

^1H NMR and GC/MS metabolomics of earthworm responses to sub-lethal DDT and endosulfan exposure

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Abstract The metabolic response of the earthworm *Eisenia fetida* to two pesticides, dichlorodiphenyltrichloroethane (DDT) and endosulfan, was characterized in contact tests using proton nuclear magnetic resonance (^1H NMR) and principal component analysis (PCA). PCA loading plots suggested that maltose, leucine and alanine were important metabolites contributing to the differences in dosed and control earthworms for both compounds at doses of 0.5, 1.0 and 2.0 $\mu\text{g}/\text{cm}^2$. Gas chromatography/mass spectrometry (GC/MS) was used to quantify the metabolites identified in *E. fetida* and determine if the changes in maltose, leucine and alanine following exposure to DDT and endosulfan (at 0.5 and 1.0 $\mu\text{g}/\text{cm}^2$) were reproducible and greater than the natural variability. Quantification by GC/MS suggested that maltose was not a reliable biomarker since it both increased and decreased in earthworms exposed to DDT and increased by just 3% with exposure to endosulfan. Leucine was not stable with the GC/MS derivitization method used in this study and could not be confirmed as a reliable biomarker. However, alanine consistently increased for both DDT and endosulfan exposed *E. fetida*. Alanine showed considerable variability in control earthworms ($\pm 41.6\%$), yet the variability in alanine to glycine ratios was just $\pm 10.5\%$. Increases in the

alanine to glycine ratio were statistically significant at the $P = 0.05$ level for the 1.0 $\mu\text{g}/\text{cm}^2$ DDT dose and both the 0.5 and 1.0 $\mu\text{g}/\text{cm}^2$ endosulfan doses, suggesting that deviations from the normal homeostatic ratio of 1.5 for alanine to glycine is a potential biomarker of DDT and endosulfan exposure warranting further study.

Keywords Alanine to glycine ratios · *Eisenia fetida* · Metabonomics · Principal component analysis

1 Introduction

Earthworms account for a large portion of the total animal biomass found in soils, contribute significantly to organic matter degradation and soil fertility and are hence an ideal organism to assess ecosystem health (Paoletti 1999; Spurgeon et al. 2003; van Rhee 1977). While there is considerable information about the impact of high concentrations of pesticides on earthworms (Edwards and Bohlen 1992), there is much less information about the effect of sub-lethal contaminant doses. However, even low-levels of contaminants have the potential to impact ecosystem health and induce stress on soil organisms (Mosleh et al. 2003; Neuhauser and Callahan 1990). As such, there is a need to identify biomarkers that are indicative of a biological response from exposure to sub-lethal levels of contaminants so that they can be used to provide an early warning of environmental damage (Bundy et al. 2002; Peakall 1994; Viant et al. 2005). Metabolomics offers a promising and innovative way to characterize the biochemical response of an organism to contaminant exposure and identify potential biomarkers of exposure (Lin et al. 2006; Robertson 2005).

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The end-products of metabolism, including amino acids and sugars, are increasingly being identified as biomarkers of contaminant exposure to earthworms in metabolomic studies (Lin et al. 2006; Robertson, 2005). Potential biomarkers in earthworms have been identified in contact tests with halogenated anilines (Bundy et al. 2002; Lenz et al. 2005; Warne et al. 2000) and 3-fluoro-4-nitrophenol (Bundy et al. 2001) using metabolomic approaches. In addition, soil studies have identified changes in the metabolic profile of earthworms exposed to pyrene (Jones et al. 2007) and metals (Bundy et al. 2004; Gibb et al. 1997). The specific metabolic responses identified in these studies are summarized in Table 1. Although metabolomic approaches have shown considerable success identifying potential biomarker metabolites in earthworms, the concentrations are rarely quantified. While the metabolomic approach is well suited for screening a large number of samples and identifying potential biomarkers, additional validation is needed to avoid false positives and ensure that changes in the biomarker are reproducible and greater than the natural variation in the test organism (Depledge and Fossi 1994; Lay et al. 2006; Viant et al. 2003). Further evaluation including, but not limited to, assessment of dose-response relationships, time-response relationships, and the specificity of the biomarker to the contaminant of interest should

also be conducted to ensure that the identified biomarkers can be applied to assess sub-lethal contaminant exposure in field situations with certainty (Svendsen et al. 2004; van der Oost et al. 2003).

In this study, the metabolic response of *Eisenia fetida* to two organochlorine pesticides with varying degrees of toxicity, dichlorodiphenyltrichloroethane (DDT) and endosulfan, was compared. While DDT usage has been largely eliminated under the Stockholm Convention, residues of DDT in agricultural soils continue to persist years after application (Kurt-Karakus et al. 2006). The World Health Organization has recommended the use of DDT in developing countries to combat malaria and its usage may therefore increase in the near future (Lubick 2007). Although endosulfan is banned in the European Union, it continues to be a commonly applied pesticide in North America and has been detected hundreds of kilometers from known application areas (Muir et al. 2004). *E. fetida* was chosen as a test species since it is the earthworm species recommended by the Organization for Economic Cooperation and Development (OECD) for ecotoxicological studies (OECD 1984). *E. fetida* is a suitable species for metabolomic investigations since the extraction method has already been developed and shows excellent reproducibility (Brown et al. 2008). In addition, Brown et al. (2008) have already identified the chemical shifts and multiplicity of the

Table 1 Metabolite changes in earthworms identified following exposure to contaminants

