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1-D and 2-D NMR-based metabolomics of earthworms exposed to endosulfan and endosulfan sulfate in soil

Jimmy Yuk, Myrna J. Simpson, André J. Simpson*

Department of Chemistry, University of Toronto, Scarborough College, 1265 Military Trail, Toronto, Ontario M1C1A4, Canada

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ABSTRACT

One-dimensional (1-D) and two-dimensional (2-D) nuclear magnetic resonance (NMR)-based metabolomics was used to investigate the toxic mode of action (MOA) of endosulfan, an organochlorine pesticide, and its degradation product, endosulfan sulfate, to *Eisenia fetida* earthworms in soil. Three soil concentrations (0.1, 1.0 and 10.0 mg/kg) were used for both endosulfan and endosulfan sulfate. Both earthworm coelomic fluid (CF) and tissues were extracted and then analyzed using ^{1}H and $^{1}H-^{13}C$ NMR techniques. A similar separation trajectory was observed for endosulfan and endosulfan sulfate-exposed earthworms in the mean principal component analysis (PCA) scores plot for both the earthworm CF and tissue extracts.

A neurotoxic and apoptotic MOA was postulated for both endosulfan and endosulfan sulfate exposed earthworms as significant fluctuations in glutamine/GABA–glutamate cycle metabolites and spermidine were detected respectively. This study highlights the application of NMR-based metabolomics to understand molecular-level toxicity of persistent organochlorine pesticides and their degradation products directly in soil.

1. Introduction

Organochlorine pesticides are a group of persistent environmental pollutants that have caused major worldwide concern as many are semi-volatile, bioaccumulative and toxic (Pandya et al., 2006). Not only are they environmentally persistent, they have potential to spread widely both regionally and globally (Wania and Mackay, 1993). Endosulfan is an organochlorine pesticide that has been commonly used in agriculture for the control of various pests (Hose et al., 2003). Due to its semi-volatility and persistent physicochemical properties, endosulfan has been observed in many environmental compartments and often in areas far away from their location of direct application (Weber et al., 2010). It is considered carcinogenic and an endocrine disruptor (Pandya et al., 2006). Endosulfan is also believed to cause central nervous system and neurodegenerative disorders in many mammals including humans (Jia and Misra, 2007). Its breakdown products include endosulfan sulfate, diol, ether, -hydroxy ether and -lactone: all of which are considered to be less toxic (Hose et al., 2003) with the exception of endosulfan sulfate.

Endosulfan sulfate is the main metabolite of endosulfan degradation in soil and sediments (Antonious and Byers, 1997). The half-life for endosulfan is around one to three months while endosulfan sulfate can persist close to two to six years depending on the soil conditions (Wan et al., 2005). Understanding the environmental impact of contaminants and their degradation products in the soil has become a major priority for the Organization for Economic Co-operation and Development (OECD) and its member countries (Mosleh et al., 2003). Currently, most studies have examined the toxicity of endosulfan sulfate in aquatic environments and results have shown similar toxicity levels to the parent compound, endosulfan (Carriger et al., 2011; Hose et al., 2003; Stanley et al., 2009). However, little focus has been placed on the toxicity of endosulfan and endosulfan sulfate in soil environments, especially to soil-dwelling organisms such as earthworms. Earthworms are important soil organisms because they play a critical role in soil development such as aeration, drainage and transportation of lower soil to the surface (Edwards and Bohlen, 1996). With these major functions, earthworms are useful biological indicators for soil fertility and soil ecosystem health (Paoletti, 1999).

Nuclear magnetic resonance (NMR)-based metabolomics is a powerful diagnostic tool in understanding the metabolic response of organisms to genetic modifications, external stimulus, and/or stressors (Griffin, 2003; Nicholson et al., 1999; Robertson et al., 2005). Environmental metabolomics is an emerging sub-

* Corresponding author.
E-mail address: andre.simpson@utoronto.ca (A.J. Simpson).

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discipline that investigates the metabolic profile of native organisms in their environment to potential stressors that they might encounter (Morrison et al., 2007). In addition, environmental metabolomics has the capability to study biochemical fluxes in endogenous metabolites after exposure to sub-lethal concentrations of contaminants (Simpson and McKelvie, 2009). This in turn allows the understanding of a contaminant's toxic mode of action (MOA), and the identification of the biomarkers that pertain to the exposure (Ekman et al., 2008; Lankadurai et al., 2011). In the soil environment, earthworms have been commonly used in NMR-based metabolomic studies to understand their response to various contaminants such as polyaromatic hydrocarbons (Brown et al., 2010; McKelvie et al., 2010), polychlorinated biphenyls (Whitfield Åslund et al., 2011) and metals (Bundy et al., 2007).

In the present study, one-dimensional (1-D) and two-dimensional (2-D)-based metabolomics will be used to understand the response of the earthworm, *Eisenia fetida* (*E. fetida*), to various concentrations of endosulfan and endosulfan sulfate in soil. Both tissue extracts and coelomic fluid (CF) will be used in tandem to investigate the toxicity and MOA of endosulfan and endosulfan sulfate. Our past metabolomic study (Yuk et al., 2010) used various 1-D and 2-D NMR techniques to examine endosulfan exposure using OECD acute toxicity tests on contact test filter paper (OECD, 1984). The results found that ^{1}H NMR Presaturation Utilizing Relaxation Gradients and Echoes (PURGE) and ^{1}H–^{13}C Heteronuclear Single Quantum Coherence (HSQC) techniques to be most effective in identifying significant metabolites of exposure between the unexposed and exposed earthworms. In a recent earthworm metabolomics study (Yuk et al., 2012), the earthworm's CF was tested as a complementary biological medium to the earthworm tissue extract after endosulfan exposure. The CF is part of a hydrostatic skeleton around the earthworm and acts as the communicator between the inner and outer environment (Cooper and Roch, 2003). The CF is responsible for cellular regulation of nutrition and excretion, which could be potential biomarkers of stress from the exposure to contaminants (Kurek et al., 2007). The CF's metabolic profile helped alleviate spectral overlap from certain metabolites, such as sugars seen in the earthworm tissue extract, to allow the detection of other metabolites in the same spectral region (Yuk et al., 2012). Previous studies focused on contact tests and method development. Thus, this study represents an important transition from traditional contact filter paper tests to endosulfan soil exposure which will provide insight into contaminant–soil interactions and soil ingestion as another mode of contact for earthworms. In addition, this study will investigate and compare the toxicity and MOA of endosulfan sulfate in soil as well.

