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RESEARCH ARTICLE

Comparison of metabolomic responses of earthworms to sub-lethal imidacloprid exposure in contact and soil tests

Vivek D. Dani[1,2] · Brian P. Lankadurai[2] · Edward G. Nagato[2] · André J. Simpson[1,2] · Myrna J. Simpson[1,2]

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Abstract

Eisenia fetida earthworms were exposed to sub-lethal levels of imidacloprid for 48 h via contact filter paper tests and soil tests. After the exposure, ^{1}H nuclear magnetic resonance (NMR) metabolomics was used to measure earthworm sub-lethal responses by analyzing the changes in the polar metabolite profile. Maltose, glucose, malate, lactate/threonine, *myo*-inositol, glutamate, arginine, lysine, tyrosine, leucine, and phenylalanine relative concentrations were altered with imidacloprid exposure in soil. In addition to these metabolites (excluding leucine and phenylalanine), fumarate, ATP, inosine, betaine, *scyllo*-inositol, glutamine, valine, tryptophan, alanine, tyrosine, and isoleucine relative concentrations shifted with imidacloprid exposure during contact tests. Metabolite changes in *E. fetida* earthworms exposed to imidacloprid showed a non-linear concentration response and an upregulation in gluconeogenesis. Overall, imidacloprid exposure in soil induces a less pronounced response in metabolites glucose, maltose, fumarate, adenosine-5′-triphosphate (ATP), inosine, *scyllo*-inositol, lactate/threonine, and tyrosine in comparison to the response observed via contact tests. Thus, our study highlights that tests in soil can result in a different metabolic response in *E. fetida* and demonstrates the importance of different modes of exposure and the extent of metabolic perturbation in earthworms. Our study also emphasizes the underlying metabolic disruption of earthworms after acute sub-lethal exposure to imidacloprid. These observations should be further examined in different soil types to assess the sub-lethal toxicity of imidacloprid to soil-dwelling earthworms.

Keywords *Eisenia fetida* · Neonicotinoids · Energy disruption · Contact exposure · Soil exposure · Gluconeogenesis

Introduction

Neonicotinoids have emerged as popular agrochemicals to manage pest populations in over 120 countries (EFSA 2012; Goulson 2013; Pisa et al. 2015). As seed treatments, neonicotinoids enter through the roots of plants and are then systemically transported to other plant parts (Tomizawa and Casida 2005; van der Sluijs et al. 2013). They induce neurotoxicity in a wide range of insects by irreversibly binding to nicotinic acetylcholine receptors, resulting in paralysis and death (Tomizawa and Casida 2005). Thus, neonicotinoids have been widely used to target pests found in a number of crops such as cotton, potato, rice, maize, sugar beets, cereals, and seeds (EFSA 2012; Goulson 2013). Due to the continuous application, neonicotinoids have been found to persist in soil, sediment, and surface waters (Bonmatin et al. 2015; Goulson 2013; van der Sluijs et al. 2013). Imidacloprid is one of the major neonicotinoids used for pest control and was the second most used agrochemical in 2008 (Pollak 2011). It is reported to have soil half-lives varying from 40 to 1230 days depending on soil properties and composition (Baskaran et al. 1999). Due to its persistence in soil, imidacloprid can be potentially taken up by succeeding crops after previous application regimes (Goulson 2013). For example, imidacloprid was detected in 97% of soil samples from untreated fields on which imidacloprid-treated corn seed was tilled 1–2 years before the soil was sampled (Bonmatin et al. 2005). Additional

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✉ Myrna J. Simpson
myrna.simpson@utoronto.ca

[1] Department of Chemistry, University of Toronto, 80 St. George St., Toronto, ON M5S 3H6, Canada

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

studies have reported that imidacloprid can be toxic to bees and birds (Gibbons et al. 2015; Pisa et al. 2015; van der Sluijs et al. 2013). For example, imidacloprid can disrupt honey bee (*Apis mellifera*) locomotion by impairing wing and leg movement (Girolami et al. 2009; Medrzycki et al. 2003) and cause mortality at concentrations as low as 0.1 μg L^{-1} (Suchail et al. 2001). Additionally, red-legged partridges (*Alectoris rufa*) were observed to have reduced fertilization, chick survival, and immune response after imidacloprid exposure (Lopez-Antia et al. 2013). Since imidacloprid is toxic to non-target organisms in terrestrial ecosystems (Gibbons et al. 2015; Pisa et al. 2015), there is a need to assess sub-lethal toxicity of imidacloprid in soil-dwelling organisms such as earthworms.

