Assessment of emerging organic contaminant toxicity to the earthworm *Eisenia fetida* using GC-MS metabolomics

Joseph Daniel Gillis

Department of Bioresource Engineering

Faculty of Agricultural and Environmental Sciences

Macdonald Campus of McGill University

Sainte-Anne-De-Bellevue, Quebec, Canada

December, 2017

A thesis submitted to McGill University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Abstract

Emerging contaminants like pharmaceuticals and personal care products are increasingly being detected in soil and water bodies as a result of the release of wastewater effluent and the application of biosolids to land. Many emerging contaminants have not undergone thorough ecotoxicity testing, since they have only recently come to the attention of scientists, government, and the public. Earthworm toxicity tests play a key role in determining the risk of a contaminant to the health of soil organisms. Metabolomics is emerging as a useful tool for assessing toxicity, since it provides a snapshot of the physiological state of an organism and yields a greater depth of knowledge than traditional endpoints. This thesis uses standard earthworm toxicity testing methods combined with targeted gas chromatography-mass spectrometry (GC-MS) metabolomics to assess the toxicity to earthworms of three emerging contaminants (triclosan, methyltriclosan, and metformin) present in biosolids. Using the 48 hour filter paper test, triclosan caused mortality at the highest exposure concentrations, and sub-lethal changes in the ratio between specific metabolites as measured by the slope of regression lines. However, no toxic effects for triclosan were observed in the 14 day test in earthworm bedding, possibly due to reduced bioavailability of triclosan in the high organic matter substrate. Methyltriclosan, the most abundant environmental transformation product of triclosan, led to significant metabolic effects at >64 times lower concentration than triclosan in the 14 day test. Succinic acid was significantly increased, suggesting a potential effect on the membrane-bound tricarboxylic acid (TCA) cycle enzyme succinate dehydrogenase caused by methyltriclosan accumulation in membranes. This mode of action has been proposed for other hydrophobic organic contaminants. Discriminant analysis revealed that metabolite profiles in the 1 and 4 $\mu g\ g^{-1}$ exposures were separate from the control and remaining treatments. The antidiabetic drug metformin caused a

reduction in glucose, malic acid, and margaric acid in the 14 day test, consistent with the mode of action (reduced gluconeogenesis and increased oxidation/reduced synthesis of fatty acids) in humans. Discriminant analysis revealed that time of exposure to metformin had a large influence on the metabolite profile, with significant discrimination between time points within a concentration at all concentrations except the highest. Discrimination within each time point based on concentration found that only Day 7 was significant, indicating that the standard 14-day exposure test may not be suitable to capture significant effects using metabolomics. In general, metabolomics detected significant effects of exposure at sub-lethal concentrations, and provided evidence to hypothesize an unknown mode of action for methyltriclosan (inhibition of succinate dehydrogenase) and to confirm a suspected mode of action for metformin (reduced gluconeogenesis and decreased synthesis/increased oxidation of fatty acids).

Résumé

Des contaminants émergents, tels que les produits pharmaceutiques et de soins personnels, sont de plus en plus détectés dans les sols et les plans d'eau, résultant de déversements des effluents d'eaux usées ainsi que de l'épandage de biosolides sur les terres. Plusieurs de ces contaminants émergents n'ont pas fait l'objet de tests approfondis d'écotoxicité, puisqu'ils n'ont que récemment retenu l'attention des scientifiques, des gouvernements et du public. Des essais de toxicité chez le ver de terre jouent un rôle clé dans la détermination des risques de contamination pour la santé des organismes du sol. La métabolomique est un outil qui s'avère utile pour évaluer une telle toxicité, puisqu'elle offre un aperçu de l'état physiologique d'un organisme et fournit une connaissance plus approfondie que les réponses mesurées traditionnelles. Cette thèse utilise des méthodes normalisées d'essais de toxicité chez le ver de terre, combinées à la chromatographie en phase gazeuse-spectrométrie de masse (GC-MS) ciblée, en métabolomique, afin d'évaluer la toxicité pour le ver de terre, venant de trois contaminants émergents (triclosan, methyltriclosan et metformine) présents dans les biosolides. Utilisant l'essai du papier filtre de 48 heures, le triclosan a causé la mort suite à une concentration d'exposition maximale, ainsi que des changements sublétaux dans le rapport entre des métabolites particuliers, mesurés par la pente des lignes de régression. Toutefois, l'essai de 14 jours n'a révélé aucun effet toxique pour le triclosan dans la litière de ver de terre, à des concentrations pouvant atteindre 64 $\mu g\ g^{-1}$, possiblement en raison d'une réduction de la biodisponibilité du triclosan à l'intérieur du substrat à teneur élevée de matière organique. Le methyltriclosan, le produit environnemental transformé du triclosan le plus abondant, a vu des effets métaboliques significatifs à 1 $\mu g\ g^{-1}$, une concentration de plus de 64 fois moins grande que le triclosan lors de l'essai de 14 jours. L'acide succinique a augmenté de façon significative, suggérant un effet potentiel sur l'enzyme succinate

désydrogénase du cycle de l'acide tricarboxylique (TCA) liée à la membrane, causé par l'accumulation de methyltriclosan dans les membranes. Ce mode d'action a été proposé pour d'autres contaminants organiques hydrophobiques. L'analyse discriminante a révélé que les profiles des métabolites à exposition de 1 et 4 $\mu g\ g^{-1}$ étaient séparés des traitements de contrôle et des traitements restants. La metformine, un médicament antidiabétique, a causé une réduction du glucose, de l'acide malique et de l'acide margarique lors de l'essai de 14 jours, ce qui est conforme au mode d'action (réduction de la gluconéogénèse et augmentation de l'oxydation/réduction de la synthèse des acides gras) chez les humains. L'analyse discriminante a révélé que le temps d'exposition à la metformine avait une grande influence sur le profil des métabolites, avec une discrimination importante entre les différents moments précis et correspondant à une concentration, et ce à toutes concentrations à l'exception de la plus élevée. La discrimination à chaque moment précis et selon la concentration n'a été significative qu'au jour 7, démontrant que l'essai normalisé d'exposition de 14 jours en métabolomique pourrait ne pas être convenable pour saisir des effets significatifs. En général, la métabolomique a détecté des effets significatifs de l'exposition à des concentrations sublétales, et a fourni des preuves pour émettre des hypothèses sur un mode d'action inconnu pour le methyltriclosan (inhibition de la succinate désyhdrogénase), et pour confirmer un mode d'action soupçonné pour la metformine (réduction de la gluconéogénèse et diminution de la synthèse/augmentation de l'oxydation des acides gras).

Acknowledgements

I must first thank my supportive wife, Rosalie Gillis-Madden, and Pollywog, who will now have my undivided attention. Thank you for waiting. Many thanks are due to Shiv for enticing me to come to McGill after defending my M.Sc. in 2011, and providing access to a wide range of subject areas for me to carve out a project that I could call my own. At McGill I was exposed to many fascinating topics within the Department of Bioresource Engineering completely outside my subject area that opened my eyes to many different ways of thinking about a problem. I owe a great deal of thanks to Gordon who, over the last 10 years, has given me the best of what a graduate student experience can be: fancy equipment with a warranty, independence to design my own research and pursue side projects, timely advice to address comments from Reviewer 2, extra funding when mine ran out, and the opportunity to attend a very generous number of conferences. I wish every graduate student could have the same experience as I have had. My sincerest thanks go to my dear friends Bailey and Stéphanie for hosting me on their couch and introducing me to their group when I first arrived in Montreal, and to Stéphanie for graciously translating my abstract on short notice. I am lucky to have a friend like Sebastian Margarit and am thankful for our lively discussions about chemistry and stats, and the reminder that there is a real world outside of academia. It was a great benefit to have laboratory technical support and a positive work environment provided by Cory J.D. Roberts, Doug Burris, and Kathy Zarayan. I greatly appreciate the help from Raghavendra Gunnaiah related to data processing, and from Rosy, Liz MacCormick, Yu Zhang, Weixi Shu, and Huifeng Qian for assistance in setting up experiments and performing laboratory work. Finally, thank you to the Dirty Friday crowd for some unproductive Saturdays: Rosy, Laura, Hossein, Carolyn[2], Sam, Liz, Tarjei, Kathleen, Dave, and Gordon.

Dedication

To my mother Dyney Catherine Gillis (1958 – 2013), who inspired my love of science. It feels like yesterday that we loaded Wiggum, Snoop, and a few bags of clothes into the car and set out on our last journey together: from Inverness, N.S., to the big city of Montreal to start my PhD.

I made it, Mom.

To Richard and Clara, you gave me a life-changing opportunity early in my education, and I have always strived to take full advantage of the doors you helped open. My words have failed in thanking you, but I hope my actions can convey the deep appreciation that I feel.

Contributions of the authors

One chapter of this thesis has been published in a peer reviewed journal (Chapter 4), one chapter has been submitted to a peer reviewed journal (Chapter 5), and one is being prepared for submission (Chapter 6). Portions of the data and ideas in this thesis have been presented at two scientific conferences in a preliminary form. The author of this thesis was the first author on the published manuscript and was responsible for the design and execution of experiments, preparation and analysis of samples by GC-MS, data processing and statistical analysis, creation of figures, and the preparation of all manuscripts and conference presentations.

The primary supervisor is Dr. Shiv Prasher, who provided guidance on the research direction and study designs. He was also involved in an editorial role in the preparation of manuscripts. Dr. Gordon Price is the co-supervisor, and also provided guidance on research direction, study designs, and editing of manuscripts. Dr. Price also supplied the maintained earthworm population, experiment materials, and laboratory instrumentation for analysis of samples. Cory J.D. Roberts and Doug Burris provided technical assistance including laboratory support and maintenance of the earthworm populations.

ResearcherID: O-5627-2014

ORCID QR Code:

Articles from PhD thesis published in refereed journals

Gillis, J.D., Price, G.W., Prasher, S.O. (2017) Lethal and sub-lethal effects of triclosan toxicity to the earthworm *Eisenia fetida* assessed through GC–MS metabolomics. Journal of Hazardous Materials 323A, 203-211 (doi:10.1016/j.jhazmat.2016.07.022)

Articles from PhD thesis in preparation or submitted to refereed journals

Gillis, J.D., Price, G.W., Prasher, S.O. (2017) Metabolomics reveals increased toxicity in earthworms (*Eisenia fetida*) of the transformation product methyl-triclosan. In Preparation

Gillis, J.D., Price, G.W., Prasher, S.O. (2017) Perturbations in the earthworm metabolite profile during a two week exposure to metformin assessed using GC-MS metabolomics. In Preparation.

Other non-thesis articles published in refereed journals during PhD program

Gillis, J.D., Price, G.W. (2016) Linking short-term soil carbon and nitrogen dynamics: Environmental and stoichiometric controls on fresh organic matter decomposition in agroecosystems. Geoderma 274, 35-44 (doi:10.1016/j.geoderma.2016.03.026)

Price, G.W., Astatkie, T. **Gillis, J.D.**, Liu,K. (2015) Long-term influences on nitrogen dynamics and pH in an acidic sandy soil after single and multi-year applications of alkaline treated biosolids. Agriculture, Ecosystems and Environment 208:1 (doi:10.1016/j.agee.2015.04.010)

Gillis, J.D., Price, G.W., and Stratton, G.W. (2014) Detection and degradation of organic contaminants in an agricultural soil amended with alkaline treated biosolids. Canadian Journal of Soil Science 94(5):595 (doi:10.4141/cjss2013-123)

Gillis, J.D. (2013) Book Review – "Water Matters:Why we need to act now to save our most critical resource, T. Lohan, AlterNet Pub, San Rafael, CA (2011). ISBN: 9780975271480". Journal of Cleaner Production 60:230 (doi: 10.1016/j.jclepro.2013.07.012).

Conference presentations from PhD thesis

Gillis, J.D., Price, G.W., Prasher, S.O. (2015) Assessment of triclosan toxicity to the earthworm *Eisenia fetida* under laboratory conditions using GC-MS metabolomics. Joint Meeting of IUSS, CSSS, and AQSSS: Soil Interfaces for Sustainable Development. July 5-10, 2017. McGill University, Montreal, QC.

Gillis, J.D., Price, G.W., Prasher, S.O. (2015) Development of a GC-MS metabolomics technique to assess earthworm (Eisenia fetida) responses to induced chemical stressors using emerging substances of concern. Dalhousie University and Canadian Water Network – Canadian Municipal Water Consortium. Symposium on the 'Impacts of alkaline stabilized biosolids application on fate and transport of emerging substances of concern in agricultural soils, plant biomass and drainage water'. June 11-12, 2015. Dalhousie University, Truro, NS.