Compound	Response	Species ^a (exposure method)	References
2-fluoro-4-methylaniline	↓ 2-hexyl-5-ethyl-3-furansulfonate, ↑ inosine monophosphate	<i>E. veneta</i> (contact tests)	Bundy et al. (2002)
4-fluoroaniline	↓ maltose	<i>E. veneta</i> (contact tests)	
3,5-difluoroaniline	↓ 2-hexyl-5-ethyl-3-furansulfonate, ↑ inosine monophosphate	<i>E. veneta</i> (contact tests)	
3-trifluoromethylaniline	↑ lactate (in coelomic fluid)	<i>E. veneta</i> (contact tests)	Lenz et al. (2005)
3-trifluoromethylaniline	↑ alanine, ↑ glycine, ↑ asparagine, ↑ glucose, ↑ citrate, ↑ succinate	<i>E. veneta</i> (contact tests)	Warne et al. (2000)
3-fluoro-4-nitrophenol	↓ acetate, ↓ malonate (both in coelomic fluid)	<i>E. veneta</i> (contact tests)	Bundy et al. (2001)
Pyrene	↓ lactate, ↓ tetradecanoic acid, ↓ hexadecanoic acid, ↓ octadecanoic acid, ↑ alanine, ↑ leucine, ↑ valine, ↑ isoleucine, ↑ lysine, ↑ tyrosine, ↑ methionine	<i>L. rubellus</i> (spiked soil)	Jones et al. (2007)
Mixed metals ^b	↑ maltose, ↑ histidine ↓ histidine, ↓ 1-methyl-histidine, ↓ maltose	<i>L. rubellus</i> (collected from contaminated soil) <i>L. terrestris</i> (collected from contaminated soil)	Bundy et al. (2004)
Copper (II)	↑ histidine	<i>L. rubellus</i> (field soil mesocosms)	Gibb et al. (1997)

^a *E.* is *Eisenia* and *L.* is *Lumbricus*

^b Cadmium, copper, lead and zinc (Filzek et al. 2004)

major metabolites extracted from *E. fetida* with phosphate buffer using proton nuclear magnetic resonance (^1H NMR). While the toxicity of DDT and endosulfan has been reported in numerous studies investigating mortality and impacts on reproduction (Doane 1962; Hans et al. 1990; Heimbach 1988; Roberts and Dorough 1984), to the best of our knowledge, metabolic biomarkers of sub-lethal exposure have not been characterized for these compounds. DDT has been classified as relatively nontoxic to *E. fetida* and the lethal concentration that kills 50 percent of the population (LC_{50}) is $>1,000 \mu\text{g}/\text{cm}^2$ for the OECD contact test (Roberts and Dorough 1984). In contrast, endosulfan has been classified as very toxic to *E. fetida* with contact LC_{50} values of $24 \mu\text{g}/\text{cm}^2$ (Roberts and Dorough 1984) and $5.7 \mu\text{g}/\text{cm}^2$ (Heimbach 1988).

The first objective of this study was to identify potential biomarkers in *E. fetida* indicative of sub-lethal exposure to DDT and endosulfan during contact tests (OECD 1984) using ^1H NMR and principal component analysis (PCA). The second objective was to quantify the metabolite concentrations in *E. fetida* and assess the reproducibility of the biomarkers identified as indicators of DDT and endosulfan exposure using gas chromatography/mass spectrometry (GC/MS). The potential of ratios between biomarkers of exposure and metabolites that are not affected during contaminant exposure was explored as a possible means of compensating for the natural variability in the *E. fetida* metabolic profile.

2 Materials and methods

2.1 Earthworm maintenance and contact tests

E. fetida were purchased from The Worm Factory (ON, Canada) and raised in earthworm bins containing sphagnum peat bedding (Magic Worm bedding; Magic Products; WI, USA) with a water content of approximately 67% water by weight (Brown et al. 2008). The earthworms acclimated for several months to reduce variations in the ^1H NMR profile due to differences in diet and other environmental factors (Brown et al. 2008). Earthworms were fed Magic Worm Food (Magic Products; WI, USA) and maintained at approximately 24°C (Brown et al. 2008). Mature earthworms with a visible clitellum and average weight of 0.59 g ($\pm 0.10 \text{ g}$; standard deviation) were depurated in groups of 5 in the dark for 96 hours on Whatman 4 Qualitative filter paper with a diameter of 9 cm (Fisher Scientific) in 500 ml jars to void their intestinal tracts (Brown et al. 2008). Earthworms were then transferred to individual 120 ml amber glass jars containing pre-treated Whatman GF/A 4.25 cm diameter glass filter paper (Fisher Scientific). To ensure that the concentrations of endosulfan and DDT applied to the filter paper during the contact tests were sub-lethal, preliminary experiments were conducted with 4 or 5 earthworms exposed to endosulfan or DDT at

concentrations of 2 and $5 \mu\text{g}/\text{cm}^2$. Earthworms exposed to DDT were alive for both concentrations, but decreased motility was noted at $5 \mu\text{g}/\text{cm}^2$. All earthworms exposed to $5 \mu\text{g}/\text{cm}^2$ of endosulfan were deceased and alive at $2 \mu\text{g}/\text{cm}^2$. In the metabolomic study, filter papers were treated with endosulfan (99% purity; Sigma Aldrich) or DDT (98%; Sigma Aldrich) to dosing concentrations of 0.5, 1.0 and $2.0 \mu\text{g}/\text{cm}^2$ for ^1H NMR and 0.5 and $1.0 \mu\text{g}/\text{cm}^2$ for GC/MS using 1 ml of chloroform (HPLC grade; Fisher Scientific) as a carrier solvent. There were ten replicates per dose. Earthworm controls ($n = 10$) were placed on filter papers pre-treated with 1 ml of chloroform. In all cases the chloroform was allowed to evaporate and 1 ml of distilled water was added prior to the addition of earthworms. Earthworms were kept in the dark for 48 hours, as recommended by the OECD contact test guidelines (OECD 1984). Earthworms were weighed prior to and following the two day exposure to contaminants. Average weight loss in control ($8 \pm 8\%$ weight loss), DDT exposed ($10 \pm 12\%$ weight loss) and endosulfan exposed earthworms ($21 \pm 23\%$ weight loss) was not significant. Earthworms were then flash-frozen in liquid nitrogen, lyophilized and stored frozen until extraction (Brown et al. 2008).