Earthworms are crucial to the terrestrial ecosystem because they maintain the physical, chemical, and biological properties of the soil (Edwards and Bohlen 1992; Pisa et al. 2015). Since imidacloprid can persist in the soil, earthworms living within the soil may be exposed to imidacloprid by passive diffusion or ingestion (Edwards and Bohlen 1992). Numerous studies have been performed to assess imidacloprid toxicity on earthworms (Cang et al. 2017; Dittbrenner et al. 2011; Luo et al. 1999; Wang et al. 2016, 2012; Zang et al. 2000; Zhang et al. 2014). For example, Luo et al. (1999) and Zang et al. (2000) reported sperm deformities in *Eisenia fetida* when exposed to imidacloprid in soil at concentrations as low as 0.5 mg kg^{-1} soil, while Dittbrenner et al. (2011) observed that earthworms *Aporrectodea caliginosa* and *Lumbricus terrestris* altered burrowing behavior in imidacloprid-contaminated soil. After exposing *E. fetida* to neonicotinoids, antibiotics, pyrethroids, carbamates, and organophosphates, Wang and co-workers (Wang et al. 2012) found that neonicotinoids had the lowest LC_{50} (concentration at which 50% mortality is observed) and thus were the most toxic class of these agrochemicals. Furthermore, sub-lethal concentrations of imidacloprid were observed to induce oxidative stress on *E. fetida* earthworms (Zhang et al. 2014). More recently, it has also been reported that imidacloprid can cause DNA damage in *E. fetida* with acute exposure (Wang et al. 2016) which induces an overall antioxidant response. As such, there is a further need to ascertain how imidacloprid exposure may alter the molecular-level function of earthworms, such as *E. fetida*, especially at sub-lethal exposure concentrations.

Metabolomics has been used to investigate the sub-lethal toxicity induced by a range of soil contaminants to earthworms (Brown et al. 2010; Bundy et al. 2002; Dani et al. 2018; Lenz et al. 2005; McKelvie et al. 2013, 2010, 2011; Simpson and McKelvie 2009; Tang et al. 2018; Whitfield Åslund et al. 2013; Whitfield Åslund et al. 2012; Whitfield Åslund et al. 2011; Yuk et al. 2013). Metabolomics is able to monitor rapid fluctuations in metabolites such as sugars and amino acids in response to an external stressor. ^{1}H nuclear magnetic resonance (NMR) earthworm metabolomics has been previously applied to assess earthworm responses after sub-lethal contaminant exposure by identifying metabolites and elucidating the toxic mode of action (Brown et al. 2010; Bundy et al. 2009; Dani et al. 2018; Lankadurai et al. 2012; McKelvie et al. 2013, 2010, 2011; Simpson and McKelvie 2009; Whitfield Åslund et al. 2013, 2012, 2011; Yuk et al. 2013). For example, Lankadurai et al. (2012) found that perfluorooctane sulfonate may interrupt adenosine-5′-triphosphate (ATP) synthesis in *E. fetida* earthworms after observing decreases in the sugars glucose and maltose and ATP, while endosulfan and endosulfan sulphate were observed to induce neurotoxicity in *E. fetida* as a result of increases in the amino acids glutamate and glutamine (Yuk et al. 2013). Tang et al. (2018) reported that low levels of mercury in soil disrupted metabolic pathways related to osmoregulation, amino acid, and energy metabolism. Consequently, NMR-based metabolomics studies are enabling the assessment of how low levels of contaminants alter earthworm metabolism and health that are not detected using other more traditional endpoints (Lankadurai et al. 2013b).

In this study, *E. fetida* earthworms were exposed to imidacloprid for 48 h via contact filter paper tests as well as in soil tests, as recommended by the Organization for Economic Co-operation and Development (OECD 1984). The 48-h study duration was selected, to be consistent with previous metabolomics studies, which detected metabolic responses to the sub-lethal level of contaminants using ^{1}H NMR metabolomics (Brown et al. 2010; Lankadurai et al. 2012; Whitfield Åslund et al. 2011; Yuk et al. 2011). Our objective was to assess how sub-lethal exposure of imidacloprid in both contact filter paper and soil tests may alter the metabolic profile of *E. fetida* using ^{1}H NMR metabolomics. Additionally, freely available concentrations of imidacloprid may be reduced in soil because of sorption to soil (K_{OC} = 210–336 L kg^{-1} soil) by interacting with clay mineral surfaces or partitioning into soil organic matter (Cox et al. 1998; Semple et al. 2004). Imidacloprid is relatively water-soluble (solubility = 0.6 g L^{-1}; log K_{OW} = 0.57; Bonmatin et al. 2005) so depending on the imidacloprid concentration, its ability to perturb metabolic pathways in earthworms may vary. As such, we hypothesize that exposure to imidacloprid in soil will also induce similar but perhaps less pronounced metabolic responses to that observed in the contact filter paper test.

Materials and methods

Earthworm exposure experiments

Eisenia fetida earthworms were cultured and only adult earthworms with a visible clitellum were selected for metabolomic experiments as described in Brown et al. (2008) and detailed in the Supplementary Materials. Preliminary experiments (see Supplementary Materials)

were used to select sub-lethal imidacloprid exposure concentrations based on the reported LC_{50} value of 27 ng cm^{-2} (Wang et al. 2012). After preliminary experiments (Supplementary Materials), imidacloprid concentrations of 3.36, 1.68, 0.84, and 0.42 ng/cm^2 (approximately 1/8th, 1/16th, 1/32nd, and 1/64th of the LC_{50}, respectively) were selected for the metabolomics experiment. For contact tests, 4.25-cm-diameter glass filter papers (Whatman GF/A; Fisher Scientific Canada) were pre-cleaned with dichloromethane (Fisher Scientific Canada) to remove any residual impurities and were then allowed to evaporate in a fume hood. The filter papers were subsequently baked in an oven for 1 h at 80 °C to remove any residual dichloromethane. The pre-cleaned filter papers were placed inside 120-mL amber glass jars and were then treated with imidacloprid (98%, Toronto Research Chemicals Ltd., Toronto, ON) solutions (1 mL in acetone, Fisher Scientific Canada). For the control treatments, 1 mL of acetone was added to the filter paper. All jars were then placed in a fume hood for 4 h to evaporate the solvent and were subsequently moistened with 1-mL distilled water prior to the addition of earthworms. An earthworm was placed in each treatment jar (10 replicates per treatment) and the jars were placed in the dark for 48 h as described by the OECD guidelines (OECD 1984). No fatalities were visually observed after the 48-h exposure. After the 48-h exposure, the earthworms were flash-frozen with liquid nitrogen to halt enzymatic activity and metabolism, lyophilized, and stored at − 20 °C until extraction for NMR-based metabolomics.