2. Materials and methods

2.1. Soil spiking

An artificial soil was prepared according to the OECD Earthworm acute toxicity test protocol (OECD, 1984). It was prepared using 10% sphagnum peat (Ward's Natural Science, ON, Canada), 20% kaolin clay (Ward's Natural Science) and 70% sand (Ward's Natural Science). Approximately 125 g (dry weight) of the artificial soil was placed in each of seven 1 L clear glass jars. Six jars were then spiked with 10 ml of endosulfan (99.9% purity; Sigma Aldrich, St. Louis, MO, USA) or endosulfan sulfate (98.8% purity; Sigma Aldrich, St. Louis, MO, USA) using three different spiking concentrations (5 mg/L, 50 mg/L and 500 mg/L) in acetone (HPLC grade, Fisher Scientific). The exposure concentrations for endosulfan and endosulfan sulfate mimicked a comet assay endosulfan study done on earthworms by Liu et al. (2009) using the OECD soil. Since no study has analyzed endosulfan sulfate to earthworms in soil environments, the exposure concentrations were kept the same as endosulfan for a direct comparison. The last glass jar was the unexposed control treatment and was spiked with 10 ml of acetone (Fisher Scientific) only. All the spiked jars were left in the fume hood to vent for 16 h to allow all the acetone to evaporate (Whitfield Åslund et al., 2011). Soil (at 375 g) was then added to each of the seven spiked soil glass jars and mixed thoroughly for a total endosulfan or endosulfan sulfate concentration of 0.1, 1.0 and 10.0 mg/kg (dry weight) for the pesticide-exposed treatments. To ensure proper moisture content (OECD, 1984), all the soils were adjusted using deionized water to 35% moisture content of the soil dry weight. All spiked endosulfan or endosulfan sulfate soil concentrations were confirmed after earthworm exposure using soxhlet extraction and quantification via gas chromatography/mass spectrometry (discussed in detail in Supplementary material section S1 and Table S1).

2.2. Earthworm exposure

E. fetida earthworms were purchased from The Worm Factory (Perth, ON, Canada) and were maintained according to Brown et al. (2008). Twenty mature earthworms with a visible clitellum were added to each of the endosulfan, endosulfan sulfate (3 each) and control soil (7 total soil treatments). All the earthworms had a mean weight of 0.48 ± 0.015 g and there were no significant differences in the average mass of the earthworms between the controls and any of the endosulfan and endosulfan sulfate treatment groups ($p = 0.953$) before exposure (data not shown).

According to the OECD soil exposure tests (OECD, 1984), earthworms were kept in lightly closed jars for 7 days at 21 °C in natural light (Eijsackers et al., 2001), then removed and depurated for 96 h on damp filter paper to remove any excess soil in their gut (Brown et al., 2008). Earthworms used for the CF extraction were separated from the earthworms used for the tissue extraction (detailed procedures are outlined below). For the highest endosulfan and endosulfan sulfate exposure concentration (10.0 mg/kg), six and two earthworms died respectively after depuration and were removed from the study.

2.3. Earthworm coelomic fluid and tissue extraction and preparation for NMR

Each earthworm's CF after exposure (control or exposed) were extracted non-invasively using electrical stimulation described in Yuk et al. (2012) (Section S2). The earthworms that were separated for tissue extraction were immediately flash frozen in liquid nitrogen, lyophilized and prepared according to Yuk et al. (2011) (Section S2). All samples were frozen immediately after preparation and each was thawed prior to NMR analysis.

2.4. 1-D and 2-D NMR spectroscopy

All NMR spectra were acquired using a Bruker Avance III 500 MHz spectrometer with a ^{1}H–^{19}F–^{15}N–^{13}C 5 mm broadband Quadruple Inverse (QXI) probe fitted with an actively shielded *Z* gradient (Bruker BioSpin, Rheinstetten, Germany). The ^{1}H 90° pulse was calibrated for each sample in the study. ^{1}H NMR experiments were performed using PURGE water suppression (Simpson and Brown, 2005) and 512 scans, a recycle delay of 3 s, and 65 K time domain points. All 1-D NMR spectra were manually phased and calibrated to the DSS internal reference methyl singlet, set to a chemical shift (δ) of 0.00 ppm.

^{1}H–^{13}C HSQC NMR experiments were optimized experimentally in terms of the relaxation delay (d1) and the number of increments in the indirect dimension (F1) as described in Yuk et al. (2010). All HSQC NMR spectra were collected in phase-sensitive mode using echo/anti-echo gradient selection, a ^{1}J ^{1}H–^{13}C (145 Hz), and a relaxation delay of 0.5 s. Twenty scans and 2048 data points were collected for each of the 196 increments in the F1 dimension. The F2 dimension was processed using an exponential function corresponding to a line broadening of 15 Hz, while the F1 dimension was processed using a sine-squared function with a $\pi/2$ phase shift. Both dimensions were zero-filled by a factor of two while forward linear prediction using 32 coefficients was applied in the F1 dimension. All 2-D NMR spectra were manually phased and calibrated to the DSS internal reference methyl singlet, set to a chemical shift (δ) of 0.00 ppm.

2.5. Data and statistical analysis

Principal Component Analysis (PCA) was performed on the 1-D and 2-D NMR spectra of the earthworm's CF and tissue extracts using an *Analysis of Mixtures* (AMIX) statistics package (version 3.9.8, Bruker BioSpin). The earthworm CF ^{1}H NMR spectra were divided into width bins of 0.02 ppm from the region 0.25–9.0 ppm using *Analysis of Mixtures* (AMIX) statistics package (version 3.9.8, Bruker BioSpin) and the region from 4.35 to 5.21 ppm was excluded due to residual H_2O/HOD signals present in this region. The earthworm tissue extract ^{1}H NMR spectra were divided into width bins of 0.02 ppm from the region 0.25–9.0 ppm and the region from 4.75 to 4.90 ppm was not included due to residual H_2O/HOD signals present in this region. For the earthworm tissue extract ^{1}H–^{13}C HSQC spectra, the carbon spectrum (F2) was divided into width bins of 0.50 ppm from the region of 10.0–140.0 ppm and the ^{1}H spectrum (F1) was divided into width bins of 0.05 ppm from the region 0.25–9.0 ppm. The region of 4.75–4.90 ppm on the ^{1}H spectrum with their associated carbon spectrum from the region 10.0–140.0 ppm was excluded due to the residual H_2O/HOD signals present in this region. The "sum of intensities" was used as the integration mode and the scaling was set to "total intensity" for all the NMR spectra. PCA was performed at the 95% confidence level and any variances that represented less than 1% or 2.5% in the bins for 1-D and 2-D NMR spectra were excluded (Brown et al., 2010; Yuk et al., 2011). Therefore in this study, each treatment group and unexposed control group had ten earthworms each for both CF and tissue extract experiments except for the highest endosulfan concentration (10.0 mg/kg) which

had six earthworms in the earthworm CF group and eight earthworms in the tissue extract group and for the high endosulfan sulfate concentration (10.0 mg/kg) which had eight earthworms in the tissue extract group. Individual and mean PCA scores with their associated standard errors for control and exposure concentrations were calculated and graphed to understand the differences between the unexposed and exposed earthworm groups. Dunnett's multiple comparison tests were conducted on the PC scores to indicate which treatment groups were significantly different from the control group ($p < 0.05$). *T*-test and Dunnett's multiple comparison tests were performed using SPSS 19.0 (IBM, Somers, NY, USA).

