (Figure 3B) showed that NMR resonances associated with the metabolites leucine ($\delta= 0.95$ ppm), alanine ($\delta= 1.47$ ppm), valine ($\delta= 1.03$ ppm), glycine ($\delta= 3.55$ ppm) and glutamate ($\delta= 2.33$ ppm) had the greatest influence on the separation of the exposed daphnids from the controls in the PCA scores plot (Figure 1B). The PC3 and PC4 loadings plot for carbamazepine exposure (Figure S3B in Supplementary Material) did not identify any additional metabolites to those already identified in the PC1 and PC2 loadings plot.

The most prominent *D. magna* metabolic response to carbamazepine exposure is the depleted levels of the following amino acids: glycine, alanine, proline, leucine, glutamate, glutamine, arginine, serine and the aromatic amino acids tryptophan, phenylalanine and tyrosine (Figure 5). In addition, a significant increase in glucose is observed with the 5.25 mg/L carbamazepine exposure concentration (Figure 5). Instead of breaking down glycogen to sustain glucose levels needed for survival, daphnia have genes that encode enzymes involved in gluconeogenesis (Campos et al., 2013) and most of the downregulated amino acids (e.g. the aromatic amino acids, alanine, glycine, glutamate, glutamine, arginine, serine) can be catabolized to intermediates of

the Krebs cycle or pyruvate (Kokushi et al., 2012). Therefore, the depletion of glucogenic amino acids and the concurrent rise in glucose may be caused by an increase in free amino acid incorporation in the Krebs cycle for glucose production without a renewal of free amino acids by proteolysis. For example, alanine is a major source material for gluconeogenesis and the significant decrease in alanine at carbamazepine exposures ≤ 7 mg/L can be an indicator of its utilization in this pathway (Figure 5; Southam et al., 2008). The precise mode of action of carbamazepine in aquatic invertebrates is currently unclear, but this likely increase in energy demand in *D. magna* may be related to the molecular ability of carbamazepine to block voltage gated sodium and calcium channels, induce gene transcription and inhibit histone deacetylases in mammals (Beutler et al., 2005). The general reduction of amino acids may also be attributed to an increase in synthesis of antioxidant defense proteins in response to toxicant induced stress (Knops et al., 2001; Smolders et al., 2005). Amino acid abundance is associated with zooplankton growth (Yebra and Hernández-León, 2004) and the depletion of amino acids with carbamazepine exposure may have a detrimental impact on *D. magna* growth and reproduction. Indeed, the water flea *Daphnia pulex* exposed to 200 μ g/L carbamazepine had delayed maturity (Lürling et al., 2006) and the water flea *Ceriodaphnia dubia* exposed to 196.7 μ g/L carbamazepine showed decreased fecundity in the F0 and F1 generations and a 264.6 μ g/L carbamazepine treatment also decreased adult body length in the F2 generation (Lamichhane et al., 2013).

The high carbamazepine exposure concentrations of 10 mg/L and 14 mg/L did not show significant decreases in a majority of amino acids, and this non-monotonic response was notable at the highest exposure concentration of 14 mg/L (Figure 5). This suggests that there may be a two-phased, concentration-dependent toxic mode of action of carbamazepine in *D. magna* with