Soil exposure tests were carried out based on the OECD recommended method (OECD 1984) for *E. fetida*. Preliminary experiments were conducted to determine the sub-lethal concentrations of imidacloprid (Supplementary Materials) based on the reported LC_{50} value of 2.82 mg kg^{-1} soil (Wang et al. 2012). Based on these experiments, imidacloprid exposure concentrations from 1, 0.75, 0.5, 0.375, 0.25, 0.25, and 0.05 mg kg^{-1} soil were used. Glass jars containing 250 g of artificial soil were spiked with 10 mL of imidacloprid (dissolved in water) and 10 mL of distilled water was added to the control jars. An additional 250 g of artificial soil was added to each jar and were mixed thoroughly. All soils were then wetted to a moisture content of 35% soil dry weight and were allowed to absorb the water for 24 h (OECD 1984). Ten earthworms with a visible clitellum were then introduced into each of the jars and the jars were immediately closed. The closed jars were kept at 21 °C and placed under constant light for the duration of the 48-h exposure (OECD 1984). After the exposure, the earthworms were allowed to depurate individually for 96 h on a damp Whatman 1 filter paper (Fisher Scientific Canada) in 20-mL glass vials (Brown et al. 2008). No fatalities were visually observed after the 96-h depuration period. The depurated worms were then flash-frozen using liquid nitrogen to halt the enzymatic activity, lyophilized, and stored at − 20 °C until extraction for NMR-based metabolomics.

NMR metabolomics and data analysis

Polar metabolites were targeted and isolated using the method of Brown et al. (2008) which has been used to assess the perturbation to different metabolic pathways (Brown et al. 2010; Lankadurai et al. 2013a). Metabolites were isolated using a 0.2 M monobasic sodium phosphate buffer solution ($NaH_2PO_4 \cdot 2H_2O$, 99.3%; Fisher Scientific Canada). The buffer was prepared in D_2O (99.99% purity; Cambridge Isotope Laboratories) and contained 10 mg/L of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS, 97%; Sigma-Aldrich) as an internal calibrant (Brown et al. 2008) and 0.1% (*w*/*v*) sodium azide (99.5%; Sigma-Aldrich) as a preservative. Additionally, the pD was adjusted to a value of 7.4 using NaOD (30% *w*/*w* in 99.5% D_2O; Cambridge Isotope Laboratories). The lyophilized earthworm tissue samples were placed in a 1.5-mL microtube and then homogenized using a 5-mm stainless steel spatula. The homogenized tissue samples were extracted with 1.20 mL of the D_2O phosphate buffer (as described previously in this section) by first vortexing the samples for 30 s using a VX 100 vortexer (Labnet, NJ, USA) and then sonicated for 15 min using a FS60 sonicator (Fisher Scientific Canada). The extracted samples were then centrifuged for 20 min using an Eppendorf 5804 R centrifuge (Eppendorf Canada) at 14,000 rpm (21,000*g*) at 4 °C to obtain the supernatant. The supernatant was then transferred to a new 1.5-mL centrifuge tube after which the procedure was repeated twice to remove any residual particles. The final supernatant (750 μL) was transferred into 5-mm High Throughput^{plus} NMR tubes (Norell Inc.: NJ, USA) for ^{1}H NMR analysis.

^{1}H NMR spectra of the earthworm polar extracts were acquired using a Bruker BioSpin Avance III 500 MHz spectrometer using a ^{1}H-^{19}F-^{15}N-^{13}C 5-mm Quadruple Resonance Inverse (QXI) probe fitted with an actively shielded Z gradient at a temperature of 298 K. Presaturation Using Relaxation Gradients and Echoes (PURGE) water suppression (Simpson and Brown 2005) was used to acquire NMR spectra. Spectra were collected using 128 scans, a recycle delay of 3 s and 16 k time domain points (Brown et al. 2008). 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 two. All NMR spectra were manually phased and calibrated to the methyl protons of the trimethylsilyl group of the DSS internal reference (δ = 0.00 ppm).

The ^{1}H NMR spectra (exhibited resonances between 0.5 and 10 ppm (Supplementary Fig. S1) consist of overlapping resonances which make metabolite identification difficult from the ^{1}H NMR spectrum alone. Thus,

metabolite identification was performed by using the distinct resonances that were previously assigned to metabolites in *E. fetida* tissue extracts using two-dimensional NMR spectroscopy which increases spectral dispersion and facilitates conformation of metabolite resonances in the ^{1}H NMR spectrum (Brown et al. 2008; Bundy et al. 2002; Yuk et al. 2012). These identified resonances used for metabolite quantification are listed in Supplementary Table S1. Furthermore, the Analysis of Mixtures (AMIX; version 3.9.7; Bruker BioSpin, Rheinstetten, Germany) statistics tool was used to divide the spectra into buckets of 0.02 ppm width (total of 475 buckets). The region between 4.70 and 4.90 ppm was excluded to omit any residual signals caused by H_2O/HOD. The NMR spectra were then scaled to total spectral intensity, which compensates for small differences in the overall metabolite concentration, and the integration mode was set to sum of intensities.

