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

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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 h of contaminant exposure. The PCA scores plots showed that diazinon exposures resulted in aberrant metabolomic profiles at all exposure concentrations tested (0.009–0.135 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

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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 metabolomic studies with most of these focusing on acute toxic stress (Li et al., 2015; Nagato et al., 2013, 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 (Fasulo et al., 2012; Nagato et al., 2013, 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 $^{1}$H 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 h acute toxicity test. While a 48 h 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 $CaCO_3$/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).

### 2.2. D. magna *acute toxicity exposures*

*D. magna* were exposed over 48 h to either BPA (Sigma–Aldrich, >99%), diazinon (Sigma–Aldrich, PESTANAL analytical standard, 98.5%) or malathion (Sigma–Aldrich, PESTANAL, analytical Standard, 97.5%). 48 h 50% lethality concentrations ($LC_{50}$) tests were conducted, using methods outlined by the OECD and Environment Canada (Environment Canada, 2000; OECD, 2012). Briefly, *D. magna* neonates (<24 h old), taken from isolated gravid females, were placed in clear glass jars (250 mL) filled with 200 mL of test solution (1 daphnid/20 mL). 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 $LC_{50}$ 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 $LC_{50}$ values. Based on this, $LC_{50}$ 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,