2.2 Earthworm tissue extraction

The lyophilized earthworm tissues were homogenized in a 1.5 ml centrifuge tube using a 5 mm wide stainless steel spatula (Brown et al. 2008). A D_2O -based phosphate buffer was used to extract the earthworm tissues as has been done in other metabolomic earthworm studies (Bundy et al. 2001, 2004; Brown et al. 2008). The homogenized tissue was extracted using 1 ml of a 0.2 M monobasic sodium phosphate buffer solution ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; 99.3%; Fisher Chemicals) containing 1 g/l sodium azide (99.5% purity; Sigma Aldrich) as a preservative (Brown et al. 2008).

For GC/MS samples, the buffer solution was made with distilled water and adjusted to a pH of 7.4 using NaOH (99.2% purity; Fisher Scientific). For ^1H NMR samples, the buffer solution was made with D_2O (99.9% purity, Cambridge Isotope Laboratories) and adjusted to a pD of 7.4 using NaOD (30% w/w in 99.5% D_2O , Cambridge Isotope Laboratories Inc). The buffer solution for ^1H NMR samples also contained 10 mg/l of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS; 97%, Sigma Aldrich) as an internal reference. Samples were vortexed for 30 seconds using a VX 100 vortexer (Labnet, NJ, USA) and then sonicated for 15 minutes using a FS60 sonicator (Fisher Scientific) to aid the extraction. Samples were then centrifuged at 12,000 rpm using a Hanil Micro-12 centrifuge (Rose Scientific, AB, Canada) and the supernatant was transferred into a new 1.5 ml centrifuge tube. The samples were then centrifuged for an additional five minutes. Samples for GC/MS were decanted into 2 ml GC vials

(Agilent Technologies) and samples for ^1H NMR were transferred into 5 mm High Throughput^{plus} NMR tubes (Norell Inc.; NJ, USA). All samples were frozen immediately after preparation until analysis.

2.3 ^1H NMR spectroscopy

^1H NMR spectra of the earthworm extracts were acquired with a Bruker Avance 500 MHz spectrometer using a ^1H -BB- ^{13}C Triple Resonance Broadband Inverse (TBI) probe fitted with an actively shielded Z gradient. ^1H NMR experiments were performed using Presaturation Using Relaxation Gradients and Echoes (PURGE) water suppression and 128 scans, a recycle delay of 3 s, and 16 K time domain points (Simpson and Brown 2005). Spectra were apodized through multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectrum, and a zero filling factor of 2. All spectra were manually phased and calibrated to the DSS internal reference methyl singlet, set to a chemical shift (δ) of 0.00 ppm. The AMIX 3.7.10 (Bruker BioSpin) algebra tool was used to calculate the mean ^1H NMR spectra. Peaks of interest were integrated using line shape analysis (noise factor of 3.5, line shape threshold of 0.01) and normalized to the entire metabolite region (δ 0.25–10 ppm) using the AMIX 3.7.10 multi integration tool.

2.4 Principal component analysis

PCA was performed on processed ^1H NMR data using the AMIX 3.7.10 statistics tool to identify differences in the metabolic profiles of *E. fetida* following exposure to DDT or endosulfan. The ^1H NMR spectra were analyzed between δ of 0.25 and 10 ppm and divided into buckets 0.01 ppm wide, for a total of 961 buckets. An exclusion was placed on the area between δ of 4.70–4.85 ppm to eliminate the small residual $\text{H}_2\text{O}/\text{HOD}$ signals. The integration mode was set at the sum of intensities and the spectra were scaled to the total intensity. This created a matrix in which each row represents an earthworm sample and each column contains the integrated area of the original spectra intensities contained within each bucket region. Plots of PC1 versus PC2 were used to examine if there were differences in the metabolic profiles of control and dosed earthworms since this plot represents the largest amount of variation that can be represented in two dimensions (Wold et al. 1987). The influential buckets in the PC calculation were subsequently identified using the loadings plots for PC1 which show the relative weight for each bucket. The metabolite peaks contained within influential bucket regions were identified using a database of the ^1H NMR spectra of a series of standard metabolites that were previously identified in *E. fetida* (Brown et al. 2008).

2.5 Derivatization and analysis of metabolites by GC/MS

Aqueous standards of the eleven amino acids identified in the *E. fetida* metabolic profile by Brown et al. (2008) using ^1H NMR (alanine, arginine, glutamic acid, glutamine, glycine, leucine, lysine, phenylalanine, serine, tyrosine and valine) were made at concentrations of 10 g/l each to test the GC/MS method. Aqueous standards of glucose, inositol, mannitol and maltose were also made at aqueous concentrations of 10 g/l each. All standard compounds were >98.5% purity (Bioshop Canada). Aliquots of 5, 10, 20, 30 and 40 μl were placed in 2 ml GC vials and evaporated to dryness under a gentle stream of nitrogen gas. The extracted worm samples were lyophilized again to remove all water from the buffer solution.

Preliminary experiments with control earthworms were carried out using three different derivitization procedures to determine suitability for analysis of earthworm metabolites: (i) *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) (Jeong et al. 2004); (ii) *N*-methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) (Jeong et al. 2004); and (iii) hydroxylamine hydrochloride addition followed by hexamethyldisilazane (HMDS) and trifluoroacetic acid (TFA) derivitization (Katona et al. 1999). The third method was chosen because it allows the simultaneous analysis of sugars, sugar alcohols, acids and amino acids (Katona et al. 1999) and yielded the greatest number of metabolites for the earthworm samples.