Table of contents

List of tables

List of figures

List of abbreviations

ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
AMPK	5' Adenosine Monophosphate-activated Protein Kinase
ANOVA	Analysis of Variance
BH	Benjamini-Hochberg
CCME	Canadian Council of Ministers of the Environment
CEPA	Canadian Environmental Protection Act
CI	Confidence Interval
DA	Discriminant Analysis
DDT	Dichlorodiphenyltrichloroethane
DW	Dry Weight
EC_{50}	Effective Concentration in 50% of the population
ESOC	Emerging Substance of Concern
FA	Fatty Acid
FDR	False Discovery Rate
GC	Gas Chromatography
HPLC	High Performance (Pressure) Liquid Chromatography
K_{OW}	Octanol-Water Partition Coefficient
LC	Liquid Chromatography
LC_{50}	Lethal Concentration in 50% of the population

MANOVA	Multivariate Analysis of Variance
MeOX	Methoxamine
MS	Mass Spectrometry
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
MTCS	Methyltriclosan
MW	Molecular Weight
NMR	Nuclear Magnetic Resonance (Spectroscopy)
OECD	Organization for Economic Cooperation and Development
PC	Principal Component
PCA	Principal Components Analysis
PLS	Partial Least Squares or Projection to Latent Structures
QC	Quality Control
RT	Retention Time
SSP	Sum of Squares and Products
TCS	Triclosan
TIC	Total Ion Chromatogram
USEPA	United States Environmental Protection Agency
WWTP	Wastewater Treatment Plant
XIC	Extracted Ion Chromatogram

Chapter 1: Introduction and literature review

1.1. Introduction

Ecotoxicology is the field of science that studies the impacts from toxic chemicals on living systems, ranging from the large-scale ecosystem level down the cellular and molecular processes taking place within an organism (Timbrell 2008). There are over 100,000 synthetic organic chemicals in use today, and only a fraction have had their environmental fate and ecotoxicology thoroughly assessed (Timbrell 2008; Clarke and Smith 2011). Land application of biosolids is recognized as a frequent source of soil contamination by chemicals that enter the municipal wastewater stream and accumulate in the solids produced during wastewater treatment (Webber and Lesage 1989; Bright and Healey 2003; Kinney et al. 2006). However, it is also recognized that the documented presence and measured concentrations of chemicals in soil are inadequate to predict toxicity, since not all soil contaminants are bioavailable or toxic (Alexander 2000; Dean and Scott 2004).

Toxicity testing with model organisms is necessary for environmental risk assessment of a chemical to an ecosystem (European Commission 2003; Chalew and Halden 2009a; Clarke and Smith 2011), and earthworms are frequently used to assess the toxicity of organic contaminants in the soil environment (Spurgeon et al. 2003). The standard observation-based endpoints of mortality, weight loss, and reproduction are suitable to determine toxicity thresholds for risk assessment, but do not yield information on the mode of action (MOA, the biochemical interaction that leads to its activity) which can be applied more generally to other organisms with similar biochemical pathways (Aliferis and Jabaji 2011; Lankadurai et al. 2011a). Exposure of an organism to compounds with bioactive properties causes alterations in their metabolism that are

reversible or not depending on the MOA, exposure dose, exposure time, environmental variables, and the condition of the organism (Aliferis and Jabaji 2011). Metabolomics is an emerging method to assess the biochemical response of organisms exposed to a variety of stimuli (Bundy et al. 2009). It is the study of small molecules and metabolites within an organism, tissue, or biofluid, a comprehensive dataset that is called the metabolome (Viant 2008). Metabolomics now compliments traditional ecotoxicology studies on earthworms (Simpson and McKelvie 2009), yielding a greater depth of information that can reveal sub-lethal metabolic perturbations pointing to a toxic MOA for a test substance (Guo et al. 2009; Lankadurai et al. 2011a).

There is accumulating evidence that exposure of earthworms to sub-lethal concentrations of a compound can lead to metabolic alterations that are measurable with GC-MS metabolomics. McKelvie et al. (2009) measured eleven metabolites in *Eisenia fetida* exposed to two pesticides, endosulfan and DDT. The alanine to glycine ratio could distinguish between the control 1.0 μg cm^{-2} for DDT and at 0.5 and 1.0 μg cm^{-2} for endosulfan. Jones et al. (2008) analyzed *Lumbricus rubellus* earthworms exposed to increasing doses of pyrene in a sterilized soil, and identified up to 51 metabolites using GC-MS. Using Partial Least Squares-Discriminant Analysis (PLS-DA), they could separate control vs. treated worms at concentrations of 40, 160, and 640 mg kg^{-1}, but not at 10 mg kg^{-1}. Guo et al. (2009) recently showed that earthworm samples exposed to sub-lethal concentrations of three contaminants (cadmium, atrazine, and fluoranthene) differing in their mode of action could be distinguished from each other in a concentration-dependent manner using a variety of multivariate statistical techniques. The mode of action has been hypothesized for several toxic substances in earthworms using metabolomics, including possible inhibition of the enzyme succinate dehydrogenase disrupting energy metabolism for methyltriclosan (Chapter 5 of this thesis) and phenanthrene (Lankadurai et al. 2011b), increase in

oxidation of fatty acids and reduced ATP synthesis and for perfluorooctane sulfonate and perfluorooctanoic acid (Lankadurai et al. 2012; Lankadurai et al. 2013a), and induction of oxidative stress by nanoparticles of TiO_2 (Whitfield Åslund et al. 2011b). These examples show the potential of metabolomics to improve the depth of information gained from toxicity testing.

1.2. Emerging organic contaminants: a contemporary environmental issue

In our modern society, we depend heavily on advancements in chemical synthesis to develop new medicines to cure diseases or improve quality of life, new pest control products to enable food production for a surging world population, and new consumer products that are desirable for a specific purpose and generate economic activity (e.g non-stick cookware or nuisance insect repellent). It is estimated that over 100,000 synthetic chemicals are in use today (Timbrell 2008). For comparison, this is more than the 70,000 known or suspected metabolites listed in the Human Metabolome Database (Wishart et al. 2013), and roughly half of the estimated 200,000 primary and secondary metabolites produced by plants (Hartmann 2007). For many products, ultimate disposal after use is directly (soap, shampoo, toothpaste, other 'down the drain' products) or indirectly (excreted pharmaceuticals, products washed off skin) to the wastewater treatment systems. In municipal collection systems, the contaminated wastewater from households, industries, hospitals, and storm water collection is aggregated for treatment to reduce nutrients, suspended solids, and pathogens before release into water bodies. The sewage sludge from wastewater treatment is typically treated to reach pathogen and metal quality guidelines before spreading on land (CCME 2010), although incineration occurs in some locations like Montreal (Bruemmer 2015). Conventional wastewater treatment processes do not remove all of the diverse synthetic compounds introduced by humans, and many different

contaminants remain dissolved in treated wastewater effluent (Loos et al. 2013) and sorbed to organic matter in biosolids produced from the residual solids (Monteith et al. 2010).

Over the last 60 years or so, researchers have sought to measure concentrations of organic contaminants in the environment, and methods for the routine detection and identification of contaminants have seen rapid advancements in the last 20 years (Noguera-Oviedo and Aga 2016). As novel methods are developed, many new anthropogenic substances are being detected in the environment, which have collectively been termed emerging contaminants (ECs), emerging substances of concern (ESOCs), or other similar names. Many emerging contaminants are pharmaceuticals, including antibiotics, antidepressants, and lipid or glucose regulators. Others are personal care products like synthetic fragrances, UV filters in sunscreen, insect repellents, or antibacterial ingredients of soaps or toothpaste. Brominated flame retardants added to textiles and fluorinated non-stick coatings on cookware have also been detected. The land application of biosolids produced during wastewater treatment is a significant source of emerging contaminants in the soil environment (Wu et al. 2010a; Clarke and Smith 2011; Clarke and Cummins 2015; Meng et al. 2016). Risk assessments based on human or environmental health pathways using the limited available occurrence and toxicity data indicate that some contaminants in biosolids can exceed levels that would cause a detrimental effect (Fuchsman et al. 2010; Langdon et al. 2010; Snyder and O'Connor 2013; Prosser and Sibley 2015; Verlicchi and Zambello 2015; García-Santiago et al. 2016). Once present in soil, contaminants are subject to transport and degradation processes that affect their fate, which is described in Section 2.2. The assessment of detrimental effects with earthworm toxicity testing and the novel measure of toxicity (metabolomics) used in this thesis are outlined in Sections 2.3 to 2.5.

1.3. Environmental fate of organic contaminants in biosolids applied to land

In the soil matrix, organic contaminants are subject to transport, sorption/desorption, and degradation processes that are influenced by the chemical structure of the substance and the properties of the soil system (Peijnenburg 2004). Persistence is inversely related to the ability of a substance to be degraded by both abiotic and biotic processes. Geochemical processes like hydrolysis, oxidation-reduction, or condensation with soil organic matter reduce the persistence of some contaminants (Berkowitz et al. 2014). Soil microorganisms begin to decompose added organic matter within hours after incorporation, and can consume a large proportion of the material added to soil within a few weeks (Gillis and Price 2016). This microbial activity will also degrade or transform some contaminants depending on their chemical structure (Hesselsøe et al. 2001). Microorganisms can degrade contaminants with common biochemical functional groups, including carboxylic acids, alcohols, amines, amides, alkanes, cycloalkanes, and phenolic rings (Hickey 2005). Many substances like pharmaceuticals contain functional groups that are foreign to known life forms. These groups may be toxic to targeted organisms (e.g. pesticides) or have desirable effects (e.g. pharmaceuticals), but they impede degradation in soil or water, increasing persistence. Examples include halogen, nitro, cyano, and sulfonic acid functional groups, branched alkanes, and rings with O, N, or S (Hickey 2005). Microbial transformation requires modification of these groups before the compound can be utilized as a substrate, requiring specific detoxifying enzymes (Hickey 2005). In some cases, transformation products are more persistent and more toxic than the parent compound (McCormick et al. 2011).

Substances that persist in soil have the potential to migrate from their original location in soil water depending on the properties of the chemical and nature of the soil (Huang et al. 2003). Water solubility determines the maximum amount of a substance that can be dissolved in water

to be transported. In addition, soil organic matter contains many different polar and nonpolar functional groups that are attracted to chemicals dissolved in soil water, forming hydrogen bonds or other noncovalent interactions that temporarily or permanently remove the substance from solution (Huang et al. 2003). Substances partition between soil water and soil organic matter based on the strength of the association with organic matter and the solubility in water, although substances can also become physically trapped within the organic matrix and irreversibly bound (Huang et al. 2003). The ratio at equilibrium between the amount of substance adsorbed to soil and the amount dissolved in water is termed the solid-water distribution coefficient (K_D). Since the majority of sorption takes place to organic matter, the sorption coefficient can be normalized to the soil organic carbon fraction to express sorption as the organic carbon-water distribution coefficient (K_{OC}). These properties are able to predict the transport of low polarity contaminants in soil under certain conditions (Huang et al. 2003), although ionisable compounds that are affected by soil pH are poorly described (Cunningham 2008). Substances with higher water solubility and lower K_{OC} are more easily transported to deeper soil layers in percolating water, while those with lower water solubility and stronger sorption to organic matter have limited mobility. Soils with high organic carbon content increase the sorption of contaminants compared to low organic matter soils (Spark and Swift 2002; Wu et al. 2015a), although it is also related to the quality (polarity and aromaticity) of soil organic matter (Xing 1997).

Soil contaminants that are persistent and partition into the organic carbon fraction have the potential to bioaccumulate in soil-dwelling organisms that occupy the contaminated space and consume the organic matter. Earthworms have been used in bioassays of contaminant bioaccumulation in soil, due to their direct physical contact and ingestion of large amounts of soil during their lifetime (Lanno et al. 2004). However, the assessment is complicated by

behavioural differences between species, and by artifacts of laboratory assays using homogenized and sieved soil that do not reflect field conditions (Jager et al. 2005). Bioaccumulation in earthworms is dependent on the desorption of contaminants from the soil matrix into pore water, which is influenced by sorption and degradation processes (Lanno et al. 2004; Carter et al. 2014) and can lead to different patterns of bioaccumulation resulting from low or high desorption rates (Jager et al. 2005). Elimination of a compound occurs if the earthworms are moved to fresh material (Jager et al. 2005). The bioaccumulation and elimination processes have been described by first order kinetics, with the rate of change in organism concentration equal to the rate of intake from pore water minus the rate of excretion of accumulated substance (Carter et al. 2014). Other approaches have used modifications to account for additional processes affecting organism concentration, such as distinguishing between passive elimination and biotransformation (Ma et al. 1998). Some authors have calculated bioaccumulation factors by measuring contaminant concentrations in soil and in earthworm tissues from agricultural sites amended with either biosolids or animal manure (Kinney et al. 2008). Once a contaminant enters an organism, it has the potential to cause toxic effects, as discussed in the next section.

1.4. Emerging contaminants in biosolids

In Canada, the management of toxic substances is under federal jurisdiction, falling under the Canadian Environmental Protection Act (CEPA), which mandates that the Government of Canada will protect human and environmental health from the risks arising from the use of and release into the environment of toxic substances (Government of Canada 1999). This is achieved through the List of Toxic Substances, which enables the Minister to regulate the import, export, production, use, sale, disposal, monitoring, or other activities that are permitted to be carried out

with substances that are on the list (Government of Canada 1999). Substances may be added to the list if human activity results in the substance entering the environment and there is evidence that the substance is toxic, persistent in the environment, and bioaccumulative in humans or other organisms (Government of Canada 1999). According to the CEPA, a substance is toxic if it enters the environment in a concentration or quantity that will "(a) Have or may have an immediate or long term harmful effect on the environment or its biological diversity; (b) constitute or may constitute a danger to the environment on which life depends; or (c) constitute or may constitute a danger in Canada to human life or health" (Government of Canada 1999).