Multiple *t*-test filtered difference ^{1}H NMR and ^{1}H–^{13}C HSQC NMR spectra were constructed to identify increases or decreases in the peaks between the control and each exposure concentration set for the identification of metabolites (Ekman et al., 2009; Yuk et al., 2011). *T*-test filtered difference NMR spectra were calculated for each exposure concentration (0.1, 1.0 and 10.0 mg/kg) from the ^{1}H NMR spectra for the earthworm CF (Figs. S3 and S4, respectively) and ^{1}H NMR spectra for the tissue extract (Figs. S5 and S6, respectively), and ^{1}H–^{13}C HSQC NMR spectra for the tissue extract (Figs. S7 and S8, respectively). More detail about the *t*-test filtered difference NMR spectra and identification of metabolites are described in the supplementary material (Section S3). Percent changes for the identified metabolites in the difference spectrum of exposed earthworms relative to control were calculated by the equation: $(I_E - I_C)/I_C \times 100$. I_E is the mean bucket intensity for the exposed earthworm group and I_C is the mean bucket intensity for the control earthworm group.

3. Results and discussions

3.1. Multivariate analysis on earthworm CF and tissue extracts

Mean and individual PCA scores plots were calculated for the ^{1}H NMR and ^{1}H–^{13}C HSQC NMR spectra for the earthworm's CF and tissue extract after endosulfan (Fig. 1A–C and Fig. S1) and endosulfan sulfate exposure (Figs. 1D–F and S2). For the mean PCA scores plot of the endosulfan exposed earthworm CF (Fig. 1A), the highest separation between the first two principal components (PC1 and PC2) was seen and accounted for 64.9% of the total variance. Separation from the control group increased with endosulfan exposure concentration. The highest concentration (10 mg/kg), was found to be significantly different from the control ($p < 0.05$). The separation between the lowest and middle exposure concentration (0.1 and 1.0 mg/kg) were clustered close together and separation compared to the control group was seen. This suggests similar response to endosulfan in that range of concentrations studied. For the mean PCA scores plot of the endosulfan sulfate-exposed earthworm CF (Fig. 1D), PC1 and PC2 had the highest separation in PCA scores plot with both axes accounting for 54.2% of the total variance. The endosulfan sulfate PCA scores separation was analogous to the endosulfan PCA scores and there was comparable separation at the low and middle concentrations (0.1 and 1.0 mg/kg), and at the highest concentration (10 mg/kg) there was significant separation in comparison compared to the control group ($p < 0.05$).

For the endosulfan-exposed earthworm tissue extracts, ^{1}H NMR and ^{1}H–^{13}C HSQC NMR PCA scores plot (Fig. 1B and C) showed the greatest separation in the PC1 and PC2 axes and both accounted for 90.6% and 90.9% of the total variance respectively. The highest