To analyze the earthworm extracts in this study, an 800 μl aliquot of hydroxylamine hydrochloride (98% purity; Sigma-Aldrich) was added with 25 g/l in pyridine (99% purity, Sigma Aldrich) to dried standards and dried *E. fetida* extracts. All standards and samples were then vortexed for 30 seconds, put in the oven at 70°C for thirty minutes, then cooled. To trimethylsilylate standards and samples, 500 μl of HMDS (98%; Sigma Aldrich) and 50 μl of TFA (99%, Sigma Aldrich) were added. All standards and samples were then placed in the oven at 100°C for one hour (Katona et al. 1999). After cooling, *E. fetida* samples were centrifuged at 1,850 rpm using a Damon I.E.C. HN-S centrifuge and the supernatant transferred to new 2 ml GC vials for analysis.

Standards and samples were analyzed using an Agilent 6890 N gas chromatograph coupled to an Agilent 5973 quadrupole mass selective detector equipped with a HP-5MS column (30 m \times 0.25 mm i.d., 0.25 μm film thickness; Agilent). The column program was 65°C for 2 min, ramped 6°C/min to 230°C, ramped at 10°C/min to 310°C and held 20 min. An Agilent 7683 auto-sampler was used to inject 1 μl of the derivitized samples in splitless mode. To accommodate for a large difference in signal size between the amino acids and sugars, five replicates of each

treatment were reanalyzed with a split setting of 20:1 to avoid saturation of the sugar peaks and provide more accurate quantification. The mass spectrometer was operated in electron impact mode (EI) at 70 eV ionization energy and in full scan mode (m/z 50–650). Data were obtained and processed with Agilent Chemstation G1701DA software. Individual compounds in *E. fetida* extracts were identified by comparison with mass spectra data from the literature (Katona et al. 1999), from the standard amino acid and sugars described above, and using NIST and Wiley MS library data. Quantification of individual compounds was carried out using the external standards. The mean concentrations of amino acids and sugars in control worms and dosed worms were compared using a two-sample t-test.

3 Results and discussion

3.1 ^1H NMR spectroscopy and principal component analysis

The mean ^1H NMR spectra for *E. fetida* exposed to DDT or endosulfan (at $2.0\ \mu\text{g}/\text{cm}^2$) and the associated mean control spectrum are shown in Fig. 1. Since the D_2O buffer used to extract earthworm tissues favors polar metabolites (Brown et al. 2008), the non-polar DDT and endosulfan were not found in the dosed *E. fetida* spectrum (Fig. 1b and c). New peaks are not observed in dosed earthworms as compared to controls. It was difficult to discern the individual peaks with certainty in the ^1H NMR spectra between approximately δ 3.0 and 4.5 ppm due to overlapping amino acid and sugar resonances (Brown et al. 2008). However, increases in the characteristic peaks of leucine (δ of 0.95) and alanine (δ of 1.47) can be discerned in the DDT and endosulfan dosed earthworms (Fig. 1). The intensity of these peaks was estimated by integrating distinct peaks for leucine (δ 0.946–0.961) and alanine (δ 1.460–1.489) and then normalizing to the total intensity of all metabolites. The contribution of both leucine and alanine to the total metabolite area increased in earthworms exposed to DDT and endosulfan compared to the controls (Fig. 1). While this method of averaging provides some insight into variation of metabolite levels, it is unable to accurately assess statistical differences between datasets or comprehend the individual variability in each specimen. Therefore in order to evaluate differences in the acquired ^1H NMR spectra, data was reduced and analyzed using PCA (Bundy et al. 2002, 2004).

Examination of PCA scores plots of PC1 versus PC2 allows for identification of spectra with similar metabolic profiles. Each data point on the scores plot is the PC representation of the entire ^1H NMR spectrum. Hence, samples with similar metabolite profiles will plot close

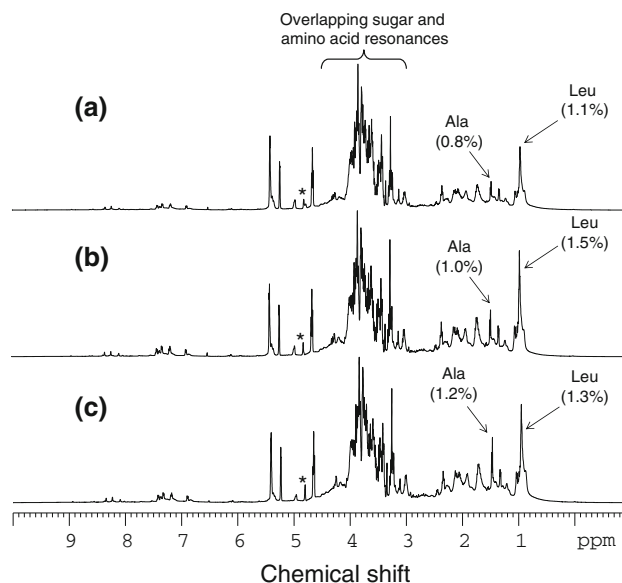


Fig. 1 Average of ten 500-MHz ^1H NMR spectra of tissue extracts for: (a) control *E. fetida*; (b) *E. fetida* exposed to $2.0\ \mu\text{g}/\text{cm}^2$ of DDT; and (c) *E. fetida* exposed to $2.0\ \mu\text{g}/\text{cm}^2$ of endosulfan. * represents the residual $\text{H}_2\text{O}/\text{HOD}$. The contribution of alanine (ala) and leucine (leu) characteristic peaks to the total metabolite area is shown in parentheses

together. The PCA scores plot of the data-reduced spectra showed differences in the metabolic profile of *E. fetida* control and exposed to $2.0\ \mu\text{g}/\text{cm}^2$ of DDT (Fig. 2a). This separation was well defined along PC1, since the majority of DDT dosed earthworms had positive PC1 values and the majority of control earthworms had negative PC1 values. In contrast, there was no apparent separation in the PCA scores plot for the two lower DDT doses (Fig. 2b, c).