Changes in the relative concentration of individual metabolites were calculated by subtracting the bucket intensity for specific resonances (Table S1) between control and exposed earthworms and subsequently dividing the difference by the bucket of the control earthworms. As such, all metabolite concentrations are reported as a percentage change relative to the metabolic profile of the control earthworms. To compare general differences between the imidacloprid-exposed earthworms and the control earthworms after the 48-h contact filter paper and soil exposure, principal component analysis (PCA) score plots were constructed. A *t* test (two-tailed, equal variance) was performed on the PCA scores to identify whether the difference in the metabolic response between the control and imidacloprid-exposed earthworms was statistically significant ($P < 0.05$; Boroujerdi et al. 2009). PCA loading plots were also constructed to identify the metabolites contributing to the separation between the scores of the control and exposed earthworms. Averaged PCA score plots were generated to investigate the separation between the control and imidacloprid-exposed classes (exposure concentration) and the relative variation between the imidacloprid-exposed classes. These plots were constructed by obtaining the scores of each earthworm class from a PCA model that contained all the data. The scores were then imported into Microsoft Excel (version 12.0.6504, Microsoft Corporation, Redmond, WA) and were averaged per class (exposure concentration) and plotted with their associated standard errors. Furthermore, partial least squares (PLS) regression analysis was also conducted to identify the relationship between the metabolic profile and imidacloprid concentration after the 48-h soil exposure (see Supplementary Materials). The *t* test–filtered ^{1}H NMR spectra were constructed to further assist in identifying metabolites that changed with imidacloprid exposure after the contact filter paper and soil exposure (Ekman et al. 2008, 2009) and is detailed in the Supplementary Materials. Both PCA loadings and *t* test–filtered ^{1}H NMR spectral results are discussed in the Supplemental Materials and shown in Supplementary Figures S6–S11.

Results and discussion

Principal component analysis of control and exposed earthworms

Individual and averaged PCA scores plots after the contact filter paper (Fig. 1 and Supplementary Fig. S2) and soil exposure experiments (Fig. 2 and Supplementary Fig. S3) were constructed to compare the metabolic responses of the imidacloprid-exposed earthworms to the unexposed control earthworms as used in other metabolomic studies (Brown et al. 2009, 2010; Bundy et al. 2002). The averaged PCA scores plot (Fig. 1) for imidacloprid contact test exposures revealed a significant separation between the control and all the exposed classes. However, these differences do not appear to be related to imidacloprid concentration (Fig. 1). After the soil exposure, significant separation ($P < 0.05$) from the controls was observed for the earthworms exposed to 0.125, 0.5, and 1 mg kg^{-1} soil imidacloprid concentrations (Fig. 2 and Supplementary Fig. S3). PLS regression models were also tested to assess the strength and significance of the relationship between the metabolic response and imidacloprid concentration after the 48-h soil exposure (Supplementary Materials). The PLS regression model showed that the polar metabolic profile did not linearly correlate to imidacloprid exposure concentration. The model also lacked any apparent predictive power because of a negative Q^2Y value (cross-

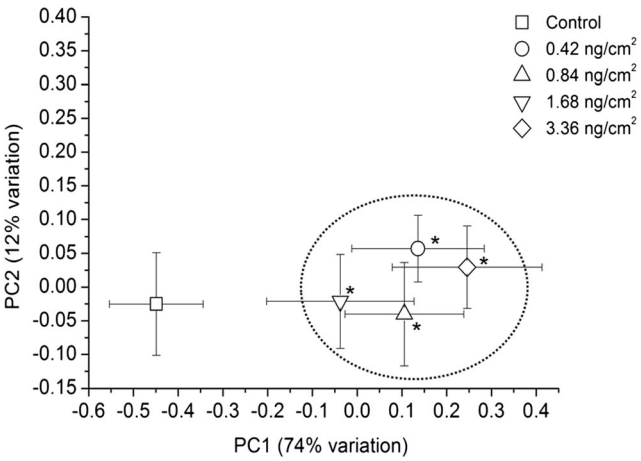


Fig. 1 Averaged principal component analysis (PCA) scores plots of PC1 versus PC2 of imidacloprid-exposed *E. fetida* after a 48-h contact filter paper exposure. Each point represents the average PCA scores for an exposure concentration and the error bars are denoted by the standard error of the average. The ellipse was used as a visual aid to group the exposed classes and the exposed classes that were significantly different from the control are labeled with an asterisk (*$P < 0.05$)