an initial phase at carbamazepine concentrations < 10.5 mg/L and a second phase at carbamazepine concentrations > 10.5 mg/L. This may be because high carbamazepine concentrations may disrupt the mechanisms responsible for generating energy production through gluconeogenesis, or there may be an onset of proteolysis due to the ability of carbamazepine to induce protein breakdown in invertebrates (Smolders et al., 2005; Contardo-Jara et al., 2011; Almeida et al., 2015). Non-monotonic dose responses were also observed with 0.01 – 100 μ g/L carbamazepine exposures to *D. magna* where high concentrations had diminished impacts on daphnid behavioural and reproduction responses (Rivetti et al., 2015). Non-monotonic responses observed with neuro-active compounds on aquatic invertebrates may be due to the onset of general toxicity mechanisms at higher concentrations irrespective of the principal mode of action of the compound (Ford and Fong, 2015; Rivetti et al., 2015). However, serine showed significant ($p < 0.05$) decreases across all carbamazepine exposure concentrations while its readily interconvertible amino acid glycine significantly ($p < 0.05$) decreased at carbamazepine exposures ≤ 10.5 mg/L (Figure 5). The consistent depletion of serine across carbamazepine exposure concentrations indicates that serine may be a very sensitive indicator for carbamazepine exposure in *D. magna*. In crustaceans and other aquatic invertebrates, glycine can be metabolized to serine, and serine is used as an energy source in the Krebs cycle through conversion to pyruvate (Shinji et al., 2012). A decrease in serine and glycine may also be due to their use in the transsulfuration pathway that can be activated for oxidative stress defense against carbamazepine exposure, with serine used to convert homocysteine to cystathionine and glycine used to convert γ -glutamylcysteine to glutathione (Long et al., 2015).

Acute carbamazepine exposure also resulted in significant decreases in the aromatic amino acids tryptophan, phenylalanine and tyrosine; phenylalanine revealed significant ($p < 0.05$)

decreases with 1.75, 5.25, 7 and 10.5 mg/L carbamazepine exposures while tyrosine significantly ($p < 0.05$) decreased at ≤ 10.5 mg/L exposures and tryptophan significantly ($p < 0.05$) decreased at ≤ 7 mg/L exposures (Figure 5). Tryptophan is a precursor for serotonin in *D. magna* (Campos et al., 2013) and phenylalanine is an essential amino acid and is the precursor to tyrosine, which is in turn involved in the production of the catecholamine neurotransmitters L-DOPA, dopamine, norepinephrine and epinephrine in *D. magna* (Ehrenström and Berglind, 1988). The significant decrease in the aromatic amino acids with acute carbamazepine exposure suggests an altered synthesis of their neurotransmitter derivatives and corresponding disturbances in dopaminergic and serotonergic signaling. Serotonin and dopamine have many physiological roles in crustaceans, for instance in the release of neurohormones (Christie, 2011), are cardio/vasoactive (Marder and Bucher, 2007), and control osmoregulation (Morris and Ahern, 2003). One of the molecular targets of carbamazepine in humans is to inhibit the enzyme adenylyl cyclase (Montezinho et al., 2007) and it was hypothesized that carbamazepine also has this mode of action in invertebrates as 0.1 and 10 $\mu\text{g/L}$ carbamazepine exposure reduced cyclic adenosine monophosphate (cAMP) levels and protein kinase A activities in tissues of the Mediterranean mussel *Mytilus galloprovincialis* (Martin-Diaz et al., 2009). Carbamazepine exposure may have altered the cAMP-dependent pathway and disrupted neuroendocrinological regulation of physiological functions (Fabbri and Capuzzo, 2010) initiating a decrease in the levels of free aromatic amino acids in *D. magna*.

3.3. NMR-based metabolomics of ibuprofen exposure

The average PC1 vs PC2 scores plot for ibuprofen exposure showed clear separation between the control and the lowest ibuprofen exposure concentrations (1.75 and 3.5 mg/L) along PC1 (54% explained variation) while the highest concentrations (10.5 and 14 mg/L) were clearly