The PCA of the data-reduced spectra showed differences in the metabolic profile of *E. fetida* controls and those exposed to $2.0\ \mu\text{g}/\text{cm}^2$ of endosulfan (Fig. 3a). This separation was well defined along PC1, with the majority of dosed earthworms having positive PC1 values and all control earthworms having negative values. The separation of control and $1.0\ \mu\text{g}/\text{cm}^2$ endosulfan dosed earthworms (Fig. 3b) was also fairly well defined, however along PC2 with the majority of dosed earthworms having negative PC2 values. There was no apparent separation in the PCA scores plot for the lowest endosulfan dose ($0.5\ \mu\text{g}/\text{cm}^2$; Fig. 3c).

3.2 Biomarker identification from PCA of ^1H NMR data

The loadings plots for PC1 (Fig. 4) were examined to determine which metabolites contributed significantly to the separation of dosed and control worms in the PCA scores plots. The largest peaks in the loadings plots identify the buckets that contribute most significantly to the PC, and

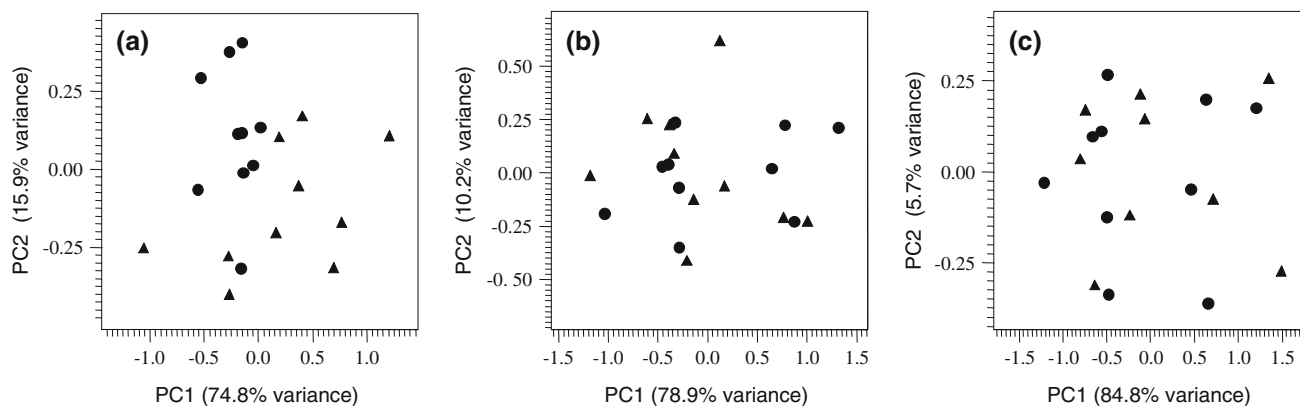


Fig. 2 PCA scores plot of PC1 versus PC2 of data-reduced ^1H NMR spectra for control *E. fetida* (●) and *E. fetida* exposed to DDT (▲) at concentrations of: (a) $2.0\ \mu\text{g}/\text{cm}^2$ ($n = 10$); (b) $1.0\ \mu\text{g}/\text{cm}^2$ ($n = 10$); and (c) $0.5\ \mu\text{g}/\text{cm}^2$ ($n = 9$; 1 died)

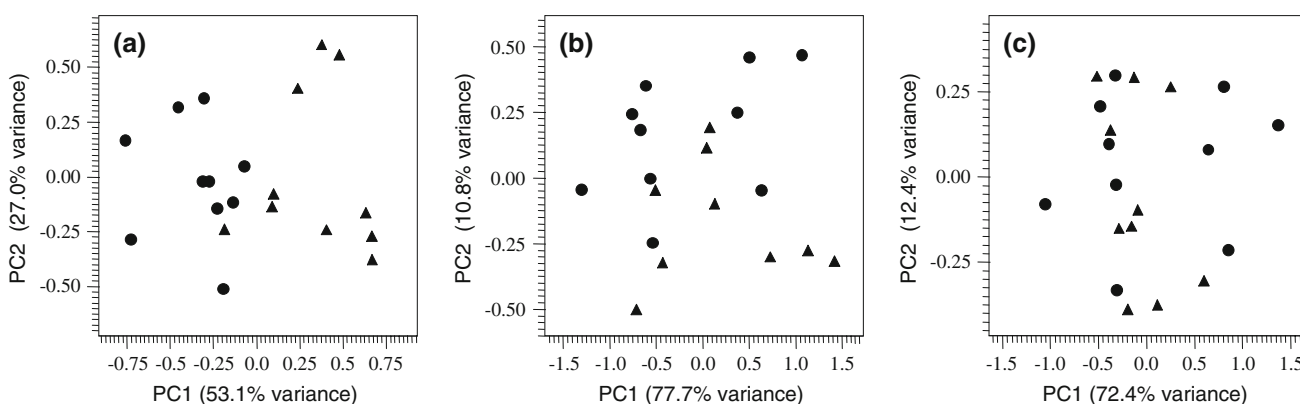


Fig. 3 PCA scores plot of PC1 versus PC2 of data-reduced ^1H NMR spectra for control *E. fetida* (●) and *E. fetida* exposed to endosulfan (▲) at concentrations of: (a) $2.0\ \mu\text{g}/\text{cm}^2$ ($n = 10$); (b) $1.0\ \mu\text{g}/\text{cm}^2$ ($n = 9$; 1 died); and (c) $0.5\ \mu\text{g}/\text{cm}^2$ ($n = 10$)

the metabolite contained within the bucket can be identified by comparison to the ^1H NMR spectra. Leucine, and to a much lesser extent alanine, were identified as important amino acids that have positive PC1 loadings for earthworms exposed to DDT (Fig. 4a) and were identified by their characteristic peaks at δ 0.95 ppm and δ 1.47 ppm, respectively. Maltose was also shown to be an important contributor as indicated by a large negative loadings peak at characteristic δ 5.41 ppm. For endosulfan, the loadings of leucine (δ 0.95 ppm) and alanine (δ 1.47 ppm) show equal importance and are large positive loadings in PC1 (Fig. 4b). Maltose also appears to be an important contributor to the separation in dosed and control earthworms (δ 5.41 ppm). All PC1 loadings plots consistently identified leucine, alanine and maltose as important metabolites for both compounds at all three dose concentrations.