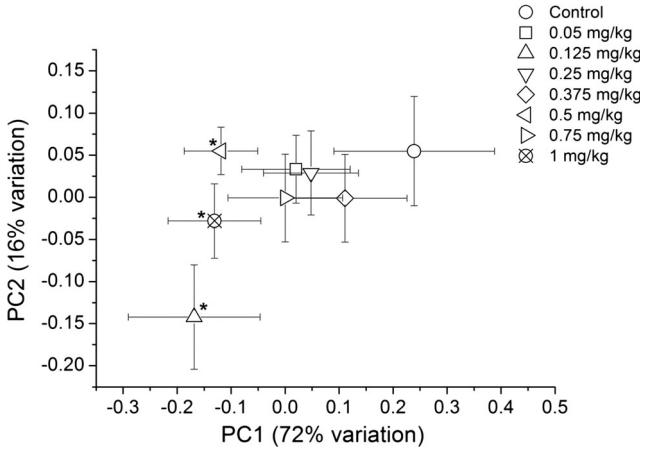


Fig. 2 Averaged principal component analysis (PCA) scores plots of *E. fetida* exposed to imidacloprid-contaminated soil after 48 h along principal components PC1 and PC2. Each point represents the average PCA scores for an exposure concentration and the error bars denote the respective standard error of the average. The exposed classes that were significantly different from the control are labeled with an asterisk (*$P < 0.05$)

validated with two components, $R^2X = 0.80$, $R^2Y = 0.06$, $Q^2X = -0.10$, $P = 0.6$ Supplementary Figs. S4 and S5). Thus, both PCA and PLS regression analyses suggest that *E. fetida* metabolic response after both contact filter paper and soil exposure is not linearly correlated with imidacloprid exposure concentrations.

Metabolic changes and perturbations with imidacloprid exposure

Metabolite percent changes have been used to provide insight into the flux of metabolites and contaminant mode of action in earthworms (Lankadurai et al. 2011; Yuk et al. 2011). A concentration-dependent exposure response was not observed in the metabolic response for the imidacloprid-exposed classes after either the contact filter paper or soil exposure (Figs. 3 and 4). Amino acids decreased in response to imidacloprid after the 48-h contact test and soil exposure although small increases in glutamine and tyrosine were observed in the 0.05 mg kg^{-1} soil exposure (Figs. 3 and 4). After the contact test study, glutamine was observed to decrease significantly for all the imidacloprid-exposed classes whereas glutamate, arginine, lysine, and tyrosine decreased significantly for all the exposed classes but the 1.68 ng cm^{-2} imidacloprid-exposed class. Valine and tryptophan significantly decreased for both the highest and lowest exposed classes but alanine and isoleucine only significantly decreased for the highest imidacloprid-exposed class (0.42 and 3.36 ng cm^{-2}; Fig. 3).

Alternatively, for the soil exposures, arginine and lysine significantly decreased for the 0.125, 0.5, and 1 mg kg^{-1} soil-exposed classes while phenylalanine and leucine decreased significantly for the 0.5 mg kg^{-1} soil-exposed class (Fig. 4). Additionally, glutamate and tyrosine significantly decreased for the earthworms exposed to an imidacloprid concentration of 1 mg kg^{-1} soil (Fig. 4). However, the significant decreases in valine, tryptophan, glutamine, alanine, and isoleucine that were reported after the contact filter paper exposure were not observed with exposure in soil. Similarly, significant decreases in phenylalanine and leucine that were observed after the soil exposure were not observed in contact tests. This difference in the amino acid profile after imidacloprid exposure may be due to the different modes of contaminant exposure (Lankadurai et al. 2013a). For example, earthworms are exposed to contaminants via passive diffusion or inhalation in contact filter paper tests (Simpson and McKelvie 2009) whereas passive diffusion and soil ingestion are modes of exposure in soil (Edwards and Bohlen 1992). Although imidacloprid is relatively water-soluble (solubility = 0.6 g L^{-1}; log $K_{OW} = 0.57$; Bonmatin et al. 2005), it has a medium-to-high sorption tendency for soil, with organic carbon normalized sorption coefficients (K_{OC}) ranging from 210 to 336 L kg^{-1} soil (Nemeth-Konda et al. 2002; Oliver et al. 2005). As imidacloprid sorbs to clay mineral surfaces and to organic matter (Cox et al. 1998), the mode of exposure may be altered due to the changes in the free form of imidacloprid.

Nevertheless, the observed decreases in amino acids in the earthworms after imidacloprid exposure after both contact filter paper and soil exposure corresponds to an upregulation in gluconeogenesis. When organisms are energy-depleted or exposed to stress, amino acids can be converted into gluconeogenic or Krebs cycle substrates to fuel energy production (Horton et al. 2006, Nelson et al. 2012). For example, glutamate, arginine, and glutamine can be eventually converted to the Krebs cycle intermediate alpha-ketoglutarate to synthesize substrate for gluconeogenesis (Newsholme et al. 2003) whereas valine, threonine, and isoleucine can also replenish Krebs cycle intermediate succinyl-CoA (Bender 2012; Nelson et al. 2012). Leucine, isoleucine, lysine, phenylalanine, tryptophan, and tyrosine can be used to replenish acetyl-CoA stores to fuel the Krebs cycle (Horton et al. 2006; Nelson et al. 2012). Additionally, tyrosine and phenylalanine can also enter the Krebs cycle by converting into fumarate (Horton et al. 2006, Nelson et al. 2012). Alanine can also be converted to pyruvate which is catalyzed by the alanine aminotransferase enzyme (Horton et al. 2006). As such, we hypothesize that the decreases in amino acid concentrations were due to an upregulation in gluconeogenesis brought on by imidacloprid exposure.