399 separated along PC2 (28% explained variation; Figure 1C). The mid ibuprofen exposure
400 concentrations (5.25 and 7 mg/L) did not show clear separation from the control in the PC1 vs
401 PC2 scores plot (Figure 1C). This suggests that the low (1.75 and 3.5 mg/L) and high (10.5 and
402 14 mg/L) ibuprofen concentrations may cause a more prominent metabolic response in *D. magna*
403 compared to the mid ibuprofen concentrations (5.25 and 7 mg/L). The PC3 vs PC4 scores plot
404 (9% explained variation) showed separation of ibuprofen exposed daphnids from the control with
405 a statistically significant ($p < 0.05$) separation of exposure concentration 7 mg/L along PC4, but
406 this does not dominate the metabolic variation from the control (Figure S1C in Supplementary
407 Material). The PLS-regression model for ibuprofen exposure had high predictive power and a
408 strong linear relationship between the metabolic profile and the ibuprofen exposure
409 concentration (cross-validated PLS-regression with 5 components, $R^2X=0.91$, $R^2Y=0.80$,
410 $Q^2Y=0.64$, $P=3 \times 10^{-8}$; Figure 2C). This suggests that the *D. magna* metabolic profiles are related
411 to ibuprofen concentration and show variation between treatment groups that can be
412 discriminated by PLS regression, indicating that ibuprofen exposure may have a mode of action
413 that is dependent on the level of exposure. Comparable results were obtained with a PLS model
414 that was reconstructed using 80% of the total data and then validated with the remaining 20% of
415 the data as an independent prediction set (Figure S2C in Supplementary Material). PCA loadings
416 plots (Figure 3) identified the metabolites that contribute to the separation between the control
417 and the ibuprofen exposed daphnids in the corresponding PCA scores plots (Figure 1; Nagato et
418 al., 2015). The PC1 and PC2 loadings plot for ibuprofen exposure (Figure 3C) showed that
419 leucine ($\delta= 0.95$ ppm), valine ($\delta= 1.03$ ppm), alanine ($\delta= 1.47$ ppm), glycine ($\delta= 3.55$ ppm) and
420 glutamate ($\delta= 2.33$ ppm) contributed to the variation from the control. The PC3 and PC4

loadings plot also showed that leucine, valine and alanine contributed to the variation in the metabolic response from the control (Figure S3C in Supplementary Material).

The mode of action of ibuprofen in mammals inhibits the synthesis of eicosanoids by reversibly inhibiting the catalytic sites of the enzyme cyclooxygenase through competition with arachidonic acid (Charlier and Michaux, 2003). Eicosanoids act as local hormones and are key regulators of immunity, ion flux, and reproduction in both vertebrates and invertebrates (Deridovich and Reunova, 1993; Stanley, 2000; Varvas et al., 2009). Non-steroidal anti-inflammatory drugs such as ibuprofen can also interrupt eicosanoid synthesis in invertebrates and previous studies suggest that the eicosanoid system is present in daphnids, and that ibuprofen can reduce *D. magna* reproduction and survival in a concentration-dependent manner (Rowley et al., 2005; Heckmann et al., 2007; 2008a; Hayashi et al., 2008). The percent change of several metabolites with acute ibuprofen exposure suggests that *D. magna* metabolic changes are dependent on ibuprofen concentration over the concentration range studied (Figure 6). Glycine, tyrosine, asparagine and serine generally decreased relative to the control with low-to-mid ibuprofen concentrations of 1.75 – 7mg/L (Figure 6). There were significant ($p < 0.05$) decreases in serine at the 1.75, 3.5 and 7 mg/L exposures, significant ($p < 0.05$) decreases in asparagine at the 5.25 and 7 mg/L exposures, and significant ($p < 0.05$) decreases in glycine and tyrosine at the 7 mg/L exposure (Figure 6). Leucine, arginine and lysine showed significant ($p < 0.05$) decreases at the two lowest ibuprofen exposure concentrations of 1.75 and 3.5 mg/L, and the magnitude of this response was attenuated with exposure towards the level of the control (Figure 6). Leucine, arginine, and lysine are essential amino acids in crustaceans and are usually found in low abundance, therefore their significant decreases may impede functions associated with molting, growth and osmoregulation (Augusto et al., 2007). Lysine is a precursor of carnitine and it is

important for fatty acid biosynthesis (Maity et al., 2012), and the relative decrease in lysine can suggest an altered generation of metabolic energy.