3.3 Quantification of metabolites using GC/MS

The concentration of amino acids and sugars in the earthworm extracts measured using GC/MS are listed in Table 2. Arginine, glutamic acid and mannitol standards did not derivitize

consistently with the method used in this study and did not produce detectable peaks in the earthworm extract. Leucine standards derivitized well and provided a linear calibration curve, however, leucine was not detected in the earthworm extracts suggesting this compound does not derivitize well in the earthworm extract or was unstable. Alanine was the most abundant amino acid measured in the control *E. fetida*, followed by glycine and lysine. Maltose and glucose concentrations were an order of magnitude larger than the other metabolites in the profile. The concentrations in the control earthworms are considered to be representative of the homeostatic metabolic profile of *E. fetida* and served as the baseline to which dosed earthworms were compared.

cBiomarkers for assessing earthworm exposure to contaminants should be reproducible at different concentrations and should consistently increase or decrease. Alanine increased in both DDT and endosulfan dosed worms for the two dosing concentrations measured (0.5 and $1.0\ \mu\text{g}/\text{cm}^2$). However, the variability in alanine concentrations was large, with a relative standard deviation (RSD) of $\pm 41\%$ in the control earthworms, and the only treatment that differed significantly (at the $P = 0.05$ level) from the control was the

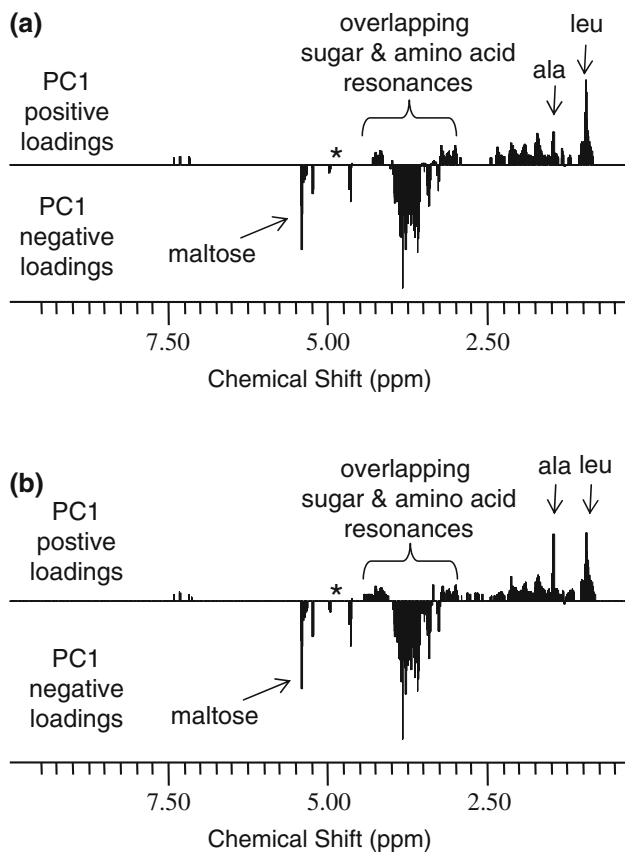


Fig. 4 PCA loadings for PC1 versus the ^1H NMR chemical shift region for *E. fetida* exposed to $2.0 \mu\text{g}/\text{cm}^2$ of: (a) DDT; and (b) endosulfan. The chemical shifts (ppm) refer to buckets from 0.25 to 10.00 ppm at intervals of 0.01 ppm. * indicates the approximate location of the residual $\text{H}_2\text{O}/\text{HOD}$ excluded region (4.70–4.85 ppm). ala = alanine, leu = leucine

highest endosulfan dose ($1.0 \mu\text{g}/\text{cm}^2$). Maltose showed an increase and decrease for the 0.5 and $1.0 \mu\text{g}/\text{cm}^2$ DDT doses, respectively, suggesting it is not a reliable biomarker for this compound. Similarly, the slight increase in endosulfan dosed worms ($\sim 3\%$) compared to controls was not sufficient to be a reliable biomarker for endosulfan exposure when the reproducibility of maltose in control earthworms is $\pm 26\%$ (RSD). Although maltose is not considered a reliable biomarker of DDT or endosulfan based on the findings of this study, it has been identified previously as a biomarker for 4-fluoroaniline (Bundy et al. 2002) and a potential biomarker of metal exposure warranting further study (Bundy et al. 2004; Table 1). Decreased glucose was significant (at the $P = 0.05$ level) for the endosulfan $0.5 \mu\text{g}/\text{cm}^2$ dose, however glucose is not a consistent biomarker for endosulfan since it increased in the $1.0 \mu\text{g}/\text{cm}^2$ dose.

3.4 Ratios of alanine to glycine from GC/MS

Although the metabolite concentrations in Table 2 account for the dry weight of the earthworms, there was still

considerable variability in the metabolite concentrations. Hence other factors such as body composition (i.e. protein or fat content) may play a role in the metabolite levels. To compensate for natural variability in metabolite levels, ratios of metabolites have been used in previous studies to help minimize variation (Bundy et al. 2004; Saude et al. 2007; Viant et al. 2003). This assumes that a given ratio of metabolites is expected in the normal baseline of the test species and that deviations from this baseline are indicative of a metabolic response. While alanine showed considerable variability in control earthworms ($\pm 41.6\%$ RSD), the variability in alanine to glycine ratios was just $\pm 10.5\%$ (RSD). The relationship of alanine to glycine is demonstrated in Fig. 5. The control group showed an excellent relationship of alanine to glycine with a slope of 1.5 and an r^2 of 0.94 (Table 3, Fig. 5). For the DDT $1.0 \mu\text{g}/\text{cm}^2$ and both endosulfan doses, the slope of alanine to glycine is outside the 95% confidence interval of the controls with r^2 values greater than 0.87 except for the highest endosulfan dose ($1.0 \mu\text{g}/\text{cm}^2$) which had an r^2 of just 0.50 (Table 3). The poor relationship in this dose may reflect acute toxicity and a gross deterioration in the earthworm health, since these dosages are near the LC_{50} value.