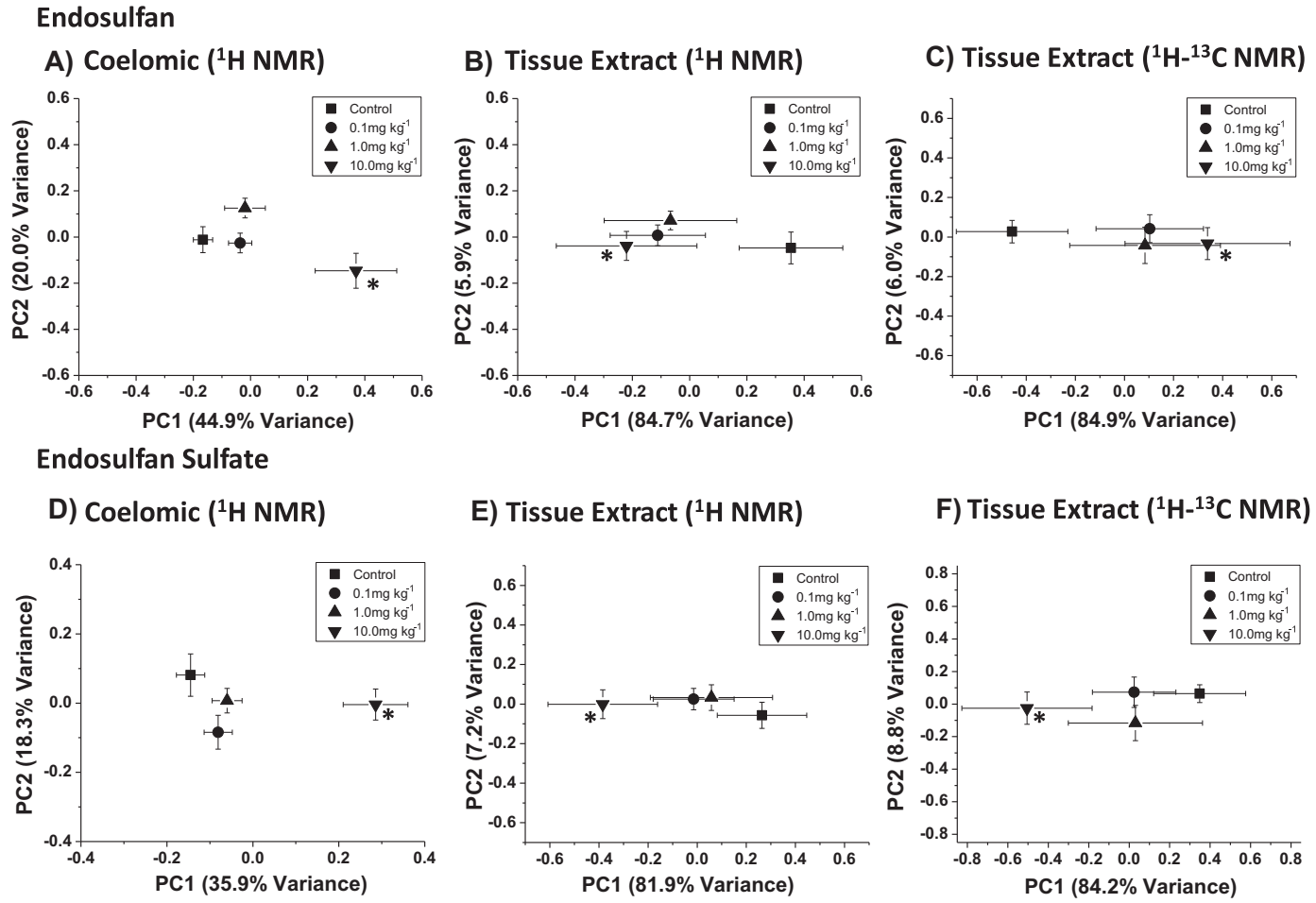

Fig. 1. Mean PCA scores plots of PC1 vs. PC2 of endosulfan-exposed *E. fetida*: A) coelomic fluid using 1-D NMR, B) tissue extracts using 1-D NMR, C) tissue extracts using 2-D HSQC NMR spectra and endosulfan sulfate exposed *E. fetida*: D) coelomic fluid using 1-D NMR, E) tissue extracts using 1-D NMR and F) tissue extracts using 2-D HSQC NMR spectra. Each point represents the mean PC score for each exposure concentration and the error bar represents the standard error of the mean. The legend indicates the exposure concentrations for each point. The "*" represents mean concentrations that were significantly different from the control ($p < 0.05$) using Dunnett's multiple comparison test.

endosulfan concentration (10 mg/kg) was significant at $\alpha = 0.05$ level for both 1-D and 2-D NMR PCA scores plots. The endosulfan sulfate-exposed earthworm tissue extracts ^{1}H NMR and ^{1}H–^{13}C HSQC NMR PCA scores plots (Fig. 1E and F) had similar separation trends along PC1 and PC2 axes and accounted for 89.1% and 93% of the total variance respectively. The low and middle concentrations (0.1 and 1.0 mg/kg) were close together in both 1-D and 2-D NMR mean PCA scores plots. However, the highest endosulfan sulfate concentration (10 mg/kg) was significantly different at the $p < 0.05$ level.

Earthworm CF was more significant to endosulfan and endosulfan sulfate soil exposure compared to the tissue extracts, especially at the highest exposure concentration (10 mg/kg) from comparison of their significance value ($p = 4.0 \times 10^{-4}$ vs. $p = 0.04$ for endosulfan and $p = 5.1 \times 10^{-7}$ vs. $p = 0.04$ for endosulfan sulfate, respectively). The earthworm's CF has a primary role in regulating homeostasis and is the first immune defense against external stimuli (Kurek et al., 2007). With various hemolytic, proteolytic and cytotoxic enzymes in the CF to constantly and actively protecting the organism from any foreign substances (Kauschke et al., 1997), it is plausible that the CF may be more sensitive to immediate changes in the soil environment by the presence of contaminants.

3.2. Metabolic response after endosulfan and endosulfan sulfate exposure

From Table S2, the advantage of analyzing both the earthworm's CF and tissue extract using 1-D and 2-D NMR can be seen as a number of response metabolites can be identified to be significant due to endosulfan and endosulfan sulfate exposure. From the relative percent change for each identified metabolite in the CF (^{1}H NMR) and tissue extracts (^{1}H and ^{1}H–^{13}C NMR) for each endosulfan exposure concentration (Fig. 2 and Fig. 3A and B, respectively) and endosulfan sulfate exposure concentration (Fig. 4 and Fig. 5A and B, respectively), the same metabolites of response were identified in both endosulfan and endosulfan sulfate exposed earthworms with similar increases or decreases at the various exposure concentrations. The similar metabolic trends observed for both contaminants suggest a similar toxic MOA for earthworms. Duplicate metabolites identified in the tissue extract by ^{1}H–^{13}C HSQC that were already detected in the ^{1}H NMR were added to the supplementary material (Fig. S9 for endosulfan and Fig. S10 for endosulfan sulfate). For the earthworm ^{1}H and ^{1}H–^{13}C NMR spectra, the reader is recommended to our previous earthworm metabolomic study where the NMR spectra with the identified metabolites are shown (Yuk et al., 2012).