In contrast to the significant decreases in amino acids with low to mid ibuprofen concentrations, there were significant ($p < 0.05$) increases in alanine, tryptophan and isoleucine at the 10.5 mg/L ibuprofen exposure concentration (Figure 6). There were also significant ($p < 0.05$) increases in methionine with ibuprofen exposure concentrations of 10.5 and 14 mg/L (Figure 6), suggesting impairment in the molting process that may be related to an onset of reproduction inhibition with the higher ibuprofen exposures (Subramoniam, 2000; Maity et al., 2012). Similarly, two weeks of ibuprofen exposures of 0, 20, 40 and 80 mg/L to *D. magna* resulted in a significant concentration-dependent reduction in reproduction and population growth rate, and the 14-day reproduction EC_{50} (half maximal effective concentration) was 13.4 mg/L (Heckmann et al., 2007). Threonine showed different dynamics than the other amino acids as there was a significant ($p < 0.05$) decrease in threonine at the highest ibuprofen exposure concentration of 14 mg/L (Figure 6). Overall the metabolite responses to higher ibuprofen exposure concentrations (10.5 and 14 mg/L) differed from responses observed at lower concentrations (1.75 and 3.5 mg/L) and these non-monotonic metabolite responses may arise from molecular-level events of ibuprofen competing with arachidonic acid for enzymes in eicosanoid synthesis in *D. magna* (Rowley et al., 2005; Heckmann et al., 2007).

4. Conclusions

In this study, ^1H NMR-based metabolomics was able to detect and distinguish *D. magna* responses to sub-lethal triclosan, carbamazepine and ibuprofen exposures. Sub-lethal triclosan exposure suggests that the daphnids were under general oxidative stress as noted by the increased levels of a majority of amino acids. The branched-chain amino acids, glutamine, glutamate and

methionine emerged as potential bioindicators for sub-lethal triclosan exposure. Amino acids largely decreased with sub-lethal carbamazepine exposures < 10.5 mg/L and increased to control levels with exposures >10.5 mg/L, which suggests a concentration-dependent relationship between the *D. magna* metabolic response and carbamazepine exposure concentration. The decreased levels of most amino acids after carbamazepine exposure may indicate their increased catabolism for energy generation, and the aromatic amino acids, serine, glycine, and alanine are potential bioindicators of sub-lethal carbamazepine exposure. The *D. magna* metabolic profile after sub-lethal ibuprofen exposure seemed to be dependent on ibuprofen concentration as the lower exposure concentrations caused contrasting metabolite changes compared to the higher exposure concentrations. Ibuprofen exposure caused changes in important amino acids such as serine, methionine, lysine, arginine and leucine which are potential bioindicators of sub-lethal ibuprofen exposure. Untargeted metabolic profiling with ¹H NMR spectroscopy discovered different biochemical responses of *D. magna* after short duration exposures to triclosan, carbamazepine and ibuprofen and was able to elucidate potential toxic modes of action for each individual contaminant. The specific metabolite findings here give the incentive for additional, targeted analyses of *D. magna* responses to triclosan, carbamazepine and ibuprofen, as well as advocate for the metabolomic analysis of exposure to additional pharmaceuticals and personal care products that are being increasingly detected in the environment. The results demonstrate the ability of ¹H NMR spectroscopy to delineate mechanisms of toxicity of these emerging contaminants and to identify new bioindicators that can be informative when developing environmental monitoring tests for freshwater ecosystems.

Acknowledgements

490 The authors thank the Krembil Foundation for supporting this research. We are thankful to Dr.
491 Ronald Soong, Dr. Brian Lankadurai, Edward Nagato and Vivek Dani for technical assistance
492 and valuable discussions. Vera Kovacevic also thanks the Natural Sciences and Engineering
493 Research Council of Canada for a Canada Graduate Scholarship.