Overall, the results suggest that deviations from the normal homeostatic ratio of 1.5 for alanine to glycine may be a useful indicator of DDT and endosulfan exposure. The relative toxicities of DDT and endosulfan may be reflected in the magnitude of their deviations from the normal alanine to glycine ratio. Endosulfan is very toxic at low concentrations and attacks the central nervous system of earthworms and acute intoxication can result in muscular twitching and convulsions (Mosleh et al. 2003). In contrast, there have been many studies on the effects of DDT on earthworms and it has been generally concluded that at the normal rates of application to field sites this insecticide does not harm earthworms (Edwards and Bohlen 1992). The relatively nontoxic nature of DDT (Roberts and Dorough 1984) is further confirmed by studies which indicate that on a dry-weight basis, earthworms can accumulate five times more DDT than what is found in the surrounding soil (Beyer and Gish 1980). Alanine concentrations were only statistically higher for the $1.0 \mu\text{g}/\text{cm}^2$ DDT dose. Hence, investigation of the metabolic response elicited by higher DDT doses is warranted to confirm that alanine is a reliable biomarker of DDT exposure and shows a consistent increase with increased DDT exposure. Similarly, investigation of lower doses of endosulfan is recommended to evaluate at which concentration elevated alanine is no longer observed. Furthermore, understanding the changes to the metabolic profile in contaminated soil is suggested to ensure that the earthworms exhibit similar metabolite changes when exposed to DDT and endosulfan in soil.

Table 2 Mean and standard deviation (sd) of amino acids and sugars in mg/g (dry weight) measured using GC/MS of *E. fetida* extracts for control earthworms and those exposed to DDT and Endosulfan (Endo.) at concentrations of 0.5 $\mu\text{g}/\text{cm}^2$ and 1.0 $\mu\text{g}/\text{cm}^2$ in contact tests

Compound	Retention Time (min)	Control Mean \pm sd (mg/g)	DDT 0.5 $\mu\text{g}/\text{cm}^2$ Mean \pm sd (mg/g)	DDT 1.0 $\mu\text{g}/\text{cm}^2$ Mean \pm sd (mg/g)	Endo. 0.5 $\mu\text{g}/\text{cm}^2$ Mean \pm sd (mg/g)	Endo. 1.0 $\mu\text{g}/\text{cm}^2$ ^a Mean \pm sd (mg/g)
Alanine	10.2	1.04 \pm 0.43	1.22 \pm 0.45	1.21 \pm 0.46	1.50 \pm 0.66	1.84 \pm 0.62 ^b
Glycine	12.0	0.71 \pm 0.26	0.78 \pm 0.32	0.71 \pm 0.21	0.76 \pm 0.25	0.68 \pm 0.18
Valine	12.2	0.24 \pm 0.12	0.26 \pm 0.10	0.27 \pm 0.13	0.27 \pm 0.15	0.24 \pm 0.07
Serine	14.1	0.46 \pm 0.22	0.50 \pm 0.21	0.51 \pm 0.21	0.56 \pm 0.29	0.45 \pm 0.14
Phenylalanine	19.6	0.22 \pm 0.13	0.24 \pm 0.10	0.24 \pm 0.15	0.24 \pm 0.18	0.16 \pm 0.06
Glutamine ^c	19.7	0.31 \pm 0.21	0.33 \pm 0.24	0.30 \pm 0.26	0.39 \pm 0.32	0.19 \pm 0.30
Lysine	22.2	0.69 \pm 0.48	0.94 \pm 0.42	0.90 \pm 0.46	1.06 \pm 1.74	0.56 \pm 0.25
Tryosine	25.0	0.22 \pm 0.20	0.28 \pm 0.14	0.29 \pm 0.18	0.29 \pm 0.29	0.21 \pm 0.06
Glucose	27.4/27.7 ^d	16.9 \pm 2.76	20.7 \pm 5.61	17.7 \pm 6.85	13.2 \pm 2.96 ^b	17.5 \pm 6.28
Inositol	28.7	3.66 \pm 0.84	4.07 \pm 0.96	4.19 \pm 1.05	3.36 \pm 0.76	3.93 \pm 1.18
Maltose	36.7/36.9 ^d	41.2 \pm 10.9	54.3 \pm 12.0	35.0 \pm 19.4	44.5 \pm 6.16	42.3 \pm 25.0

^a One worm died

^b Statistically different at the $P = 0.05$ level from the mean of the control

^c Below detection in control (1 worm), DDT 0.5 $\mu\text{g}/\text{cm}^2$ (1 worm), DDT 1.0 $\mu\text{g}/\text{cm}^2$ (1 worm), Endo.0.5 $\mu\text{g}/\text{cm}^2$ (5 worms), Endo. 1.0 $\mu\text{g}/\text{cm}^2$ (3 worms)