After the contact filter paper exposure, the sugar maltose appeared to increase significantly for all imidacloprid-exposed classes whereas, in the soil exposure, maltose was observed to significantly increase in the 0.5 mg kg^{-1} soil-exposed class. Maltose is usually hydrolyzed into two glucose molecules, which can undergo glycolysis to produce energy (Horton et al. 2006). Therefore, significant increases in maltose could

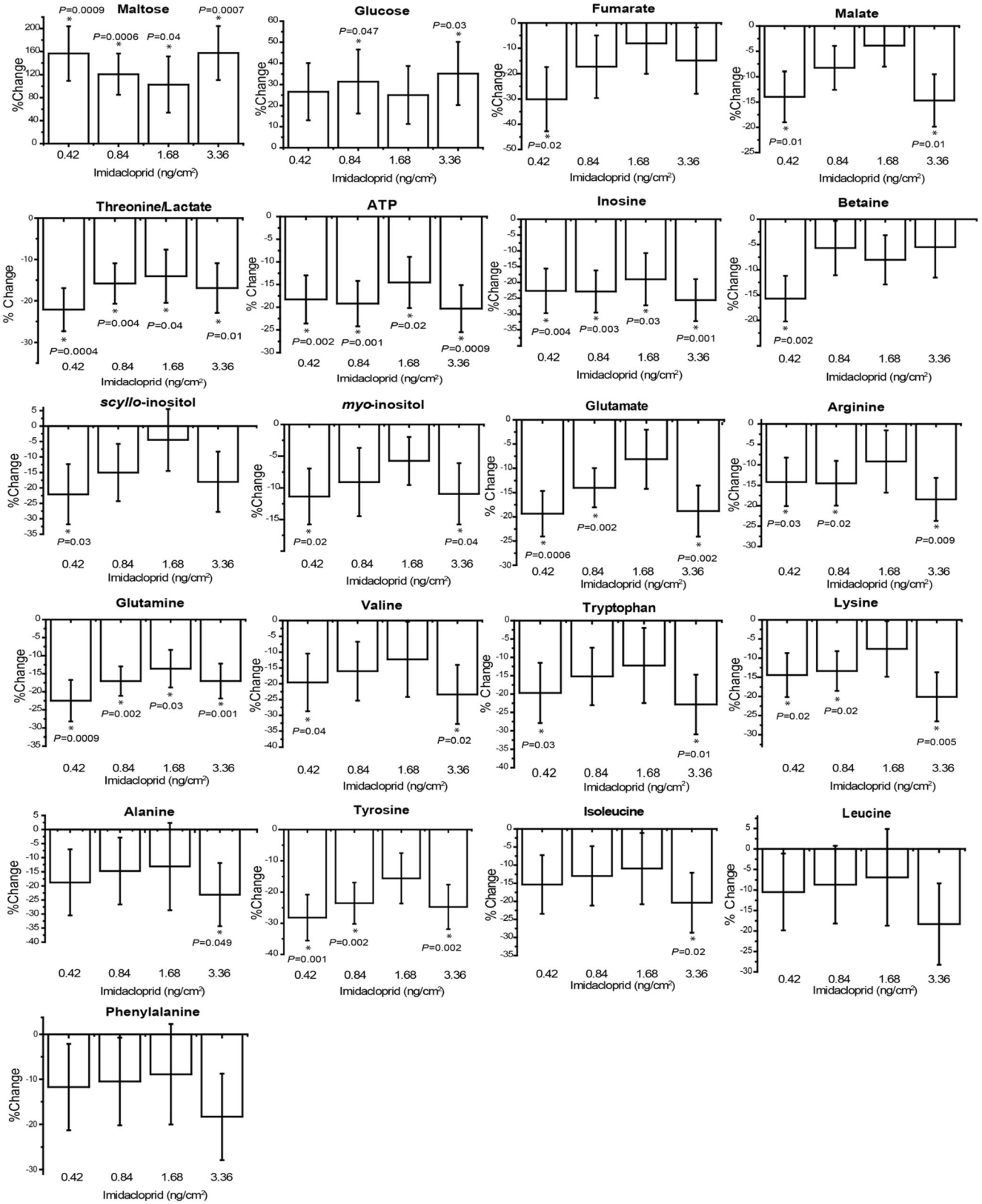


Fig. 3 Percent (%) change relative to the control of selected metabolites of imidacloprid-exposed *E. fetida* after a 48-h contact filter paper exposure (exposure concentrations used included 0.42, 0.84, 1.68, and 3.36 ng/cm^2). The % changes were obtained by first subtracting the buckets representing the metabolites in the control earthworms from the exposed earthworms and subsequently dividing the difference by the buckets of the control earthworms. The % changes that were significantly different from the control are labeled with an asterisk (*$P < 0.05$). The % changes are shown with their propagated standard error

suggest an inhibition in the enzyme that hydrolyzes maltose in *E. fetida* earthworms. Similarly, glucose was observed to increase in all the imidacloprid-exposed classes after both the contact filter paper and soil exposure. However, significant increases in glucose were only observed in the 0.84 and 3.36 ng cm^{-2} contact filter exposures and in the 0.125 mg kg^{-1} soil exposure. These increases in glucose levels also support an upregulation in gluconeogenesis.