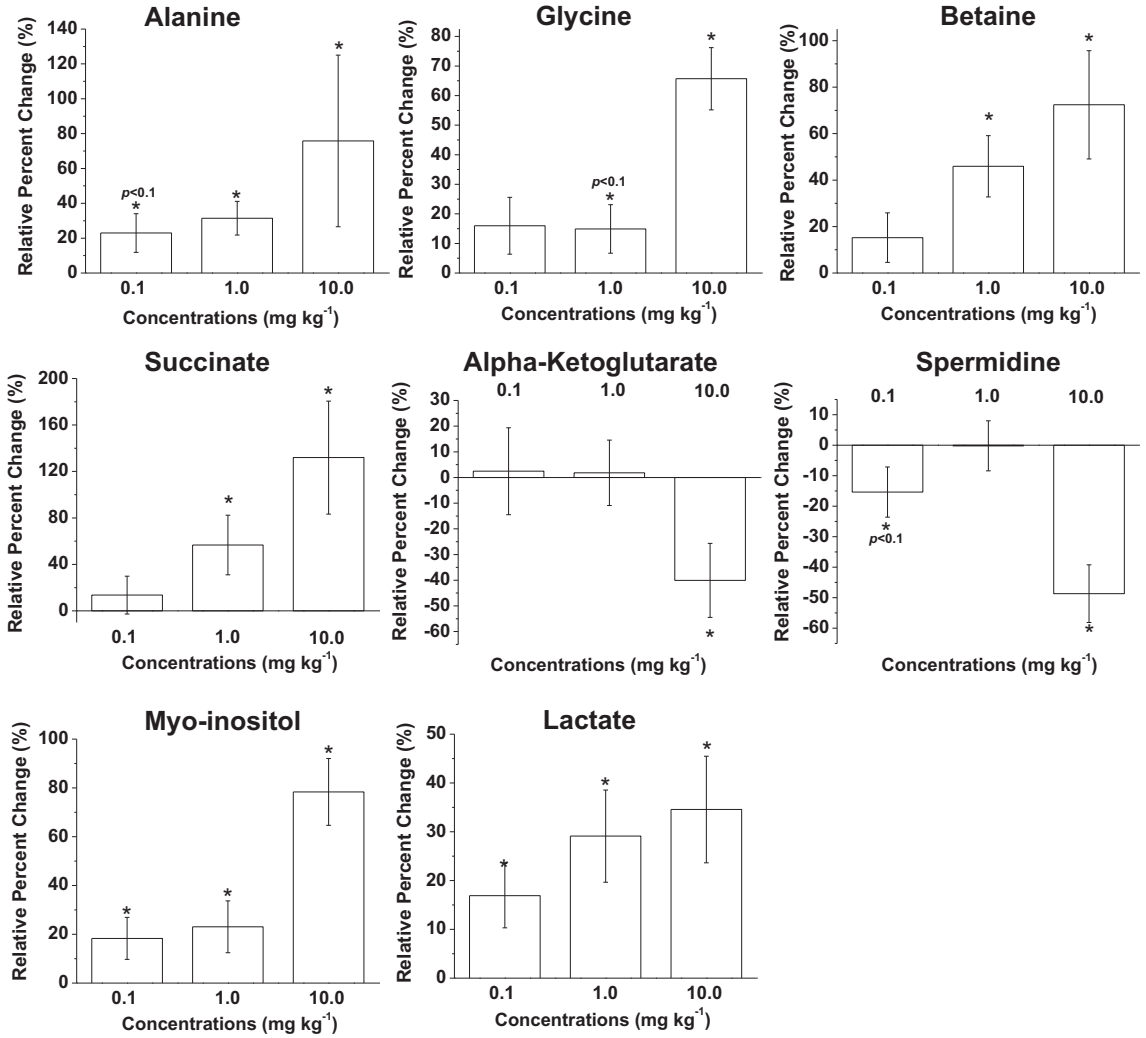

Fig. 2. Percent change (%) of all identified metabolites from the *t*-test filtered 1-D NMR difference spectra of endosulfan-exposed *E. fetida* coelomic fluid. Percent changes that were significantly different from the control ($p < 0.05$) are labeled with "*". Each percent change is shown with their associated standard error.

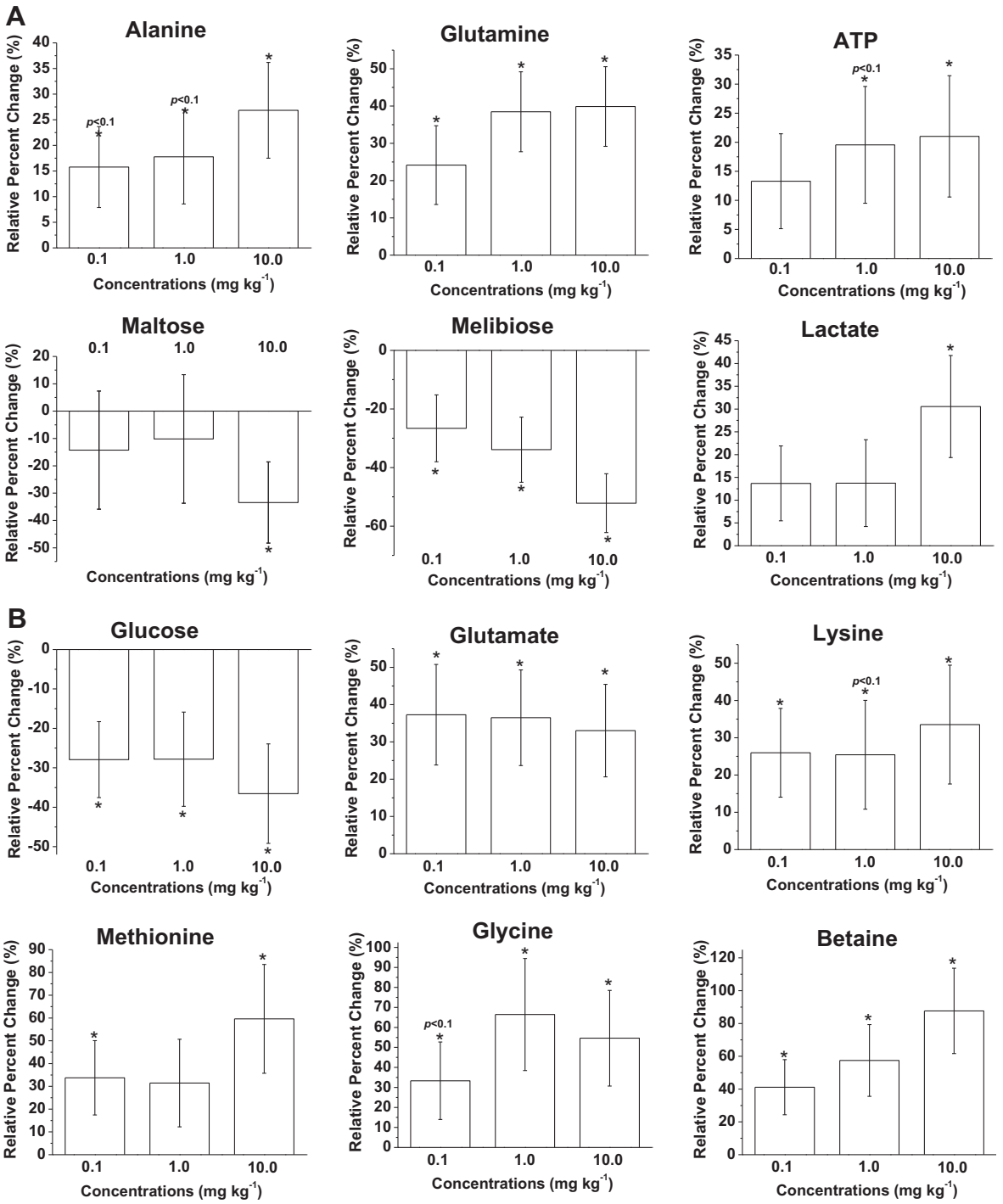

Fig. 3. Percent change (%) of all identified metabolites from the *t*-test filtered A) 1-D NMR and B) 2-D NMR difference spectra of endosulfan-exposed *E. fetida* tissue extracts. Percent changes that were significantly different from the control ($p < 0.05$) are labeled with "*". Each percent change is shown with their associated standard error.