^d Two peaks are produced as a result of derivitization

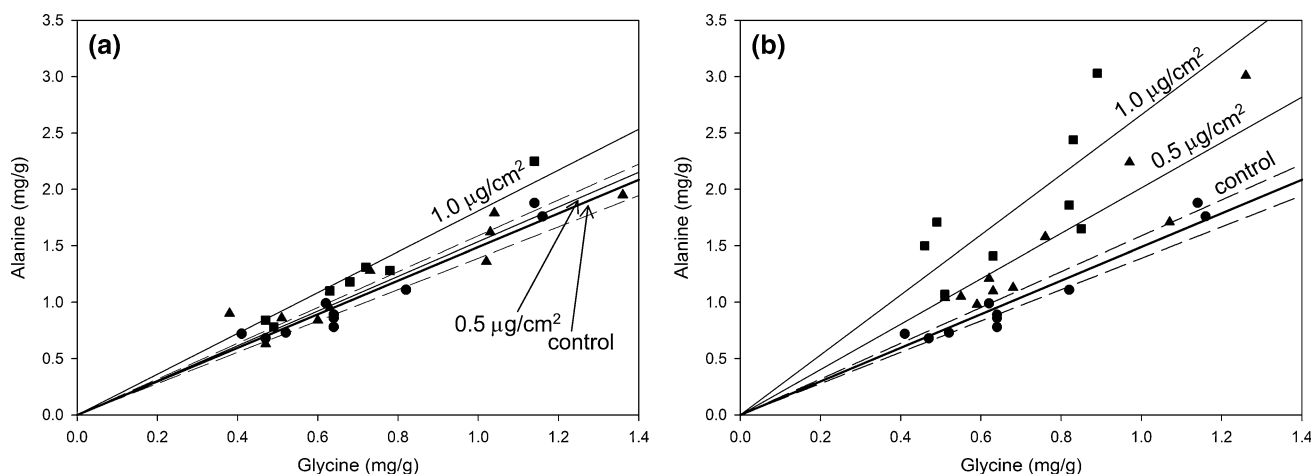


Fig. 5 Alanine versus glycine in mg/g (dry weight) for control *E. fetida* (●) and *E. fetida* exposed to: (a) DDT; or (b) endosulfan; at concentrations of 0.5 $\mu\text{g}/\text{cm}^2$ (▲) and 1.0 $\mu\text{g}/\text{cm}^2$ (■). Dashed lines

are the 95% confidence interval of the least squares regression of the control data (bold solid line). Solid lines are the least square regressions of the exposed *E. fetida*

3.5 Biomarkers of exposure

Unique biomarkers that are specific to individual contaminants would be useful for monitoring responses at field sites with mixtures of contaminants to determine which compounds are bioavailable and affecting earthworm metabolic profiles. In this study, the loadings plots consistently showed that leucine and alanine were important metabolites contributing to the separation between control *E. fetida* and those exposed to DDT and endosulfan. Unfortunately, it was not possible to verify leucine as a robust and reliable biomarker of DDT and endosulfan exposure with the GC/MS method

used in this study. Increased alanine was confirmed as a metabolic response to DDT and endosulfan in this study using GC/MS and has also been reported in earthworms following exposure to pyrene (Jones et al. 2007) and 3-trifluoromethylaniline (Warne et al. 2000).

The lack of unique biomarkers is a common criticism of metabolomics (Robertson 2005). However, even with non-unique metabolites, the use of multiple biomarkers in combination may allow for differentiation of specific responses (Connor et al. 2004). Increased alanine and leucine may be due to the breakdown or degradation of protein in the earthworm and a resultant increase in free

Table 3 Ratio of alanine to glycine in *E. fetida* extracts

Treatment	Slope (m) of alanine versus glycine in Fig. 5 (m \pm 95% confidence interval)
Controls	1.5 \pm 0.1, r^2 0.94
DDT 0.5 $\mu\text{g}/\text{cm}^2$	1.5 \pm 0.1, r^2 0.89
DDT 1.0 $\mu\text{g}/\text{cm}^2$	1.8 \pm 0.1, r^2 0.98 ^a
Endosulfan 0.5 $\mu\text{g}/\text{cm}^2$	2.0 \pm 0.2, r^2 0.87 ^a
Endosulfan 1.0 $\mu\text{g}/\text{cm}^2$	2.7 \pm 0.5, r^2 0.50 ^a

^a The ratio of alanine/glycine is statistically different at the $P = 0.05$ level from the mean of the control. The summary statistics for the two sample independent t -tests are included in the supplementary material

amino acids (Jones et al. 2007; Nath et al. 1997). Alanine is on average the most abundant amino acid in proteins, with an occurrence of approximately 9.0 mol % (Mathews et al. 2000). The average occurrence of leucine in proteins is 7.5 mol % (Mathews et al. 2000). Consequently, a substantial increase in alanine and leucine would be expected with protein breakdown. It has also been proposed that alanine may be a stress signal expressed by cells, yet the mechanism by which alanine may protect cells is unknown (Ben-Izhak Moselise et al. 2003; Forcella et al. 2007). The loadings plots for DDT and the integration of ^1H NMR spectra suggested that leucine was a more important metabolite than alanine for DDT. Quantification of leucine using an alternate approach, such as LC-MS, can confirm this hypothesis and confirm whether specific ratios of alanine to leucine can be used to attribute metabolic responses specifically to DDT or endosulfan at co-contaminated sites.

4 Concluding remarks

At field sites, contaminant concentrations generally decrease through time until a plateau is reached, at which point the remaining contaminant is considered non-bioavailable and non-toxic (Alexander 2000; Morrison et al. 2000). Using earthworms, this hypothesis can be assessed directly by introducing laboratory-raised *E. fetida* into soil collected from contaminated sites and investigating deviations in identified biomarkers from their normal baseline concentrations. A strength of metabolomics is that it enables rapid screening of large quantities of metabolites and can identify potential biomarkers easily using pattern recognition techniques (Miller 2006). However, additional verification is often necessary to confirm that the biomarkers identified are reproducible, consistent in direction and greater than the natural variability in the test organism (Lay et al. 2006). In this study, PCA of ^1H NMR spectra indicated that maltose, leucine and alanine were potential

biomarkers of DDT and endosulfan exposure to *E. fetida*. Using GC/MS it was determined that maltose was not a reliable biomarker. It was not possible to confirm leucine as a suitable biomarker with the GC/MS method used in this study. However, increased alanine was consistently measured in earthworms exposed to DDT and endosulfan. Ratios of alanine to glycine minimized the natural variations and are a potential biomarker of DDT and endosulfan exposure warranting further study.

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