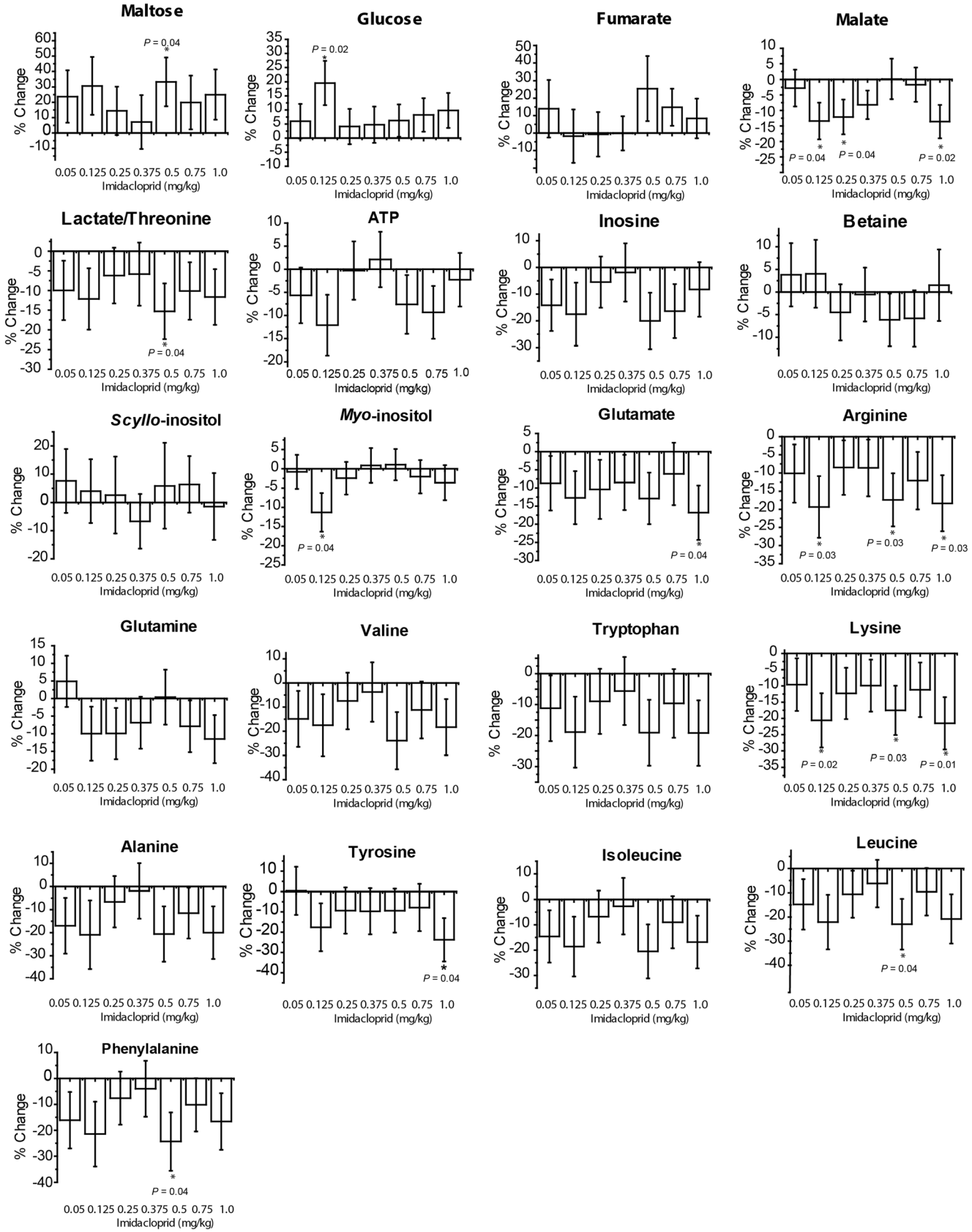
The Krebs cycle intermediate fumarate decreased significantly only for the lowest imidacloprid-exposed class whereas malate significantly decreased for the earthworms exposed to the lowest and highest concentrations (0.42 and 3.36 ng cm^{-2}) after contact filter paper exposure. Similarly, after the 48-h soil exposure to imidacloprid, the Krebs cycle intermediate malate was observed to decrease in all the imidacloprid-exposed classes though significant decreases were observed for the 0.125, 0.25, and 1.0 mg kg^{-1} soil-exposed classes. However, fumarate was observed to increase for the 0.05, 0.5, 0.75, and 1 mg kg^{-1} soil imidacloprid-exposed classes whereas subtle decreases were observed in the 0.125, 0.25, and 0.375 mg kg^{-1} soil-exposed classes. Increases in fumarate levels can be explained by the decreases in tyrosine and phenylalanine as shown in Fig. 4 (Nelson et al. 2012). Furthermore, significant decreases in lactate/threonine were reported in all the imidacloprid-exposed classes after the contact filter paper exposure. In the soil exposure, lactate/threonine was observed to decrease for all the imidacloprid-exposed classes though only significantly for the earthworms exposed to the 0.5 mg kg^{-1} soil imidacloprid concentration. Lactate and threonine can be converted into pyruvate, the primary substrate for gluconeogenesis (Bender 2012, Nelson et al. 2012). Thus, our results further suggest that imidacloprid can alter gluconeogenesis in *E. fetida* earthworms.