Endosulfan is known to inhibit the action of the neurotransmitter gamma aminobutyric acid (GABA), which is responsible for the uptake of chloride ions by neurons (Waagepetersen et al., 2003). This is a critical biological process because GABA assists in returning the neuron into its homeostatic state after depolarization. However, the inhibitory action by endosulfan results in only a partial repolarization of the neuron and leads to uncontrolled muscle contractions, convulsions and hyperactivity in organisms (Jia and Misra, 2007). A past toxicity study on endosulfan exposure in living organisms has shown physiological changes such as muscular twitching and restlessness (Ribeiro et al., 2001). In this study, all the earthworms exposed to endosulfan or endosulfan sulfate showed similar behavioral symptoms of restlessness and uncontrolled muscle contractions at lower concentrations (0.1 and 1.0 mg/kg) but body stiffness at the highest soil concentration (10.0 mg/kg). This observation was also seen in our previous endosulfan exposure study on *E. fetida* but using contact test filter paper where body stiffness and muscular twitching was also detected (Yuk et al., 2011). A number of studies, using lethality experiments analyzed the toxicity of endosulfan sulfate and

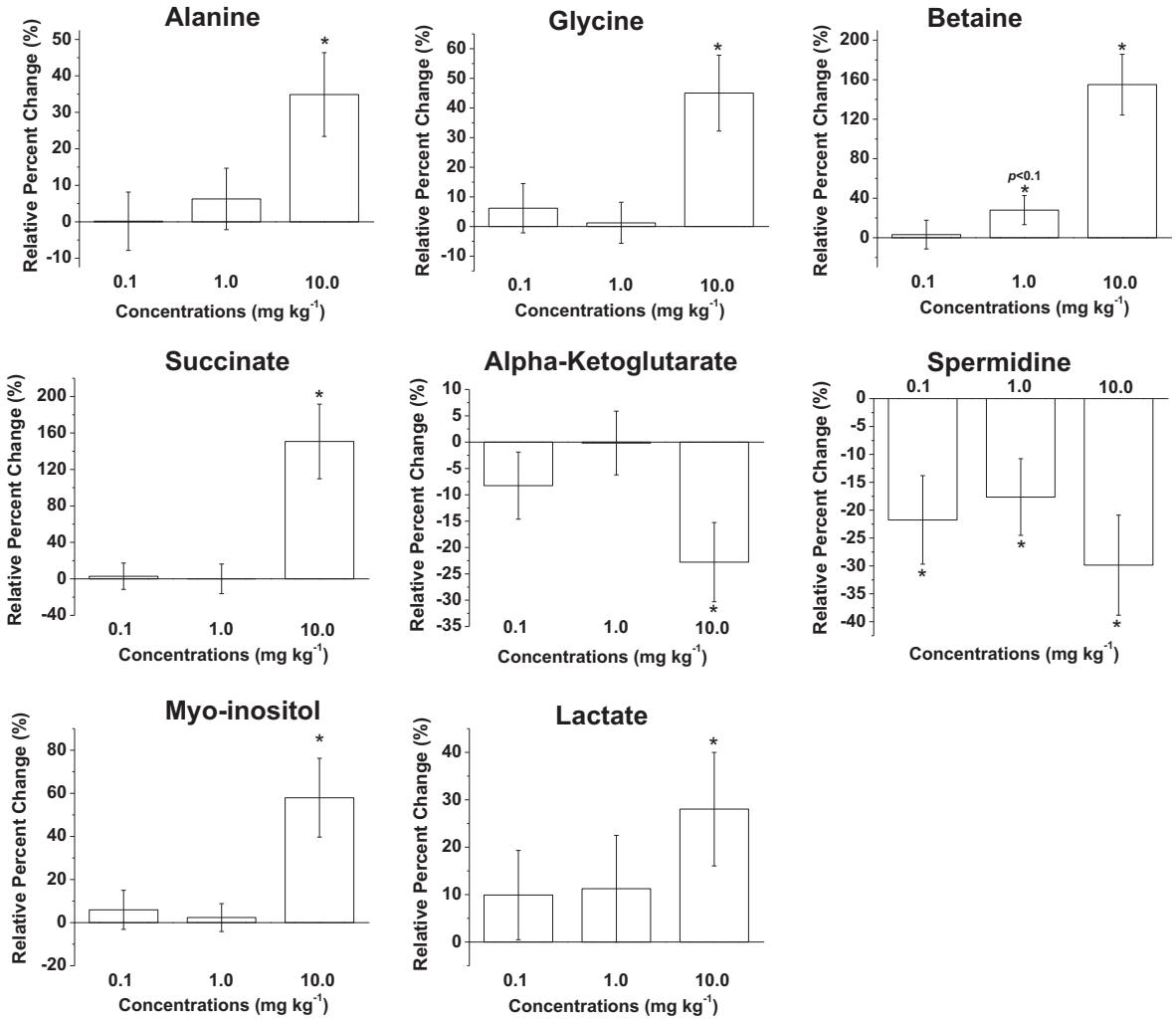

Fig. 4. Percent change (%) of all identified metabolites from the *t*-test filtered 1-D NMR difference spectra of endosulfan sulfate-exposed *E. fetida* coelomic fluid. Percent changes that were significantly different from the control ($p < 0.05$) are labeled with "*". Each percent change is shown with their associated standard error.

suggested a similar toxicity to the parent compound in aquatic organisms (Carriger et al., 2011; Stanley et al., 2009; Wan et al., 2005). In the present study, the same response metabolites were detected with similar fluctuations in both endosulfan and endosulfan sulfate exposed earthworms. This finding further suggests a similar neurotoxic MOA in soil.

Glutamine, a precursor to GABA, and glutamate, an exhibitory neurotransmitter, significantly increased compared to the control ($p < 0.05$) in all exposure concentrations for both endosulfan- and endosulfan sulfate-exposed earthworms. Neurons are considered to be metabolically challenged as they do not have the mechanisms for producing glutamate and GABA from simpler molecules such as glucose (Hertz et al., 1999). Therefore, an essential metabolic transport system called the glutamine/GABA–glutamate cycle is used to regulate their concentrations from nearby astrocytes for effective inhibitory and exhibitory transmissions (Waagepetersen et al., 2003). Extracellular glutamate levels have been reported in neurological stress conditions especially linked to environmental toxins (Obrenovitch and Urenjak, 1997) and can cause an excitotoxic response in earthworms. The production of glutamate in astrocytes comes from the citric acid cycle (CAC) intermediate alpha-ketoglutarate (Bak et al., 2006) and was detected to be significantly decreased compared to the control at the highest concentration (10.0 mg/kg) in the endosulfan and endosulfan sulfate exposed earthworm's CF. Due to the competitive inhibition of the GABA chloride channels by endosulfan or endosulfan sulfate exposure, an influx of GABA will be present in the neurons. The removal of excess GABA in the neurons is caused by catabolism to the CAC intermediate succinate (Bak et al., 2006), and this increased in the CF of both the endosulfan and endosulfan sulfate exposed earthworm. No other CAC intermediates in the exposed earthworms were significantly different compared to the control group and this illustrates the sufficient regulation of the CAC for energy production during exposure. However in our past research study (Yuk et al., 2012), the CAC intermediates alpha-ketoglutarate, succinate, malate and fumarate were significantly decreased compared to the control after sub-lethal endosulfan exposure using contact test filter paper experiments (Yuk et al., 2012). In this study, the soil environments which contain organic matter were used and can potentially provide a source of energy for the earthworms compared to their exposure on contact test filter paper. Therefore, the transition into soil environments can potentially provide the earthworms with the essential nutrients needed to sustain important aerobic energy cycles such as the CAC even in the presence of contaminants.