The legislative framework for biosolids in Canada has been reviewed in detail by the Canadian Council of Ministers of the Environment (CCME 2010). In Canada, the management of municipal biosolids (from production to disposal) is accomplished through the acts and regulations within each province or territory, unless the product is sold as a fertilizer which falls under the federal Fertilizer Act and Fertilizer Regulations (CCME 2010). Municipalities are granted the authority to regulate certain aspects of biosolids, including land application, which varies between locations. Municipalities can require permits or other approvals for the production or disposal of biosolids and can use by-laws to control the quality of effluent entering the wastewater stream (CCME 2010). The definition of a 'biosolid' varies by province, but generally refers to the solid residuals from the treatment of sewage wastewater, which have undergone some sort of further process to meet defined standards of quality. Nova Scotia and Quebec each have two categories of biosolids based on quality (NS: Class A and B; QC: Class 1 and 2).

While it varies by province, trace metals (including arsenic, cadmium, chromium, cobalt, copper, mercury, molybdenum, nickel, lead, selenium, and zinc) are generally controlled by

maximum acceptable concentrations established for biosolids (CCME 2010). Only a few provinces specify limits for organic contaminants in biosolids, which consider dioxins, furans, and polychlorinated biphenyls (CCME 2010). Nova Scotia has a limit for dioxins and furans (in Toxic Equivalency Factor, TEQ) of 17 or 50 ng TEQ kg^{-1} for Class A or B biosolids respectively, and a limit of 800 ng g^{-1} for polychlorinated biphenyls in Class A. For Class B Biosolids in Nova Scotia, the guidelines state that analysis of selected industrial chemicals, alkylphenols and ethoxylates, flame retardants, pharmaceuticals, hormones, steroids, personal care products, and other substances must be monitored by producers every 10,000 Mg generated (NSE 2010). However, this does not apply to Class A biosolids, there are no specific contaminants mentioned by name, and no limits are specified.

In response to the nation-wide lack of monitoring data for assessing risk of emerging organic contaminants in biosolids, the Canadian Council of Ministers of the Environment (CCME) conducted a survey of 71 pharmaceuticals and personal care products in biosolids from 11 WWTPs across Canada (Monteith et al. 2010). Although some compounds were not detected in any samples, the pharmaceuticals diphenhydramine, carbamazepine, and ciprofloxacin, and the personal care product ingredients miconazole, triclosan, triclocarban, HHCB, AHTN, and ATII were detected in over 90% of samples. Two antimicrobial compounds, triclosan (up to 30 μg g^{-1}) and ciprofloxacin (up to 27 μg g^{-1}), had the highest measured concentrations among all contaminants measured (Monteith et al. 2010). A similar picture emerged from the USEPA Targeted National Sewage Sludge Survey of contaminants in biosolids from across the US, with azithromycin, carbamazepine, ciprofloxacin, doxycycline, 4-epitetracycline, erythromycin, fluoxetine, gemfibrozil, miconazole, ofloxacin, tetracycline, triclocarban, and triclosan detected

in >90% of samples (USEPA 2009b). Triclosan (up to 133 μg g^{-1}) and ciprofloxacin (up to 47 μg g^{-1}) were among the highest concentrations detected in US samples as well.

1.5. Toxicity assessment using earthworms

Assessment of toxicity to an organism involves determining the effective dose or concentration at which specific impacts occur (Calow and Forbes 2003). Knowing the environmental concentration or dose at which a substance is toxic and causes harm to an organism is used for risk assessment as the threshold to which measured or predicted environmental concentrations are compared (European Commission 2003). Earthworms have been adopted as a standard organism in toxicity testing, and frequently represent soil organisms when establishing effect thresholds for environmental risk assessment (Spurgeon et al. 2003). There are two variations of the standard toxicity test: a 48 hour filter paper exposure, and 14 day 'soil' exposure (OECD 1984). The OECD method provides a recipe for a 'standard' soil for exposure, with a high sand content to minimize sorption of the test substance. There are valid criticisms of the filter paper test and of the OECD soil used in the 14 day test in that they do not truly reflect real world scenarios, but both tests still have value for investigating toxicity (Spurgeon et al. 2003). In addition, the recommended test species *Eisenia fetida* is generally not a soil dwelling earthworm, preferring organic matter rich environments more than the mineral dominated OECD soil (Spurgeon et al. 2003). Other earthworm species that live in soil have been used, but generally reproduce slower than *E. fetida* and are more difficult to culture (Spurgeon et al. 2003).

Since the OECD method was published in 1984 to measure toxicity based on weight loss and mortality (OECD 1984), many alternative measures of toxic response have been developed

using the same or modified exposure methods. Damage to earthworm DNA in response to organic contaminants and heavy metals has been assessed using the COMET assay, which measures breaks in DNA strands (Reinecke and Reinecke 2004; Liu et al. 2009; Lin et al. 2010). Activities of enzymes involved in defense against free radicals (superoxide dismutase and catalase) and metabolism of xenobiotics (glutathione-S-transferase) have also been measured in response to emerging contaminant exposure (Xue et al. 2009; Lin et al. 2010; Yang et al. 2012; Han et al. 2014). Efforts have also expanded to include proteomics (Ji et al. 2013a; Zhang et al. 2017) and metabolomics (Bundy et al. 2009; Whitfield Åslund et al. 2012; Lankadurai et al. 2013b; McKelvie et al. 2013) analysis to determine protein or metabolite biomarkers that indicate toxicity. In particular, metabolomics has shown great promise since it can detect sub-lethal changes in metabolite levels in response to stress on an organism (Bundy et al. 2009; Simpson and McKelvie 2009). Based on the affected metabolites and using knowledge of their biochemical pathways, a potential mode of action for the toxic substance can often be proposed. The use of metabolomics for toxicity assessment is described in Section 2.5.

1.6. Metabolomics as a novel measure of toxic response

1.6.1. Modes of action and mechanisms of toxicity

Aside from the regulatory definitions mentioned previously, toxicity can be generally defined as the ability of a substance to induce an adverse response or effect in an organism (Sparling 2016). All adverse effects have a biochemical basis, thus the effect is dependent on the target location within the organism where the toxicant interacts with the exposed biological system. Escher et al. (2011) define the mode of action as "a common set of physiological and behavioral signs that characterize a type of adverse biological response". The toxic mechanism

of action is defined as the critical underlying biochemical interactions or processes that give rise to a mode of action (Escher et al. 2011). Although the use of these terms is inconsistent in the literature, the mode of action is typically used to describe an observed toxic response in which the mechanism is not known, while mechanism of action implies knowledge of specific interaction between the toxic substance and one or more biochemical systems in an organism (Escher et al. 2011).

There is a baseline toxicity of all substances that is non-specific and results from disturbances to the structure and function of biological membranes (van Wezel and Opperhuizen 1995; Escher and Hermens 2002). This baseline toxicity, termed narcosis, is generally consistent for all chemicals, since the amount of substance in a biological membrane at the endpoint of lethality has been observed to be constant (Abernethyand et al. 1988; van Wezel and Opperhuizen 1995). Inert hydrophobic contaminants generally act through narcosis since they are bioaccumulative and have a high potential to accumulate in lipids, with the narcotic potency increasing with hydrophobicity expressed as log (K_{OW}) (Verhaar et al. 1992). Aside from non-specific narcosis, there can be specific membrane-related modes of action. If a substance is reactive, it can cause degradation of membrane lipids and proteins by forming reactive products that oxidize membrane lipids and proteins (Escher et al. 2011). Many important cellular processes, such as electron transport systems and cellular signalling, are controlled by membrane-bound enzymes that can be affected by disturbances to membrane characteristics or through the blockage of important sites (Escher et al. 2011). Foreign compounds within the membrane can interfere with proton shuttling mechanisms in mitochondria and lead to uncoupling (Escher et al. 2011), defined as “any process through which energy released from the combustion of a substrate (food) in the mitochondria is not conserved” (Nedergaard et al. 2005).

Blockage of receptor binding sites of membrane-bound proteins can inhibit the electron transport chain, while blockage of transport channels in the membrane can cause inhibition of ATP synthesis (Escher et al. 2011).

Other compounds can interact with proteins, peptides, DNA, or RNA, through mechanisms that have their own modes of action. Electrophilic compounds have the ability to react with nucleophilic centers and can lead to improper functioning of proteins, peptides, DNA, or RNA, through oxidative damage or the formation of adducts (Escher et al. 2011). Damage to enzyme proteins can lead to the depletion of critical metabolites, while damage to DNA or RNA can lead to errors in transcription or translation (Escher et al. 2011). In addition, compounds with polar functional groups can covalently or noncovalently bind to enzyme receptors, leading to inhibition of the enzyme or competitive binding, with consequent effects on their respective biochemical pathways (Escher et al. 2011).

1.6.2. Metabolomics and its use in toxicology

Metabolomics looks at the last link of the "omics" chain, the small molecules that are acted on by enzymes and are the biological building blocks and functional molecules that make up the chemistry of life (Ryan and Robards 2006). The Human Metabolome Database (Wishart et al. 2013) includes entries for over 70,000 confirmed or suspected metabolites in humans, while plants may have more than 200,000 (Hartmann 2007). Metabolomics, as its own field of science, is just under two decades old, and analytical methods are still being developed and standardized (Kanani et al. 2008). To date, the exhaustive characterization of the metabolome has not been reported for any organism, but tens to hundreds of targeted metabolites can be routinely quantified in a sample, allowing many different biological questions to be answered

with this technique (Viant et al. 2017). This is only a small fraction of possible metabolites, and efforts are ongoing to automate metabolite identification and annotation for a wide range of metabolites in non-targeted analysis (Viant et al. 2017).

Environmental metabolomics is the study of the interactions between organisms and their environment using a metabolomics approach, and includes the effects of chemical stimuli such as environmental pollutants (Bundy et al. 2009). Earthworms, a standard test organism in ecotoxicology (OECD 1984), have been adopted in environmental metabolomics to observe metabolic changes due to pollutant exposure. The ability to detect sub-lethal responses at lower pollutant concentrations is a significant advantage of metabolomics over mortality or reproduction based end-points (Bundy et al. 2002). There are a growing number of studies in the metabolomics literature investigating earthworm ecotoxicology using NMR (e.g. Warne et al. 2000; Bundy et al. 2002; Brown et al. 2008; McKelvie et al. 2011) or GC-MS (e.g. Jones et al. 2008; McKelvie et al. 2009; Baylay et al. 2012; Mudiam et al. 2013; Gillis et al. 2017), while LC-MS approaches have been limited to date. Metabolomics has also been used to investigate toxic responses in other organisms, including bivalves (Zhang et al. 2011; Ji et al. 2013b), fish (Samuelsson et al. 2006; Sotto et al. 2017), aquatic plants (Liu et al. 2011), and daphnids (Nagato et al. 2016; Wagner et al. 2017). A significant advantage of metabolomics in toxicology is the ability to confirm or hypothesize a mode of action for a test substance. In a metabolomics study, biomarkers of exposure to the substance are identified that characterize the toxic response, and from these it is possible to interpret the mode of action by placing the affected metabolites in the context of known biochemical pathways (Aliferis and Jabaji 2011). Knowing the mode of action of a toxic compound is important in risk assessment when deciding if toxicity can be

extrapolated between species (Schrenk 2014). If the biochemical pathway is conserved between species, they may be similarly affected by exposure.

A search of the scientific literature for various terms describing environmental metabolomics studies with earthworms yielded 34 papers which are summarized in Table 1.1. Almost 80% (27/34 studies) used exclusively Nuclear Magnetic Resonance (NMR) spectroscopy. Three used Gas Chromatography-Mass Spectrometry (GC-MS), another three used NMR and GC-MS, while Ultra Performance Liquid Chromatography-Quadrupole-Time of Flight Mass Spectrometry (UPLC-QTOF-MS) was used once, making NMR the most common approach in this field to date. The filter paper contact test was the most common test media (14/34), followed by natural soils (11/34), artificial soils (8/34), and water (1/34). In many cases the metabolite profile of different chemicals can be clearly distinguished from one another based on unique features, while there also appears to be commonly affected metabolites (e.g. alanine) that may be general indicators of stress. In 25 studies, the authors were able to directly or indirectly hypothesize a mode of action to explain the observed effects on the metabolome. Effects on energy metabolism pathways were common and include the tricarboxylic acid cycle metabolites, glucose and other carbohydrates, as well as ATP. Enzyme production or protein catabolism explanations for increases or decreases in amino acid abundance were also commonly reported. Increases in osmoregulators such as betaine were attributed to the cellular response to membrane instability caused by some toxins. Only a few substances have been studied in more than one experiment, including phenanthrene (8/34) and endusulfan (5/34), and there exists a vast knowledge gap in understanding the metabolic effects of chemical toxins to earthworms. There is great potential to understand the mode of action of contaminants using metabolomics, and a long list of environmental contaminants for which the toxicity is poorly understood.

Table 1.1. Summary of earthworm toxicology studies using metabolomics, including biomarkers identified and mode of action hypothesized if applicable. ND – Not Determined; NR – Not Reported; NS – Not Significant.