In the contact filter paper exposure, the energy molecule ATP and the nucleoside inosine were observed to decrease significantly for all the earthworms exposed to imidacloprid. However, all of the imidacloprid-exposed earthworms after the soil test were observed to have depleted ATP levels except for the 0.375 mg kg^{-1} soil-exposed class, though none of these changes were significant relative to the control. When *E. fetida* earthworms were exposed to sub-lethal levels of perfluorooctane sulfonate for 48 h, earthworms were observed to have increased levels of ATP (Lankadurai et al. 2013a) while having depressed ATP levels after a 48-h contact test (Lankadurai et al. 2012). This contrast in the earthworm response was explained by the difference in the mode of exposure in contact tests and soil tests (Lankadurai et al. 2013a). Therefore, the difference in the mode of exposure associated with contact tests and soil tests can most likely explain the varied response in ATP. Similarly, inosine was observed to decrease for all the earthworms exposed to imidacloprid-contaminated soil, though this change was not statistically significant. Inosine can assist in extracellular signaling, neuroprotection, and also replenish ATP stores during stressful conditions (Haskó et al. 2004; Shafy et al. 2012). Although its function is not fully understood in earthworms, Bundy et al. (2002) suggested that increases in inosine storage could be used to generate ATP during stressful conditions. Thus, decreases in inosine levels most likely suggest a conversion to inosine monophosphate, a substrate used in ATP synthesis.

The osmoregulator betaine is observed to regulate metabolic function with decreases in betaine suggesting depressed metabolic activity (Craig 2004; Eklund et al. 2005; Ratriyanto et al. 2009; Tuffnail et al. 2009). Betaine was observed to decrease in all the earthworms exposed during the contact filter paper exposure though only significantly for the lowest exposed class (Fig. 3). This result potentially indicates a decrease in metabolic activity in the imidacloprid-exposed classes or a depression in cellular processes that require betaine. Both *myo*-inositol and *scyllo*-inositol were observed to decrease in the imidacloprid-exposed worms after the contact filter paper exposure but significant decreases were observed for the lowest exposed class (0.42 ng cm^{-2}) and the lowest and highest exposed classes (0.42 and 3.36 ng cm^{-2}) for *myo*-inositol and *scyllo*-inositol, respectively (Fig. 3). Additionally, a significant decrease in *myo*-inositol was only observed in the earthworms exposed to 0.125 mg kg^{-1} soil of imidacloprid (Fig. 4). Both *myo*-inositol and *scyllo*-inositol have osmoregulatory and cell signaling functions (Horton et al. 2006; Moreno and Arús 1996; Strange et al. 1991); therefore, concentration decreases likely indicate alterations in cellular mechanisms that involve these molecules (Moreno and Arús 1996).

Conclusions

^{1}H NMR metabolomics was used to elucidate *E. fetida* sub-lethal responses after a 48-h imidacloprid contact filter paper and soil exposure. The non-monotonic response in *E. fetida* earthworms and the perturbations to specific metabolites likely involved an upregulation in gluconeogenesis with imidacloprid exposure in both contact filter paper and soil tests. Additionally, *E. fetida* responses after imidacloprid soil exposure for several metabolites (glucose, maltose, fumarate, inosine, ATP, betaine, *scyllo*-inositol, lactate/threonine, and tyrosine) were not as pronounced as compared with the *E. fetida* responses observed with the contact exposure test. In contact tests, earthworms are directly exposed to imidacloprid on a filter paper through either passive diffusion or respiration (Simpson and McKelvie 2009) whereas in soil exposure studies, earthworms are exposed through passive diffusion or soil ingestion. Thus, this less pronounced metabolic perturbation after exposure to imidacloprid-contaminated soil was most likely due to the different modes of exposure associated with soil tests, as we hypothesized.


Maltose
P = 0.04
Glucose
P = 0.02
Fumarate
Malate
P = 0.04
P = 0.04
P = 0.02
Lactate/Threonine
P = 0.04
ATP
Inosine
Betaine
Scyllo-inositol
Myo-inositol
P = 0.04
Glutamate
P = 0.04
Arginine
P = 0.03
P = 0.03
P = 0.03
Glutamine
Valine
Tryptophan
Lysine
P = 0.02
P = 0.03
P = 0.01
Alanine
Tyrosine
P = 0.04
Isoleucine
Leucine
P = 0.04
Phenylalanine
P = 0.04
% Change
0.05 0.125 0.25 0.375 0.5 0.75 1.0
Imidacloprid (mg/kg)

◀ **Fig. 4** Percent (%) change relative to the control of selected metabolites of *E. fetida* in response to imidacloprid soil exposure (concentration expressed as mg kg^{-1} soil and ranges from 0.05, 0.125, 0.25, 0.375, 0.5, 0.75, and 1.0 mg kg^{-1}). The % changes were obtained by first subtracting the buckets representing the metabolites in the control earthworms from the exposed earthworms and subsequently dividing the difference by the buckets of the control earthworms. The % changes that were significantly different from the control are labeled with an asterisk (*$P < 0.05$). The % changes are shown with their propagated standard error

This highlights that the mode of exposure determines the intensity of the metabolic response with sub-lethal imidacloprid exposure. Our results also reveal the metabolic impairment of imidacloprid to *E. fetida* earthworms in acute toxicity studies. These findings should be confirmed with additional studies using imidacloprid-contaminated soils.

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