An increased energy expenditure in the earthworm was identified by the significant ($p < 0.05$) decrease in the sugar concentrations (maltose, melbiose and glucose) and increased ATP

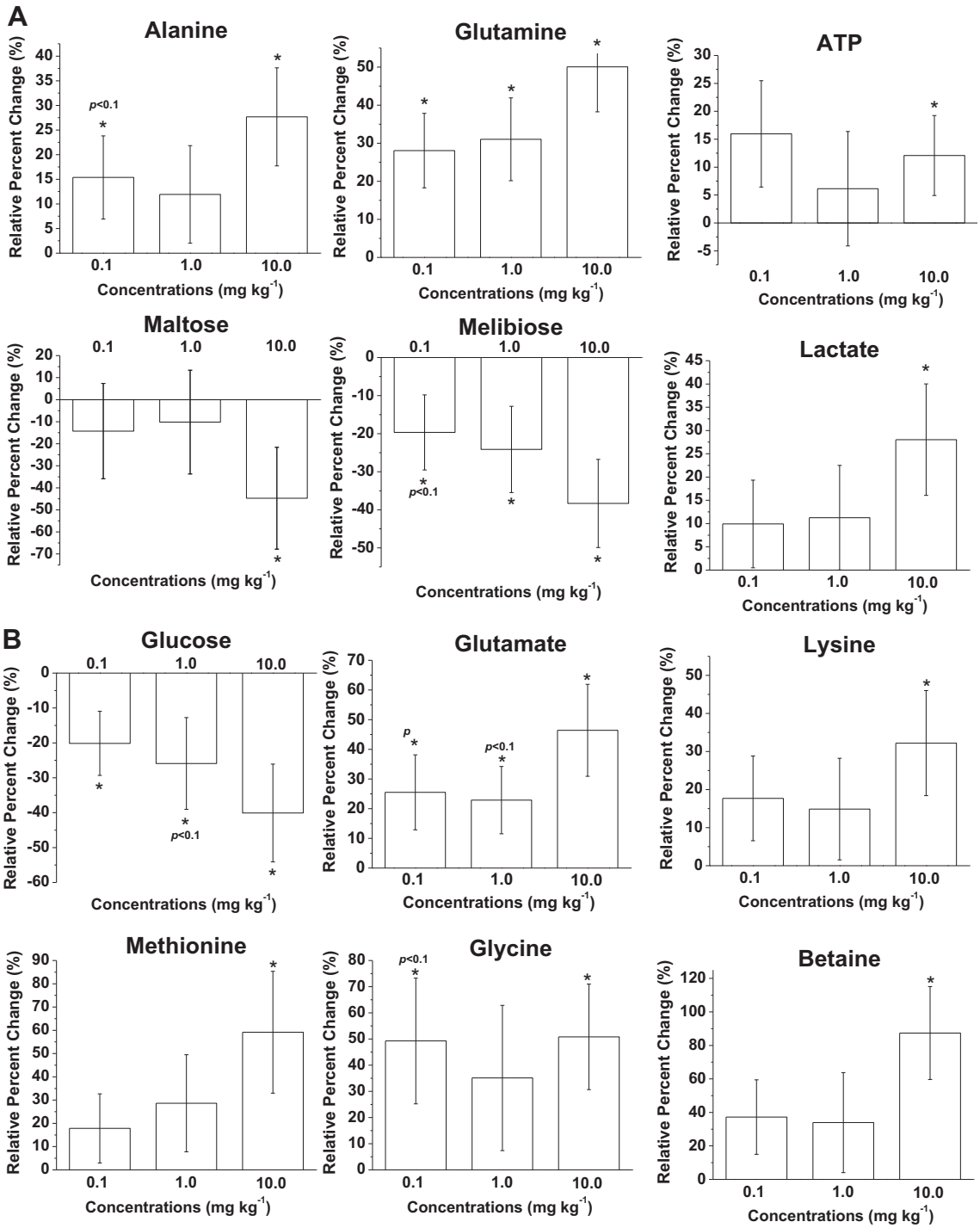

Fig. 5. Percent change (%) of all identified metabolites from the *t*-test filtered A) 1-D NMR and B) 2-D NMR difference spectra of endosulfan sulfate-exposed *E. fetida* tissue extracts. Percent changes that were significantly different from the control ($p < 0.05$) are labeled with "*". Each percent change is shown with their associated standard error.

concentration by endosulfan and endosulfan sulfate exposure (10.0 mg/kg). Lactate, a key metabolite in anaerobic metabolism for energy production and commonly detected in organisms after extraneous muscle activity (Robergs et al., 2004), increased as well in all exposure concentrations.

The polyamine spermidine decreased to significant levels in the earthworm's CF for endosulfan- and endosulfan sulfate-exposed earthworms. Polyamines are essential for cellular proliferation and gene regulation. A past study has shown that their abnormal levels can impair cellular function (Seiler and Raul, 2005). Significant decreases in spermidine concentrations are known to cause apoptosis in cells due to external stressors (Grassilli et al., 1995). A past study (Liu et al., 2009) detected DNA damage using comet assays in *E. fetida* after endosulfan exposure in OECD soil. Significant DNA damage was detected at soil concentrations (0.1, 1.0 and 10.0 mg/kg) after 7, 14, 21 and 28 days exposure in their study ($p < 0.01$), and it was concluded that the usage of comet assays is a reliable indicator for endosulfan exposure. In this study, the results provided further biochemical insight for endosulfan and gave a first look into endosulfan sulfate exposure using the same

soil exposure concentrations as the decrease in spermidine was also detected. A decrease in spermidine was also seen in our previous earthworm metabolomic study (Yuk et al., 2012) after endosulfan exposure but using contact filter paper test. This result shows that difference in the mode of exposure to the contaminant (contact using filter paper or ingestion of soil) was irrelevant and a similar decrease of spermidine was still detected. An apoptotic MOA resulting from endosulfan and endosulfan sulfate exposure could be due to a potential defensive mechanism by a significant DNA damage in the earthworms. The rise in apoptosis will increase muscle and protein degradation in organisms (Tews, 2002) and this was detected as free amino acids, lysine and methionine, significantly increased in the endosulfan- and endosulfan sulfate-exposed earthworms compared to the control.