Reference	Earthworm Species	Test Media	Analytical Platform	Test Compound(s)	Statistical Analysis	Metabolite Biomarkers	Hypothesized Mode of Action
Baylay et al. (2012)	*Lumbricus rubellus*	Clay loam soil with 3% composted bark (28 days)	GC-MS, NMR	Imidacloprid	ANOVA, DA	NR	ND
	Lumbricus rubellus	Clay loam soil with 3% composted bark (28 days)	GC-MS, NMR	Thiacloprid	ANOVA, DA	NR	ND
	Lumbricus rubellus	Clay loam soil with 3% composted bark (28 days)	GC-MS, NMR	Chlorpyrifos	ANOVA, DA	NR	ND
	Lumbricus rubellus	Clay loam soil with 3% composted bark (28 days)	GC-MS, NMR	Nickel	ANOVA, DA	NR	ND
Brown et al. (2009)	*Eisenia fetida*	Filter Paper (2 days)	NMR	Naphthalene	PCA, DA	NS	General stress response
	Eisenia fetida	Filter Paper (2 days)	NMR	Phenanthrene	PCA, DA	NS	General stress response
	Eisenia fetida	Filter Paper (2 days)	NMR	Pyrene	PCA, DA	NS	General stress response
Brown et al. (2010)	*Eisenia fetida*	Commercial Worm Bedding (2 days)	NMR	Phenanthrene	t-test, PCA	isoleucine, alanine, glutamine, maltose	General stress response
Bundy et al. (2001)	*Eisenia veneta*	Filter Paper (2 days)	NMR	3-fluoro-4-nitrophenol	PCA	malonate, acetate, succinate, trimethylamine-N-oxide	Interference with carbohydrate metabolism
Bundy et al. (2002)	*Eisenia veneta*	Filter Paper (2 days)	NMR	4-fluoroaniline	PCA	maltose	ND
	Eisenia veneta	Filter Paper (2 days)	NMR	3,5-difluoroaniline	PCA	inosine monophoshate, 2-hexyl-5-ethyl-3-furansulfonate	May affect nucleotide synthesis
	Eisenia veneta	Filter Paper (2 days)	NMR	2-fluoro-4-methylaniline	PCA	inosine monophoshate, 2-hexyl-5-ethyl-3-furansulfonate	May affect nucleotide synthesis

Table 1.1 Continued

Reference	Earthworm Species	Test Media	Analytical Platform	Test Compound(s)	Statistical Analysis	Metabolite Biomarkers	Hypothesized Mode of Action
Bundy et al. (2007)	*Lumbricus rubellus*	Sampled from seven field sites	NMR	Metal contaminated site (likely zinc causing effect)	Correlation, PCA	histidine	Protective mechanism to reduce cytotoxicity
Ch et al. (2015)	*Metaphire posthuma*	Soil (14 days)	GC-MS	Cypermethrin	t-test, PCA, PLS-DA	22 metabolites (mainly fatty acids, sugars and amino acids)	Disturbed neural system metabolism
Dani et al. (2018)	*Eisenia fetida*	Filter Paper (2 days)	NMR	Atrazine	t-test, PCA	Maltose, fumarate, malate, threonine, lactate, ATP, betaine, scyllo-inositol, glutamate, arginine, glutamine	Reduced ATP synthesis
Gibb et al. (1997)	*Eisenia andrei*	Sandy soil (in laboratory, 28 days)	NMR	Cu(II)	PCA, PLS-R	NS	ND
	Lumbricus rubellus	Sandy soil (outdoor mesocosm, 28 days)	NMR	Cu(II)	PCA, PLS-R	histidine	Distrupted histidine catabolism and excretion, OR increased production of histidine
Gillis et al. (2017)	*Eisenia fetida*	Filter Paper (2 days)	GC-MS	Triclosan	ANOVA, Regression, PCA	mannitol:inositol, valine:inositol	ND
Guo et al. (2009)	*Lumbricus rubellus*	Loam soil (28 days)	NMR	CdCl2	Correlation, PCA, PLS-R	beta-hydroxybutyrate, fumarate, lysine, malate	ND
	Lumbricus rubellus	Loam soil (28 days)	NMR	Atrazine	Correlation, PCA, PLS-R	glucose, asparagine, DMH, Asn, betaine, succinate	Biochemical starvation
	Lumbricus rubellus	Loam soil (28 days)	NMR	Fluoranthene	Correlation, PCA, PLS-R	lysine, lactate, cytidine triphosphate	ND
He et al. (2018)	*Eisenia fetida*	Loam soil (10 days)	UPLC-QTOF-MS	(–)-PCB 91	t-test, PCA, HCA	18 identified metabolites	amino acid metabolism, energy metabolism, neurodevelopment, and nucleotide metabolism
	Eisenia fetida	Loam soil (10 days)	UPLC-QTOF-MS	(+)-PCB 91	t-test, PCA, HCA	66 identified metabolites	amino acid metabolism, energy metabolism, neurodevelopment, and nucleotide metabolism
	Eisenia fetida	Loam soil (10 days)	UPLC-QTOF-MS	(±)-PCB 91	t-test, PCA, HCA	19 identified metabolites	amino acid metabolism, energy metabolism, neurodevelopment, and nucleotide metabolism
Ji et al. (2013a)	*Eisenia fetida*	Water (4 days)	NMR	2,2′,4,4′-tetrabromodiphenyl ether (PBDE 47)	Correlation, ANOVA, PCA, PLS-DA, O-PLS-DA	betaine, glycine, 2-hexyl-5-ethyl-3-furansulfonate, glucose, ATP, maltose, succinate	Membrane destabilization; Osmotic stress; Disturbed energy metabolism

Table 1.1 Continued

Reference	Earthworm Species	Test Media	Analytical Platform	Test Compound(s)	Statistical Analysis	Metabolite Biomarkers	Hypothesized Mode of Action
Jones et al. (2008)	*Lumbricus rubellus*	Sterilized loam soil (42 days)	GC-MS, NMR	Pyrene	PCA, PLS-R, PLS-DA	lactate, tetradecanoic acid, hexadecanoic acid, octadecanoic acid, alanine, leucine, valine, isoleucine, lysine, tyrosine, methionine	Impaired glucose metabolism, increased fatty acid metabolism
Lankadurai et al. (2011a)	*Eisenia fetida*	Filter Paper (2 days)	NMR	Phenanthrene	t-test, PCA	alanine, lysine, arginine, isoleucine, maltose, ATP, betaine	Protein catabolism for energy production; General stimulation of metabolism; Induction or inhibition of Cytochrome P450
Lankadurai et al. (2011b)	*Eisenia fetida*	Filter Paper (2 days)	NMR	Phenanthrene	t-test, PCA	alanine, glutamate, maltose, cholesterol, phosphatidylcholine, succinate, fumarate	Inhibition of succinate dehydrogenase by membrane destabilization; Disrupted osmoregulation
Lankadurai et al. (2012)	*Eisenia fetida*	Filter Paper (2 days)	NMR	Phenanthrene	t-test, PCA	leucine, alanine, glutamate, arginine, lysine, phenylalanine, maltose, malate, fumarate, succinate, betaine, scyllo-inositol, myo-inositol, 2-hexyl-5-ethyl-3-furansulfonate, ATP	Inhibition of succinate dehydrogenase
Lankadurai et al. (2013a)	*Eisenia fetida*	OECD artificial soil (2, 7, 14 days)	NMR	Perfluorooctane sulfonate	t-test, PCA, PLS-DA	2-hexyl-5-ethyl-3-furansulfonate, betaine, leucine, arginine, glutamate, maltose and ATP	Elevated fatty acid oxidation; Disruption in energy metabolism; Interruption of ATP synthesis
Lankadurai et al. (2015)	*Eisenia fetida*	OECD artificial soil (2, 7, 14 days)	NMR	C60 nanoparticles	t-test, PCA	leucine, isoleucine, valine, alanine, arginine, glutamate, lysine, glycine, phenylalanine, malate, succinate, fumarate, myo-inositol, betaine, inosine, glucose, maltose, 2-hexyl-5-ethyl-3-furansulfonate	Production of enzymes for defense and repair; Increased energy consumption; Increased glycolysis
McKelvie et al. (2009)	*Eisenia fetida*	Filter Paper (2 days)	GC-MS, NMR	DDT	t-test, linear regression, PCA	alanine, alanine:glycine	Protein breakdown or degradation
	Eisenia fetida	Filter Paper (2 days)	GC-MS, NMR	Endosulfan	t-test, linear regression, PCA	alanine, alanine:glycine	Protein breakdown or degradation
McKelvie et al. (2010)	*Eisenia fetida*	Artificial soil (30 days)	NMR	Phenanthrene	t-test, PCA, PLS-R	betaine, alanine, isoleucine, leucine	ND

Table 1.1 Continued

Reference	Earthworm Species	Test Media	Analytical Platform	Test Compound(s)	Statistical Analysis	Metabolite Biomarkers	Hypothesized Mode of Action
McKelvie et al. (2011)	*Eisenia fetida*	Filter Paper (2 days)	NMR	Carbaryl	t-test, PCA	Phenylalanine, tyrosine, lysine, alanine, valine, leucine	ND
	Eisenia fetida	Filter Paper (2 days)	NMR	Chlorpyrifos	t-test, PCA	NS	ND
	Eisenia fetida	Filter Paper (2 days)	NMR	Carbamazepine	t-test, PCA	Fumarate, glutamate, valine, leucine	ND
	Eisenia fetida	Filter Paper (2 days)	NMR	Estrone	t-test, PCA	adenine, glutamine	ND
	Eisenia fetida	Filter Paper (2 days)	NMR	Caffeine	t-test, PCA	NS	ND
	Eisenia fetida	Filter Paper (2 days)	NMR	Aroclor 1254	t-test, PCA	NS	ND
	Eisenia fetida	Filter Paper (2 days)	NMR	PBDE 209	t-test, PCA	maltose, lysine, glutamate	ND
	Eisenia fetida	Filter Paper (2 days)	NMR	Nonylphenol	t-test, PCA	adenine, glutamate	ND
	Eisenia fetida	Filter Paper (2 days)	NMR	Dimethyl phthalate	t-test, PCA	phenylalanine, alanine, leucine, valine	ND
McKelvie et al. (2013)	*Eisenia fetida*	Artificial soil, 1-27% OM (2 days)	NMR	Phenanthrene	t-test, ANOVA, PCA, PLS-DA	alanine, lysine, arginine, isoleucine, maltose, ATP, betaine	ND
Mudiam et al. (2013)	*Metaphire posthuma*	Soil (7 days)	GC-MS	Carbofuran	t-test, PCA, PLS-DA, 2D-HCA	glucose, tyrosine, valine, pyroglutamic acid, phosphoric acid, glycine, leucine, 2-amino-3-phenylpropane, galactose, proline, alanine, ornithine, serine, phenylalanine, isoleucine, methionine, succinic acid	Disruption of energy metabolism; Disturbance of amino acid and carbohydrate metabolism
Shi et al. (2018)	*Eisenia fetida*	OECD Artificial Soil (14 days)	NMR	Hexabromocyclodo decane	ANOVA, correlation, PCA, O-PLS-DA	ATP, lactate, valine, lysine, betaine, glycine	Disrupted energy metabolism; Disrupted membrane stability
Warne et al. (2000)	*Eisenia veneta*	Filter Paper (3 days)	NMR	3-trfluoromethyl aniline	Correlation, PCA, HCA	glucose, glycine, asparagine, citrate, succinate, alanine, lactate	ND
Whitfield Åslund et al. (2011c)	*Eisenia fetida*	OECD artificial soil (2 days)	NMR	Aroclor 1254	ANOVA, PCA, PLS-R	ATP, lysine	Disrupted energy metabolism; Disrupted membrane stability

Table 1.1 Continued

Reference	Earthworm Species	Test Media	Analytical Platform	Test Compound(s)	Statistical Analysis	Metabolite Biomarkers	Hypothesized Mode of Action
Whitfield Åslund et al. (2011b)	*Eisenia fetida*	Sandy loam soil (20-23 weeks)	NMR	TiO2 Nanoparticles N	PCA, PLS-DA	leucine, valine, alanine, glutamate, lysine, tyrosine, phenylalanine, lactate, maltose	Oxidative stress
	Eisenia fetida	Sandy loam soil (20-23 weeks)	NMR	TiO2 Nanoparticles B	PCA, PLS-DA	leucine, valine, alanine, glutamate, lysine, tyrosine, phenylalanine, lactate, maltose	Oxidative stress
(Whitfield Åslund et al. 2012)	*Eisenia fetida*	Aged (>30 years) PCB contaminated soil	NMR	Aroclor 1254	ANOVA, Correlation, PCA, PLS-R	ATP	ND
Whitfield Åslund et al. (2013)	*Eisenia fetida*	Aged (>20 years) petroleum contaminated soil	NMR	Petroleum hydrocarbons	PCA, PLS-R	ND	ND
Yuk et al. (2010)	*Eisenia fetida*	Filter Paper (2 days)	NMR	Endosulfan	PLS-DA, MANOVA	alanine, leucine, lysine, glutamate, glucose, maltose	ND
Yuk et al. (2011)	*Eisenia fetida*	Filter Paper (2 days)	NMR	Trifluralin	t-test, PCA	Alanine, glycine, maltose, ATP	Non-polar narcosis
	Eisenia fetida	Filter Paper (2 days)	NMR	Endosulfan	t-test, PCA	weight change, leucine, phenylalanine, tryptophan, lysine, glutamate, valine, glycine, isoleucine, methionine, glutamine, alanine, maltose, glucose, meibiose, malate, fumarate, ATP	Neurotoxic
Yuk et al. (2012)	*Eisenia fetida*	Filter Paper (2 days)	NMR	Endosulfan	t-test, ANOVA, PCA	Alanine, glycine, malate, alpha-ketoglutarate, succinate, betaine, myo-inositol, lactate, spermidine, glutamine, fumarate, glutamate, maltose, melibiose, ATP	Apoptotic
Yuk et al. (2013)	*Eisenia fetida*	OECD artificial soil (7 days)	NMR	Endosulfan	t-test, PCA	alanine, glycine, betaine, succinate, aplha-ketoglutarate, spermidine, myo-inositol, lactate	Neurotoxic and apoptotic
	Eisenia fetida	OECD artificial soil (7 days)	NMR	Endosulfan Sulfate	t-test, PCA	alanine, glycine, betaine, succinate, aplha-ketoglutarate, spermidine, myo-inositol, lactate	Neurotoxic and apoptotic

1.7. Sample analysis and data processing in GC-MS metabolomics

1.7.1. Benefits and drawbacks of the GC-MS platform for metabolomics

GC-MS is a very common platform for metabolomics analysis, due to the high resolution of chromatographic peaks, high analyte sensitivity, high selectivity based on the unique mass spectra for each compound, good reproducibility of duplicate samples, and the availability of free (Golm Metabolite Database) and commercially available (Fiehn Lib, NIST Library) mass spectral libraries containing thousands of compounds to aid in the identification of unknowns in a complex sample (Hummel et al. 2007; Kind et al. 2009). The resolution of GC-MS allows clear separation of a wide range of compounds with a molecular weight less than 650 amu, although non-volatile metabolites require derivatization procedures, including methoximation of some sugars to prevent multiple peaks from open chain and cyclic isomers, and trimethylsilylation of O-H and N-H bonds to increase volatility (Kind et al. 2009). The volatility requirement decreases the metabolome coverage that is possible by GC-MS, although derivatization expands the range of possible metabolites.