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764

Figure Captions

Figure 1. Principal component analysis shows separation between control and exposed *D. magna*. Average principal component analysis (PCA) scores plot for the ^1H NMR spectra of *D. magna* extracts after 48 hour exposure to (A) triclosan ($\mu\text{g/L}$) PC1 (first PCA component) versus PC2 (second PCA component), (B) carbamazepine (mg/L) PC1 versus PC2, and (C) ibuprofen (mg/L) PC1 versus PC2. The mean scores shown with the associated standard error are labeled with the exposure condition (control or exposure concentration) and were obtained by averaging the scores of each exposure concentration ($n=10$) or control ($n=10$). Statistical significance from the control ($p < 0.05$) is indicated with an asterisk (*).

Figure 2. Partial least squares regression analysis between control and exposed *D. magna*. Average predictions of (A) triclosan (TCS), (B) carbamazepine (CBZ), and (C) ibuprofen (IBP) concentrations ($\hat{y}_i$) given spectra i by the partial least squares (PLS) model derived from the leave-one-out cross-validation procedure for PLS models constructed with the chemical exposure concentrations as the Y variable and the ^1H NMR bucket intensities as the X table. The solid line is a linear regression between the actual and predicted values. The error bars represent the standard error of the mean.

Figure 3. PCA loadings plots indicating the metabolites that were major contributors to the separation observed in the average PCA scores plot of the *D. magna* extracts after 48 hour exposure to (A) triclosan PC1 (first PCA component) and PC2 (second PCA component), (B) carbamazepine PC1 and PC2, and (C) ibuprofen PC1 and PC2. The numerical labels refer to the ^1H NMR chemical shift of each 0.02 ppm bucket.

788

789 Figure 4. Percent change in selected metabolites of 48 hour triclosan exposed *D. magna* extracts
790 (n=10) compared with the control *D. magna* (n=10). Metabolite percent changes were obtained
791 by subtracting the NMR bucket intensity values of the exposed group from the bucket intensity
792 values of the control, then dividing by the control bucket value (Nagato *et al.*, 2013). The percent
793 changes are shown with their associated standard error and statistical significance ($p < 0.05$) is
794 indicated with an asterisk (*).

795

796 Figure 5. Percent change in selected metabolites of 48 hour carbamazepine exposed *D. magna*
797 extracts (n=10) compared with the control *D. magna* (n=10). Metabolite percent changes were
798 obtained by subtracting the NMR bucket intensity values of the exposed group from the bucket
799 intensity values of the control, then dividing by the control bucket value (Nagato *et al.*, 2013).
800 The percent changes are shown with their associated standard error and statistical significance (p
801 < 0.05) is indicated with an asterisk (*).

802

803 Figure 6. Percent change in selected metabolites of 48 hour ibuprofen exposed *D. magna* extracts
804 (n=10) compared with the control *D. magna* (n=10). Metabolite percent changes were obtained
805 by subtracting the NMR bucket intensity values of the exposed group from the bucket intensity
806 values of the control, then dividing by the control bucket value (Nagato *et al.*, 2013). The percent
807 changes are shown with their associated standard error and statistical significance ($p < 0.05$) is
808 indicated with an asterisk (*).

$$[\text{TCS}]_{\text{predicted}} = 0.56 * [\text{TCS}]_{\text{exposed}} + 17.1$$

$$R^2Y = 0.40$$

PLS model parameters

No. of components = 9

$R^2X = 0.98$; $R^2Y = 0.78$; $Q^2Y = 0.34$

P-value (permutation test) = 9×10^{-4}

$$[\text{CBZ}]_{\text{predicted}} = 0.89 * [\text{CBZ}]_{\text{exposed}} + 0.65$$

$$R^2Y = 0.87$$

PLS model parameters

No. of components = 9

$R^2X = 0.97$; $R^2Y = 0.96$; $Q^2Y = 0.87$

P-value (permutation test) = 2×10^{-6}

$$[\text{IBP}]_{\text{predicted}} = 0.72 * [\text{IBP}]_{\text{exposed}} + 1.73$$

$$R^2Y = 0.64$$

PLS model parameters

No. of components = 5

$R^2X = 0.91$; $R^2Y = 0.80$; $Q^2Y = 0.64$

P-value (permutation test) = 3×10^{-8}