Alanine and glycine also increased in the endosulfan- and endosulfan-exposed earthworm's CF and tissue extract compared to the unexposed earthworms. Alanine and glycine are both known for their cytoprotective action in cells against stress damage by contaminants (Nissim et al., 1992) and are known to increase in cells to induce gene expression for stress protein synthesis (Forcella et al., 2007; Howard et al., 2010). Osmolytes, betaine and myo-inositol, also increased in endosulfan- and endosulfan sulfate-exposed earthworms compared to the unexposed earthworms. Hydrophobic contaminants such as endosulfan and endosulfan sulfate can cause fluctuations in the intracellular solute content and stability in biological membranes (Nelson et al., 1990). Betaine and myo-inositol are both common organic osmolytes in biological systems that assist in maintaining osmotic balance in cells (Strange et al., 1991; Yancey et al., 1982). Increases in betaine and myo-inositol were also identified in *E. fetida* from exposure to other hydrophobic contaminants such as polyaromatic hydrocarbons (Lankadurai et al., 2011) and also in our previous endosulfan study using contact filter paper test (Yuk et al., 2012).

3.3. Comparison of endosulfan- and endosulfan sulfate-exposed earthworm CF and tissue extracts using multivariate analysis

To investigate the relative toxicity between endosulfan and endosulfan sulfate, an overall mean PCA scores plot was constructed using all exposure concentrations from the 1-D and 2-D NMR spectra of the earthworm CF and tissue extract (Fig. S11). Examining the separation trajectory using various contaminant exposure groups provides insight into differences in their toxicity or MOAs. For example, in our previous earthworm NMR-based metabolomics study (Yuk et al., 2011), an overall PCA scores plot showed separate trajectories for two different pesticides (trifluralin and endosulfan) using three sub-lethal exposure concentrations. Different metabolites of response were detected for both trifluralin- and endosulfan-exposed earthworms and explained two different toxic MOAs. In another study (McKelvie et al., 2011), two pesticides (carbaryl and chlorpyrifos), three pharmaceuticals (carbamazephine, estrone and caffeine), two persistent organohalogens (Aroclor 1254 and PBDE 209) and two industrial compounds (nonylphenol and dimethyl phthalate) were investigated on their sub-lethal exposure to *E. fetida* using an overall PCA scores plot. Their results were able to identify contaminant specific biomarkers from the various MOAs using 1-D NMR-based metabolomics.

In the overall mean PCA scores plot of the 1-D NMR spectra of the earthworm's CF (Fig. S11A), the endosulfan- and endosulfan sulfate-exposed earthworms had similar separation trajectories on the PC1 and PC2 axis as both axis represented a total variance of 57.5%. The low and middle concentrations (0.1 and 1.0 mg/kg) were closely grouped for both contaminants and were similar in their separation to the control group on the PC1 axis. The two highest concentrations of endosulfan and endosulfan sulfate (10 mg/kg) were significantly different compared to the control group at $p < 0.05$. For the overall mean PCA scores plot of the 1-D and 2-D NMR spectra of the earthworm tissue extract (Figs. S11B and S11C, respectively), a similar separation trajectory from the exposed earthworms to the control was seen on the PC1 and PC2 axes with a total variance of 89.2% and 92% respectively. Higher separation by the exposed groups were seen for both 1-D and 2-D NMR PCA scores plots to the control group as the concentration increased for both contaminants. The highest exposure concentrations of both the endosulfan and endosulfan sulfate exposure groups were significantly different than the control ($p < 0.05$).

A similar separation trajectory, by the endosulfan and endosulfan sulfate exposure groups to the control group in the PCA scores plot, shows the similarity of their toxicity. In addition, this result explains the identical response metabolites detected in the *t*-test filtered difference NMR spectra and confirms their MOA. There were no significant differences between each of the endosulfan and endosulfan sulfate exposure groups even at the highest exposure concentration (10 mg/kg) in the earthworm CF and tissue extract. The results in this study confirm past endosulfan and endosulfan sulfate toxicity studies where only aquatic species were examined using growth/survival bioassays (Wan et al., 2005) or half maximal effective concentrations (EC_{50}) (Stanley et al., 2009). The use of NMR-based metabolomics provided a rapid and in-depth approach in probing soil environments using earthworms as biological indicators in comparing the toxic MOA of these two contaminants. Generally, the degradation of pesticides in soil has a lower toxicity to native organisms compared to the parent compound (Andreu and Pico, 2004). However, in this study, the main degradation product, endosulfan sulfate, was detected to be just as toxic as the parent compound, endosulfan, in soil. Since endosulfan sulfate is more persistent in the soil environment than endosulfan (Rand et al., 2010), higher priority should be given to the identification of endosulfan sulfate in contaminated soils during bioremediation efforts because the results from this study show that toxicity does not change as the parent compound, endosulfan, degrades.

4. Conclusions

Understanding the environmental consequences by persistent organohalogen pesticides on ecosystem health is a major priority for many environmental agencies such as the OECD and United Nations Environment Program (Liu et al., 2009). Our study demonstrates the potential of 1-D and 2-D NMR-based metabolomics in delineating the toxic MOA of a persistent environmental contaminant, endosulfan, and its main degradation product, endosulfan sulfate, at various exposure concentrations in soil for seven days. The results displayed a similar toxicity for both contaminants by the same increase in separation from the control group as the exposure concentration increased in the PCA scores plot. A similar neurotoxic and apopotic MOA was observed in both endosulfan and endosulfan sulfate exposed earthworms as identical metabolites of response were detected compared to the unexposed earthworms. However, in this study, unaged, spiked soil with only seven days of exposure were used as an initial experiment to understand the toxicity of contaminants and their degradation product to native soil organisms. Future studies will use aged spiked soils and longer exposure times (>seven days) to assess contaminant toxicity as past studies have shown the decrease in the bioavailability of contaminants over time in soil (McKelvie et al., 2010; Semple et al., 2003) and a change in the earthworms metabolic response to contaminants over various times of exposure (Lankadurai et al., 2011). In addition, biological assays will be conducted to understand more about the neurotoxic

and apoptotic MOA. For example, the detection of DNA damage can be conducted using comet assays (Liu et al., 2009) to monitor the earthworm's exposure to aged soils and contaminants over time and correlate to the decrease in spermidine concentrations. Nevertheless, this is the first metabolomics study that utilizes 1-D and 2-D NMR techniques and tests both earthworm CF and tissue extracts to compare their biochemical response to a concerning environmental contaminant and its main degradation product in soil. NMR-based metabolomics can be a powerful ecotoxicology tool to provide vital toxicity information for assessing contaminated soil sites using earthworms as biological indicators.

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Appendix A. Supplementary material

Supplementary materal related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2012.12.007.

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