The sample preparation process to extract metabolites from earthworm tissue and produce a sample suitable for GC-MS analysis contains many steps that have the potential to introduce errors (technical variability) into the analysis (see Section 1.7.2.). Additional biological variability within the population under study, due to differences such as life stage, genetic code, or growth environment, can mask treatment effects depending on the magnitude of the biological differences (see Section 1.7.3.). The complexity of metabolomics analysis requires a number of steps to minimize or account for variability due to technical and biological sources (see Section 1.7.6.). These steps vary greatly between studies, and the methods used for earthworm sample

preparation, analysis, and data normalization for GC-MS metabolomics have not been standardized (De Livera et al. 2012; Liebeke and Bundy 2012).

1.7.2. Sources of technical variability

The general protocol for sample preparation in a metabolomics experiment (Fig. 1.1.) typically includes quenching metabolism through freezing and/or lyophilization, tissue disruption and homogenization of samples, solvent extraction of metabolites, centrifugation or filtration for removal of solids, evaporation of the supernatant and derivatization of the dried sample (in GC-MS metabolomics), and analysis of the sample by the instrument of choice (typically GC-MS,

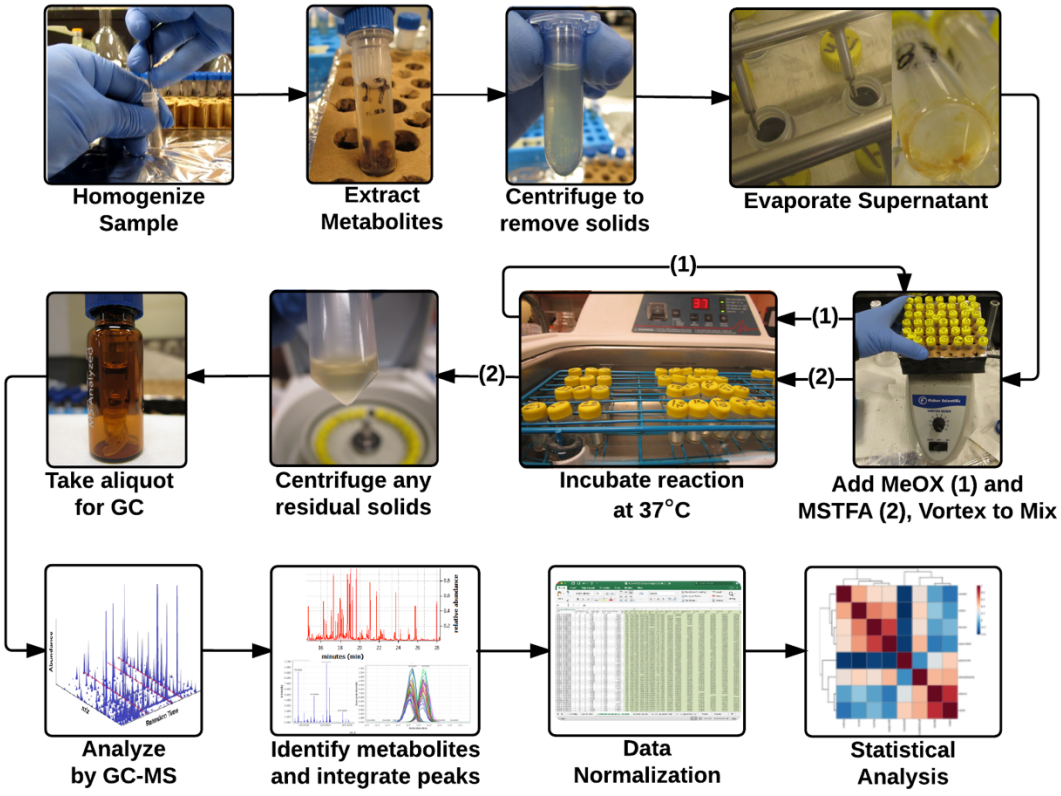

Figure 1.1. Metabolomics work flow from sample processing to data analysis

LC-MS, or NMR). While quenching metabolism in liquid N_2 is the first step after harvesting of organisms in most experiments, the remaining aspects of sample preparation are less consistent between studies. For instance, some authors lyophilize (freeze-dry) the samples prior to homogenization (Bundy et al. 2004; Brown et al. 2008; McKelvie et al. 2009; Schock et al. 2016; Gillis et al. 2017) or after samples have been ground (Bundy et al. 2008; Guo et al. 2009), while other authors extract the samples directly after flash-freezing in liquid N_2 (Lenz et al. 2002; Rochfort et al. 2009). If storage until further processing is necessary, samples can be frozen at -20°C (Bundy et al. 2002; Gillis et al. 2017) or -80°C (Bundy et al. 2008; Guo et al. 2009; Rochfort et al. 2009).

The homogenization of samples is an important step, since the stored samples are first exposed to ambient temperatures at this stage, potentially allowing degradation of sensitive metabolites. Some authors have performed tissue homogenization over ice (Lenz et al. 2005; Alvarez et al. 2010), dry ice (Jones et al. 2008), or liquid N_2 (Bundy et al. 2008; Rochfort et al. 2009; Liebeke and Bundy 2012) to prevent the activity of degradative enzymes, while other authors did not use this technique (Bundy et al. 2002; Brown et al. 2008; McKelvie et al. 2009; Schock et al. 2016; Gillis et al. 2017). Tissue disruption can be accomplished with a manual implement such as a mortar and pestle (Bundy et al. 2002; Bundy et al. 2004; Jones et al. 2008; Rochfort et al. 2009; Schock et al. 2016) or metal micro spatula (Brown et al. 2008; McKelvie et al. 2009; Gillis et al. 2017). Automated methods include electronic homogenizing equipment that can increase sample throughput and reduce variability (Lenz et al. 2002; Liebeke and Bundy 2012), although this equipment is not available in all laboratories. Liebeke and Bundy (2012) tested different tissue disruption techniques for earthworm samples (mortar and pestle, cryogenic impact mill, cryogenic milling plus bead beater). They found that the mortar and pestle had the

highest variability, while the cryogenic milling and bead beating methods clustered similarly using both Principal Components Analysis and hierarchical clustering. However, there were reductions in a few metabolites after cryogenic milling plus bead beating compared to just cryogenic milling, suggesting a degradative effect from bead beating (Liebeke and Bundy 2012).

Sample extraction is perhaps the most influential step in sample preparation. There are multiple solvents (e.g. water, methanol, ethanol, isopropanol, acetonitrile, chloroform) used in different combinations that all yield a suitable extraction, and the chosen solvent system can greatly influence the metabolome coverage, abundance of extracted metabolites, and variability in measured abundances (Alvarez et al. 2010; Duportet et al. 2012; Liebeke and Bundy 2012). Other aspects of the extraction procedure can also influence the analysis. When water is added to frozen tissue, dramatic changes in certain metabolites can be observed after as little as 30 to 60 seconds due to the re-solubilization of degradative enzymes that are present in the tissues (Liebeke and Bundy 2012). Heating the sample during extraction can denature degradative enzymes and prevent changes in the metabolite profile during sample preparation (Liebeke and Bundy 2012; Schock et al. 2016). Mixing of the sample during extraction is most commonly done by vortexing (McKelvie et al. 2009; Rochfort et al. 2009; Alvarez et al. 2010; Liebeke and Bundy 2012; Gillis et al. 2017), although ultrasonic extraction has also been used (McKelvie et al. 2009; Gillis et al. 2017). Some authors mix the sample by adding solvent during the homogenization procedure (Lenz et al. 2005; Guo et al. 2009). Following extraction, samples are typically centrifuged to remove suspended materials, but some studies have included a filtration step to minimize interferences by producing a cleaner sample that is less susceptible to degradation (Guo et al. 2009; Liebeke and Bundy 2012).

For GC-MS metabolomics, it is necessary to derivatize polar metabolites into a chemically stabile form that is volatile at the instrument operating temperatures. The derivatization is typically a two-step process. First, aldehyde and ketone groups are methoximated (R=O to R=N-O-CH3), primarily to ensure carbohydrates are in the linear rather than cyclic form, which reduces the number of chromatographic peaks generated for a single metabolite (Dettmer et al. 2007). The methoximation reaction is normally accomplished by adding methoxamine HCl dissolved in pyridine and incubating at elevated temperature for a period of time. Various temperature and incubation time combinations have been reported, such as 37°C for 90 min (Liebeke and Bundy 2012) and 70°C for 30 min (McKelvie et al. 2009). Second, functional groups containing acidic hydrogens are derivatized to trimethylsilyl or tert-butyl dimethylsilyl forms, reducing the reactivity and polarity of these groups to increase stability and volatility for GC-MS analysis (Dettmer et al. 2007).

Instrument conditions can strongly influence the variance within a batch and between batches analyzed on different days. Analyzing dirty samples (e.g. unfiltered derivatized earthworm extracts) leads to deposits of non-volatile and reactive substances in the front of the column that have active sites and can bind analytes, reducing the amount striking the detector. This is corrected by trimming the column at the receiving end by 1 meter between analysis runs, but it reduces the retention time of each run slightly as shown by the shift in retention time between each run in Fig. 1.2. Fouling of the ion source also occurs when dirty samples are analyzed, leading to reduced sensitivity of the instrument over time as shown in the reduction in average abundance between runs 1-2 and 4-5 in Fig. 1.2. The ion source can be cleaned periodically to restore the performance, as indicated in the increase in average abundance

between runs 3-4 in Fig. 1.2. This introduces a bias into the analysis that needs to be accounted for, to prevent artifacts of the sample analysis overshadowing any treatment effects.

1.7.3. Sources of biological variability

The analysis of metabolite profiles in biological samples provides a snapshot of the physiological state at the time of sampling. This state may ultimately be influenced by experimental treatments applied to the organism, but is also dependent on characteristics of the individual organism including genetics, food consumption, disease, age, and weight. Brown et al. (2008) note that it can take up to one month for earthworms to adjust to laboratory conditions and have a stable metabolite profile when a new population is established, since changes in diet,

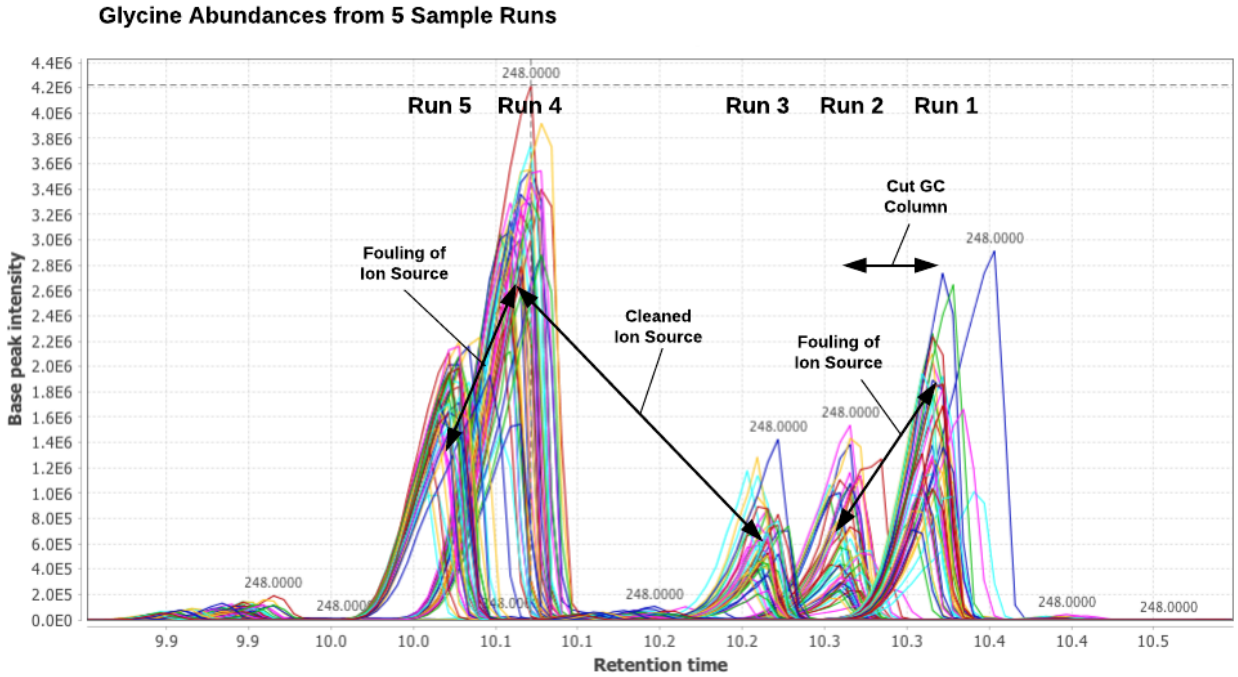

Figure 1.2. Effect of instrument deterioration (ion source fouling) and corrective actions (trimming column, cleaning ion source) on ion abundance and retention time between runs.

growth environment, and stress from shipping lead to unhealthy worms. To ensure a consistent organism response to the test substance in toxicity testing with *E. fetida* earthworms, the standard method recommends using visibly healthy adults at least 2 months old, weighing 0.3 to 0.6 g (0.45±0.15 g), and with a visible clitellum (OECD 1984). This minimizes potential differences due to age and developmental stage of the organism in the observed toxic response. This range is used by many authors in metabolomics studies (McKelvie et al. 2011; Whitfield Åslund et al. 2012; Lankadurai et al. 2013a; Gillis et al. 2017), and has been sufficient to minimize biological variability and detect toxic effects in metabolomics experiments.

Animals such as earthworms are unable to synthesize certain amino acids (including valine, leucine, and phenylalanine), and must obtain these essential amino acids from their diet (Pokarzhevskij et al. 1989; Pokarzhevskii et al. 1997; Costa et al. 2015). Amino acids are generally limited in soil and plant litter, but much more abundant in microorganisms (Pokarzhevskij et al. 1989). Earthworms obtain the majority of their essential amino acids through microorganisms in their diet and from the gut microbiome (Pokarzhevskii et al. 1997; Larsen et al. 2016). A similar influence of the gut microbiota exists for the earthworm fatty acid (FA) profile, where FAs measured from gut tissue reflect a bacterial and fungal origin, and differ greatly from bulk soil (Sampedro et al. 2006). The standard method for reproduction toxicity testing recommends feeding of oatmeal, cow manure, or horse manure as suitable foods (OECD 2004), while some authors use commercially available worm food (Brown et al. 2008; McKelvie et al. 2009; Yuk et al. 2010). Lowe and Butt (2007) recommend manure that is urine-free, from animals that have not been recently medicated, and that it is dried, ground, and re-wetted before feeding for maximum palatability. It is not known how diet affects the earthworm metabolome or the response to toxic substances. Some authors recommend a depuration period where

earthworms are allowed to evacuate gut contents for 24 to 96 hours prior to testing since metabolite variability between samples is lower (Warne et al. 2000; Lenz et al. 2005; Brown et al. 2008), but Warne et al. (2001) found that starvation effects on the metabolite profile can occur during periods of food deprivation. Brown et al. (2008) note that the abundance and variability in the sugar region of NMR spectra decreases after 96 hours of depuration, but this may not be desirable if information on sugars is required.

1.7.4. Structure of GC-MS datasets

Datasets generated from GC-MS analysis have three dimensions: retention time (t_R), fragment ion mass to charge ratio (m/z), and abundance (Fig. 1.3). Analytes elute from the column at different rates, providing separation between analytes and tentative identification based on the length of time required to pass through the column. The analyte retention time depends on the chemical properties of the compound, including polarity and molecular weight, which influence the interaction with the chromatography column. The t_R is measured at point of highest abundance for each peak, but in reality the peak extends over a period of time, as can be seen in Fig. 1.2. Analytes can generally be separated based on t_R, although compounds with similar properties can elute at the same time. Retention times for a particular analyte will vary slightly between runs (Fig. 1.2) due to small variations in the instrument operating characteristics such as mobile phase flow rates and temperature changes. The retention time is also affected by analyte concentration, as well as mobile and stationary phase composition. The mass spectrum, composed of the sampled m/z range and the abundance measured for each interval of m/z, is sampled several times per second over the GC-MS run to capture chromatographic peaks and

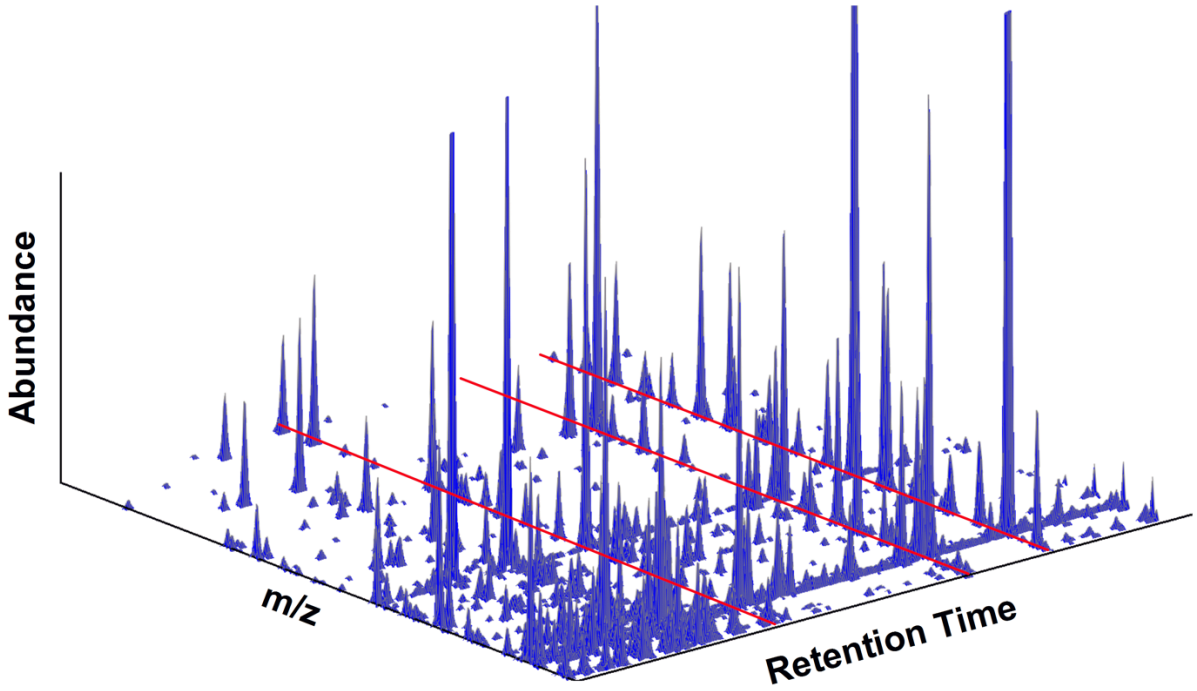

Figure 1.3. Example GC-MS analysis showing the three-dimensional data structure. Peaks falling along the red lines reflect the mass spectra for analytes eluting at the retention times shown. Abundances below a certain threshold are excluded from this figure to highlight the larger peaks.

produce the third data dimension. The m/z values correspond to the masses of ion fragments divided by the formal charge on the fragment. Mass spectra of three example analytes eluting at different retention times are indicated with red lines in Fig. 1.3. With a typical scan range of 50-600 amu, scan rate of 2/s, and run time of 40 minutes (2400 seconds), over 2,000,000 data points are acquired during each GC-MS run from a metabolomics experiment. Millions of data points in each sample must be reduced to a few dozen numbers that represent the quantity of each specific metabolite measured, which are further summarized through dimension reduction and statistical analysis to yield inferences about the system under study.

Different MS designs can measure m/z to a greater or lesser accuracy, depending on the sampling speed and resolving power of the MS. More economical designs (e.g. single

quadrupole MS used in this thesis) can only distinguish between nominal masses (rounded to the nearest atomic mass unit or amu), with more advanced designs measuring masses to an accuracy of 0.001 (e.g. time of flight MS) to 0.00001 amu (e.g. Orbitrap MS). Additionally, some MS designs (e.g. ion trap MS) have a second step, in which a specific ion m/z is isolated and fragmented again to generate a mass spectrum for the isolated fragment. This is useful for the discrimination between co-eluting analytes that produce mass fragments with the same m/z but different chemical structure, since the fragmentation pattern is structure-dependent. In a typical GCMS metabolomics dataset, there may be hundreds of chromatographic peaks, some with some partially or completely overlapping retention times, so a combination of features is required to unambiguously identify metabolites in an unknown sample.

Analytes are identified based on the unique combination of t_R and the m/z for characteristic ions produced during fragmentation of a specific compound, which are determined from the analysis of pure standards. The retention time and fragmentation pattern (fragment ions and their relative abundances) produced for a specific analyte is generally consistent between analysis runs performed under the same instrument conditions (Fig. 1.4). The most abundant m/z is typically selected as the quantifying ion, although some high abundance m/z such as 73 (trimethylsilyl group present in all derivatized analytes) may not be useful since they can be difficult to resolve from neighbouring chromatographic peaks. In addition, two or more m/z with high abundance and good resolution are selected as qualifying ions that must be present with the quantifying ion at the same retention time to confirm the identification. Once established for each analyte, the identifying information can be used to extract data on analyte presence and abundance in unknown samples.

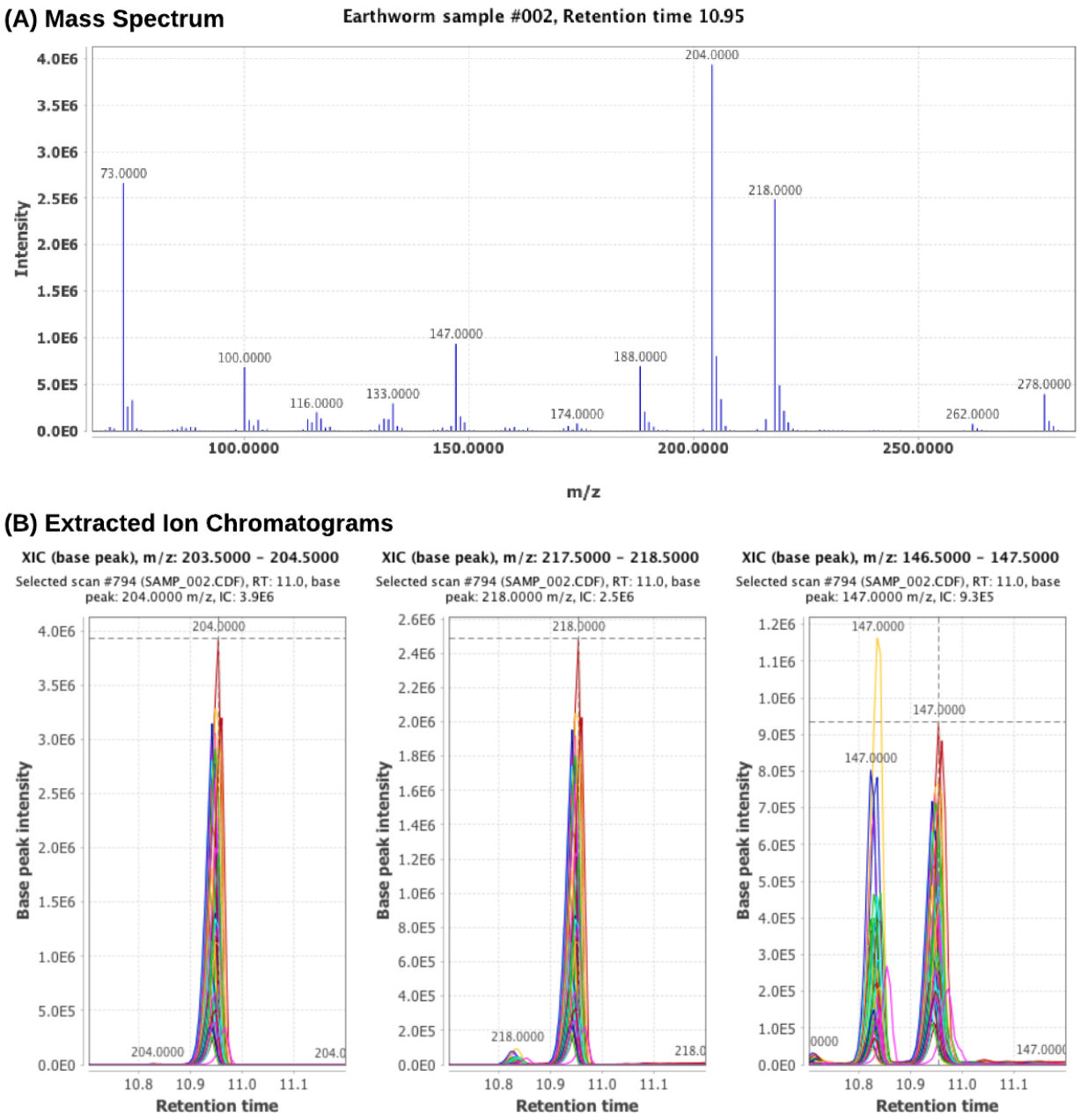

Figure 1.4. Mass spectrum (A) and extracted ion chromatograms for three characteristic ions (B) of the amino acid serine extracted from a batch of earthworm samples, which all elute around the same retention time of 10.95 minutes. There is slight variability in the retention time from run-to run, but the peaks can be clearly resolved from any neighbouring analytes. The ion intensities for m/z 204, 218, 147 in (A) correspond to the peak heights shown in (B).

Abundance or intensity reflects the amount of each fragment ion striking the detector within each sampling interval across the chromatographic peak. Abundance of a chromatographic peak can be measured as peak height (highest value measured across the peak), or peak area (the sum of all values measured across a peak). The abundance produced per mass of analyte added is termed the response factor, and indicates the sensitivity of the instrument for detecting a specific compound. The sensitivity is compound-specific due to the varying ability of the ion source to fragment the compound and produce ions that can be accelerated to strike the detector.

1.7.5. Data extraction

The general process for extracting data for a suite of metabolites from a batch of GC-MS runs includes peak detection, integration, peak alignment, and export of the data for further normalization and statistical analysis. Several free software packages are available to extract data from GC-MS chromatograms, including XCMS (Gowda et al. 2014), MAVEN (Clasquin et al. 2002), Metabolite Detector (Hiller et al. 2009), and MZmine 2 (Pluskal et al. 2010). In this thesis, MZMine 2 was used for all data extraction, integration, alignment, and export. MZMine 2 parses the nominal m/z × retention time × abundance matrix (Fig. 1.3.) and generates extracted ion chromatograms (retention time × abundance for a single m/z, Fig. 1.4B.). Peaks in each m/z are identified based on local maxima in abundance, or a recursive threshold method in which abundance exceeds a defined threshold value for a specified number of consecutive time points (Katajamaa and Orešič 2005; Pluskal et al. 2010). For targeted metabolomics where the retention time and characteristic ions are known for an array of metabolites, these identifying parameters are passed to the program in a .csv file for targeted peak detection, which searches the specified

m/z values for an identifiable peak at the specified retention time range. Sample data files are extracted in batches, generating a peak list for each sample containing the abundance and retention time of the detected peaks, which are then aligned across all samples based on the m/z and retention time combination (Katajamaa and Orešič 2005). This aligned peak list comprises the raw data, which can be normalized using algorithms included in MZMine 2 (Katajamaa and Orešič 2005; Pluskal et al. 2010), or exported for further processing. In some cases, the peak finding algorithm will not detect a peak and there will be gaps in the dataset, which complicates statistical analyses that will follow. MZMine 2 includes a gap-filling algorithm that searches through the original data files using less restrictive parameters attempting to find a peak for the missing values (Katajamaa et al. 2006).

1.7.6. Data normalization

Along with the desired variance caused by treatment or group differences in a metabolomics experiments, there are additional sources of biological and technical variance as discussed in previous sections. Biological variance can arise from natural fluctuations in metabolite concentrations based on organism age, development stage, health, weight, nutrition, genotype, or other causes, many of which are unknown or not measurable (Livera et al. 2015). The analysis of samples depends heavily on the functioning of the analytical instrument, which is subject to technical variation over long runs and between groups of samples analyzed on different days (Livera et al. 2015). Many of these sources of variability cannot be controlled or measured. In these cases, randomization is the only option to prevent bias due to a disproportionate influence of the variance on one treatment versus another. Randomly assigning treatments to the experimental units, randomizing the order of sample processing, and

randomizing the run order of samples will distribute biological and technical variance across all samples. Unwanted variability is unavoidable, and is confounded with treatment effects during statistical analysis which can reduce the ability to detect significant effects or lead to spurious results (Livera et al. 2015). Methods for data normalization (the overall removal of unwanted variation in a dataset) are included with most metabolomics data processing software (Clasquin et al. 2002; Pluskal et al. 2010; Gowda et al. 2014; Xia et al. 2015).

If the source of unwanted variance can be measured or controlled, it is desirable to remove this unwanted variation either pre- or post-analysis, and a variety of methods have been developed to deal with this problem (Livera et al. 2015; Wu and Li 2016; Chen et al. 2017). However, the strategy for normalization is not standardized, and the chosen method can influence which metabolites differ between experimental treatments (Chen et al. 2017). Biological variance is best controlled prior to the experiment where possible by careful selection of generally uniform organisms of a consistent weight, development stage, diet, or other parameters (Brown et al. 2008; Wu and Li 2016). In some cases, biological variability can be normalized post-analysis by scaling the data to a reference variable. In urine samples, measured metabolite levels are often scaled to the level of creatinine or osmolality to account for dilute or concentrated urine that fluctuates based on water consumption and excretion (Warrack et al. 2009). Variance due to weight differences within individual samples can also be reduced post-analysis by scaling the instrument response to the weight of tissue extracted (Wu and Li 2016).

Technical variance within an experiment results from aspects of the sample preparation and analysis, such as gradual changes in instrument response over time due to instrument contamination or deterioration of parts, abrupt changes in instrument response between analysis days due to instrument maintenance, or differences in the performance of personnel conducting

sample preparation, both between people and between days (van der Kloet et al. 2009; De Livera et al. 2012). These sources of variability can often be measured and quantified, allowing the structure of the error to be determined and a strategy implemented to remove it. Batch effects include intra-batch (run order) and inter-batch (day to day) variance, and methods for removal include scaling based on the median (Wang et al. 2003), mean, or dispersion (van den Berg et al. 2006), spiked internal standards (Bijlsma et al. 2006), and linear regression modelling (Wang et al. 2013). More than one normalization approach is typically applied (van der Kloet et al. 2009).

The most common method of normalization in metabolomics is through the use of one or more isotopically labelled internal standards spiked into each sample to scale metabolite abundances, assuming that the unwanted variance in the dataset matches the variance explained by differences in internal standard abundances between samples (De Livera et al. 2012). This may be the case if, for example, the completeness of derivatization varies between samples but is reflected in the abundance of the internal standard. However, internal standards do not account for variance introduced prior to the internal standard, such as during the extraction phase of sample preparation if the internal standard is added after extraction prior to derivatization. Using a single internal standard is the simplest approach, but it is unlikely that all metabolites behave similarly to the internal standard. Adding multiple internal standards representative of different classes of metabolites and normalizing within each class has also been employed (Bijlsma et al. 2006; Sysi-Aho et al. 2007). Statistical methods have been developed in R that can further improve data normalization by modelling the unwanted variance explained by multiple internal standards (De Livera et al. 2012).

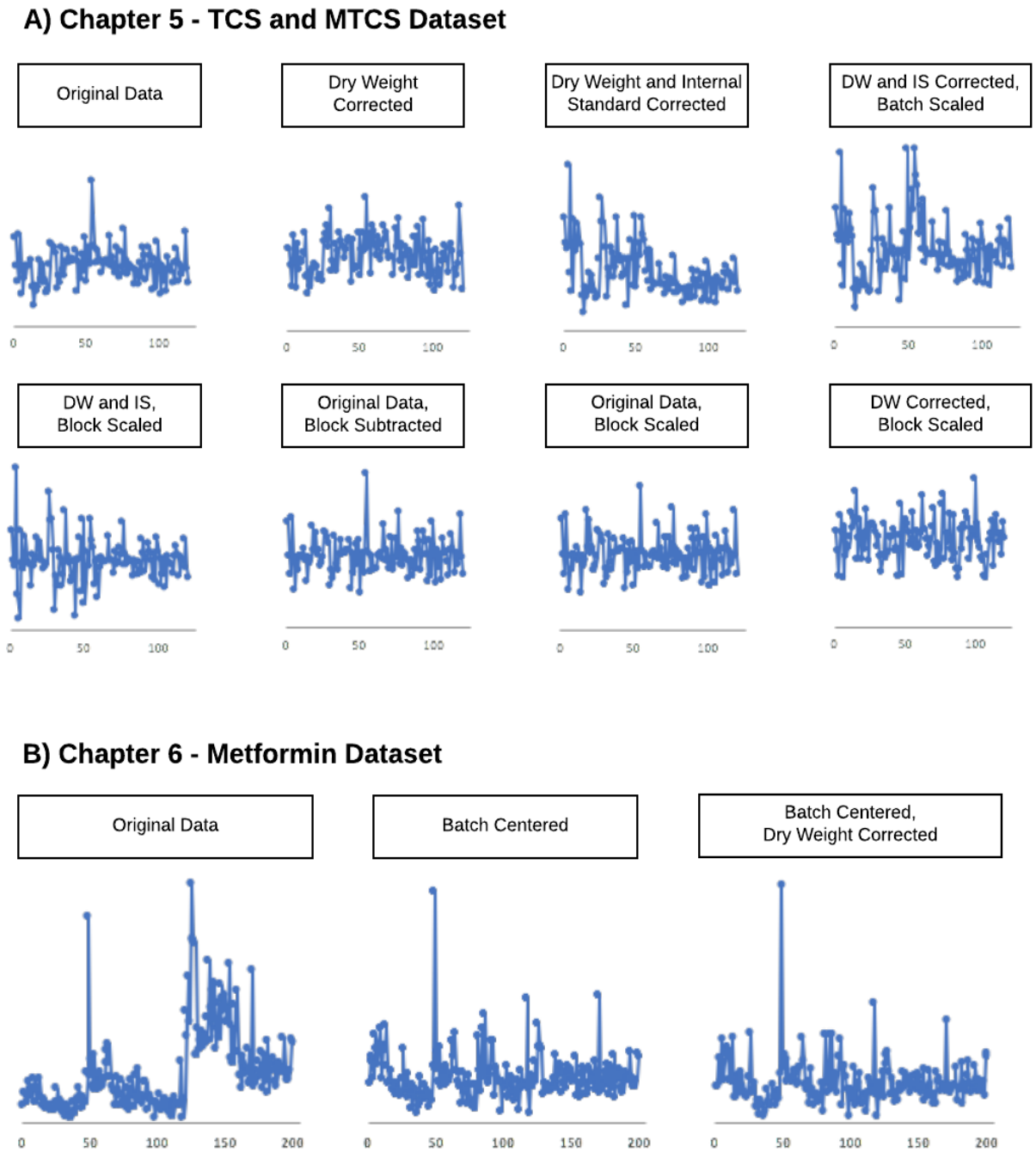

Figure 1.5. Effect of data correction on the abundances of succinic acid in earthworms from two experiments exposing earthworms to triclosan or methyltriclosan (A) or to metformin (B). Samples for the TCS and MTCS study (n=120) were analyzed in 10 randomized blocks of 12 samples corresponding to one replicate of the experimental design, analyzed over 3 days (4 blocks on day 1, 3 blocks on days 2 and 3). Samples for the metformin study (n=200) were analyzed in randomized groups of 40 samples (not corresponding to experimental design) on 5 separate days. Samples within each group were scaled by a correction factor calculated for each group based on the ratio of the group mean to the mean of the largest group.

An alternative normalization technique involves analysis of quality control (QC) samples at the beginning and end of a batch, as well as interspersed every 10 samples or less throughout the run (Sangster et al. 2006; Kamleh et al. 2012). The QC sample is ideally composed of a suitable standard reference material extracted along with the experimental samples, although standardized materials are not always available. An alternative method involves generating a pooled sample by combining a fixed aliquot of extract from each sample extracted in the batch, and preparing aliquots of the pooled sample as QC samples (Gika et al. 2008; Lai et al. 2009). Analysis of the QC samples over the course of the run allows tracking of intra-batch effects and correction of samples based on a curve fit to the QC data (Dunn et al. 2011). Assuming that measurement errors within an experiment are distributed randomly throughout a batch and by randomizing samples between batches, the pooled QC sample can be used to scale batches to a reference batch (e.g. the highest abundance) or to the mean or median of all batches to remove inter-batch variance (Bijlsma et al. 2006; van der Kloet et al. 2009; Draisma et al. 2010).

1.8. Statistical analysis of metabolomics datasets

The datasets generated in metabolomics experiments are inherently multivariate with an array of tens to hundreds of metabolites. Univariate statistical analyses (t-test, ANOVA, Regression Analysis) are suitable to examine one metabolite at a time for a limited number of metabolites, but the amount of effort for analysis and interpretation grows with the number of variables. As the number of independent tests for each metabolite grows, the probability of a false positive increases, requiring a correction to the α-level used to determine significance. In addition, there is often a high degree of correlation between certain pairs of metabolites and

univariate tests to not make use of this information describing the relationship between metabolites.

Multivariate techniques that consider all metabolites at once and account for the covariance between metabolites have been used extensively to analyze metabolomics data, including multivariate ANOVA (MANOVA), Principal Components Analysis (PCA) and Discriminant Analysis (DA). There is no single standard approach used throughout the field, and it is recommended to use multiple approaches since each analyses will have different strengths and weaknesses, and will reveal different information about the data (Goodacre et al. 2007). Multivariate ANOVA provides a significance test for all variables at once based on the sum of squares and cross products matrix, and eliminates the need to correct for false positives due to multiple testing. A problem with multivariate data sets is multicollinearity, the tendency for some of the variables to be highly correlated and not independent. Both PCA and PLS-DA are dimension-reduction tools that eliminate multicollinearity by generating new, independent variables called canonicals that are linear combinations of the original variables (Goodacre et al. 2007). Principal Components Analysis produces canonical variables (Principal Components, PCs) that describe as much variance in the original variables as possible. It is known as an unsupervised technique since it does not take into account pre-existing knowledge of the experimental treatments. The post-hoc assignment of samples to groups can reveal treatment effects, but if other sources of variance (e.g. technical) are dominant, treatment effects can be difficult to detect. Discriminant Analysis is a supervised technique that allows the assignment of samples to groups, and the canonical variables that are generated (Discriminant Functions) maximize the separation between groups rather than the total amount of variance described

(Goodacre et al. 2007). These univariate and multivariate statistical methods are outlined in the following sections.

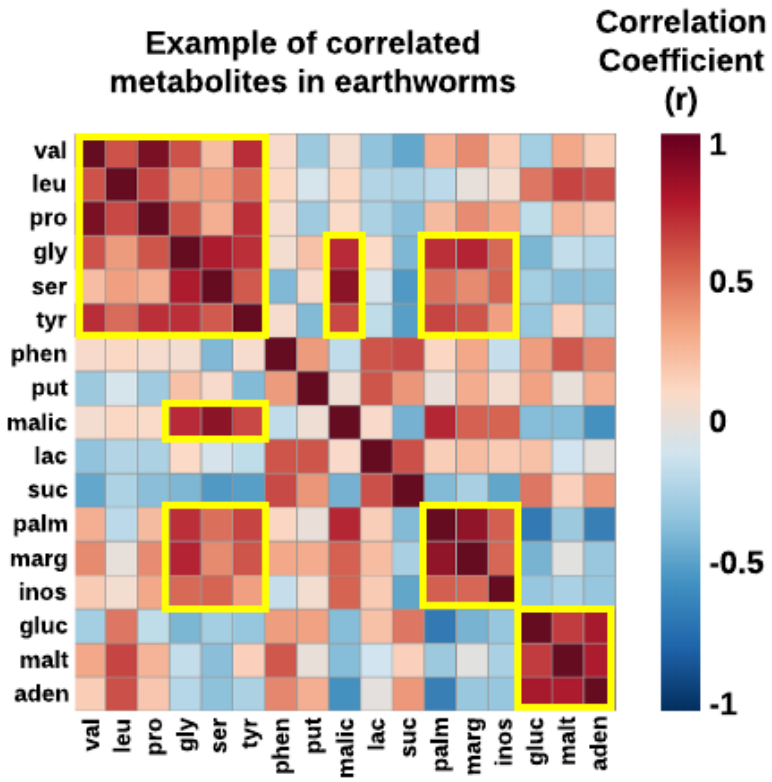

Figure 1.6. Correlations between metabolite pairs in untreated earthworm samples, highlighting groups of metabolites that show a high degree of correlation.

1.8.1. Univariate ANOVA

Univariate methods consider one metabolite at a time, and test for differences between the means of two or more groups of samples that reflect the factors and levels in the experimental design. The Analysis of Variance (ANOVA) is a statistical technique that partitions the total variability in a dataset into its component parts: the variability between treatments, and the variability within treatments. The between-treatments variability represents the amount

explained by the treatments, while the within-treatments variability is considered the error or unexplained variability. In ANOVA, the data set is modelled to estimate the effect size (treatment mean minus overall mean) for each group by minimizing the variance in the dataset not explained by the statistical model, which is structured differently based on the experimental design (Montgomery 2005). The analysis then conducts a series of F-tests based on the statistical model to determine if the effect of at least one group is different from zero, based on the ratio of treatment mean squares (variance explained by treatments divided by treatment degrees of freedom) to error mean squares (residual variance divided by error degrees of freedom). This calculated F-value is compared to a critical F-value to determine significant effects based on the desired level of significance, the treatment degrees of freedom, and the error degrees of freedom. The use of ANOVA assumes that the error terms are normally and independently distributed with a constant variance for each level of each factor. Each test is based on a pre-determined level of significance (termed the α level, generally 0.05 or below) representing the probability of a false positive (type I error) occurring (Montgomery 2005).

In a metabolomics dataset, where a large number of univariate tests may be run at α=0.05, the probability of encountering a false positive increases with each subsequent test. This increased probability of a false positive is frequently controlled in metabolomics studies by either the Bonferroni (Shaffer 1995) or Benjamini-Hochberg (Benjamini and Hochberg 1995) corrections. The Bonferroni method controls the family-wise error rate, and corrects the α level by dividing by the number of independent tests conducted (α/n, n = number of tests), requiring a larger effect size before declaring an effect significant (Shaffer 1995). It is a conservative method, eliminating all but the most significant responses as the number of tests grows from tens to hundreds or thousands. In the process, a number of potentially interesting true positive results

can be excluded if their p-values are not highly significant. The Bonferroni method gives a high degree of protection against false positives at the expense of statistical power. The Benjamini-Hochberg method is a less conservative method that controls the false discovery rate (FDR) at a pre-defined level, which is the expected proportion of false positives in a series of independent tests (Benjamini and Hochberg 1995). In the Benjamini-Hochberg (BH) procedure, the p-values from the group of tests are ranked from high to low, and the BH α-level for each test is calculated as FDR$\times i/n$, where FDR is the pre-defined false discovery rate, i = the rank for each test based on p-value, and n = number of tests. The ranked p-values are compared to the BH α-level, and those less than or equal are declared significant. The Benjamini-Hochberg method penalizes based on a greater number of tests, but the tests with the lowest p-values are penalized less than the highest as i/n approaches 1. The Benjamini-Hochberg method is preferred in other omics fields since it has greater statistical power while still controlling false positives (Glickman et al. 2014). However, the pre-defined FDR is not standardized and ranges in the literature from a conservative value of 0.05 (equal to α) to a less restrictive 0.2 (Glickman et al. 2014).

1.8.2. Multivariate ANOVA (MANOVA)

The extension of ANOVA (partitioning total variability in a dataset into component parts) to a dataset with multiple response variables is called multivariate ANOVA or MANOVA (Rencher 2003). Where ANOVA tests for differences between the means of each group for a single response variable, MANOVA tests for differences between the vector of means for each group with multiple response variables. In MANOVA, variance is partitioned into a matrix of between-group sums of squares and products (SSP) and within-group SSP. The between group SSP reflects the variance associated with treatment effects and can be expressed as a matrix of

treatment means for all variables minus the overall mean, multiplied by its transpose. The within group SSP reflects error variance and can be expressed as a matrix of differences between treatment means and the observations for all variables, multiplied by its transpose. The test for significance is called Wilks' Lambda, which is calculated as the ratio of determinants of the between SSP to the total (between plus within) SSP and compared to critical values to determine significance (Rencher 2003). A significant result can be followed up by a series of univariate F-tests to investigate treatment effects within the responses. Other tests of significance have been developed based on eigenvalues and eigenvectors of the data matrix, including Roy's Largest Root Test, Pillai's, and Hotelling's T^2 (Rencher 2003). The three methods calculate the test statistic differently and vary in their power in rejecting the null hypothesis.

1.8.3. Principal Components Analysis

In a metabolomics dataset, there can be tens to hundreds of metabolites measured in each sample, and summarizing the information to be visualized and interpreted becomes more challenging with a larger number of metabolites measured in a study. Principal components analysis (PCA) is a dimension reduction technique that transforms the dataset to derive a smaller subset of uncorrelated variables (components) that maximize the variance explained (Jolliffe 2014). For each principal component calculated, a set of coefficients are generated (one for each response variable) which are multiplied by the corresponding value in each row of data (i.e. each sample) and summed to yield a one-dimensional component reflecting the entire set of response variables in a single column. This reduces the complexity of visualizing the data, providing a reasonable representation of high-dimensional data in fewer dimensions (Jolliffe 2014). Principal components analysis is an unsupervised technique, in that sample labels (experiment treatments

or groups) are not considered in the optimization of coefficient values. This contrasts with Discriminant Analysis (discussed in the next section), another dimension reduction technique where samples are identified with a categorical variable, and the multivariate distance between groups is maximized rather than the total amount of variance explained (Klecka 1980).

In PCA, there are no tests for significance between groups, but t-tests, ANOVA, or MANOVA can subsequently be performed on the principal components to test for significant effects (Lankadurai et al. 2011a; McKelvie et al. 2011). This difference reflects an overall effect of a treatment on all metabolites as reflected in the coefficients for the linear combination of variables that makes up each principal component. By examining the standardized coefficients for each principal component, the metabolites that contribute most to the explained variance (i.e. largest coefficients) can be identified (Bylesjö 2015). In metabolomics, the largest coefficients are interpreted as important metabolites that contribute most to the variance in the dataset and to the observed separation between variables in the projected space (Worley and Powers 2013).

1.8.4. Discriminant analysis

Discriminant analysis (DA) is another dimension reduction technique that transforms a multivariate dataset into a smaller subset of uncorrelated canonical variables called discriminant functions that are a linear combination of the original variables, similar to PCA, although the objectives and parameter optimization criteria of PCA and DA differ. Where the canonical coefficients in PC are optimized to maximize the variance explained by each canonical variable, DA optimizes the coefficients to maximize separation between groups in the multivariate space (Klecka 1980). In this way, discriminant analysis is a supervised technique where the sample classification is taken into account during the optimization procedure. Discriminant analysis

projects the data onto a new multivariate space, rotating the axis for each discriminating variable (i.e metabolite) to provide the largest separation between groups along that axis. The discriminant function coefficients, which are multiplied by metabolite abundances and summed, combine the characteristics of the data in such a way that the group means calculated from the discriminant function are as different as possible (Klecka 1980).

Discriminant analysis uses the matrix of total sum of squares and products to represent the data, taking into account the inter-relationships among variables and the amount of dispersion in the dataset (Klecka 1980). The total SSP matrix represents distance from the grand centroid, which has coordinates that are the overall mean for all variables. Similarly, a group centroid can be defined as coordinates that are the group mean over all variables, and a within-group SSP matrix can be calculated for each group. If group locations are distinct from each other, then the dispersion around the group centroid will be less than the dispersion around the grand centroid, and the elements of the within-group SSP matrix will be smaller than the total SSP matrix (Klecka 1980). The difference between these is called the between-group SSP matrix, and the size of the between-group SSP relative to the within-group SSP provides a measure of how well the groups can be separated (Klecka 1980). Discriminant analysis derives coefficients using the between-group and within-group SSP matrices to achieve the maximum possible group separation (Klecka 1980).

1.9. Research objectives and hypotheses

Biosolids from municipal wastewater treatment plants (WWTPs) are frequently applied to soils in North America, and are known to be contaminated with a growing number of emerging substances of concern (ESOCs) (USEPA 2009b; Monteith et al. 2010). There is a

potential risk of exposure and harm to soil organisms that must be assessed using information gained from exposure experiments, and there are additional capabilities of metabolomics to study topics often neglected in traditional toxicity testing. This research project uses metabolomics to investigate the toxicity to earthworms of three prevalent but understudied biosolid and soil contaminants: triclosan (TCS, a ubiquitous contaminant in biosolids), methyltriclosan (MTCS, a persistent triclosan breakdown product with unknown toxicity), and metformin (MET, one of the most prescribed pharmaceuticals worldwide but lacks complete ecotoxicity information). The following questions were considered:

1) Are there toxic effects on earthworms from these three prevalent contaminants at sub-lethal or environmentally relevant concentrations?

2) Is methyltriclosan, a breakdown product, more or less toxic than its parent compound triclosan?

3) How does exposure time impact the earthworm metabolite profile observed during exposure to a contaminant?

4) What is the toxic mode of action of the three contaminants?

The first objective was to determine if there are metabolic or physiological effects induced by the selected compounds at environmentally relevant exposure concentrations. This information can be employed in screening-level environmental risk assessments as a toxicity

threshold for these substances in the soil compartment. While the toxicology of TCS has been well documented, the effects of MTCS and MET have not been studied in earthworms prior to this work. None of these compounds have been investigated in the earthworm using metabolomics. It is hypothesized that there will be metabolic effects in earthworms caused by exposure to these three contaminants, and that the effects will be concentration-dependent.

The second objective was to determine if toxicity increases or decreases when triclosan (TCS) is transformed into methyltriclosan (MTCS). This transformation occurs in wastewater treatment plants and soil, and MTCS concentrations can approach TCS concentrations in soil and earthworms. Triclosan is among the most commonly detected and highest concentration contaminants in biosolids, of which a large proportion will be transformed into MTCS following land application. There is a general lack of knowledge regarding toxicity of transformation products including MTCS to soil organisms, and the change in toxicity from the parent compound to transformation product will have relevance for other contaminants that undergo methylation in soil. It was hypothesized that the transformation product methyltriclosan is more toxic than its parent compound triclosan.

The third objective was to determine how the earthworm metabolite profile varied over time in response to contaminant exposure. This is an underlying factor in any experiment, but there are very few earthworm metabolomics studies that investigate the effect of time. Standard methods for toxicity testing with earthworms recommend a fixed time of either 2-day filter paper or 14-day soil test, but are based on assessment of mortality, weight loss, and reproduction endpoints. The metabolic impacts would be expected to shift over time, and the recommended exposure intervals may not capture the most important effects. It was hypothesized that a concentration dependent pattern in metabolite profiles will emerge as a function of time.

Using metabolite data and knowledge of the biochemical pathways in which they are involved, the fourth objective was to hypothesize an unknown or confirm a suspected mode of action for each contaminant. The mode of action has not been confirmed for TCS, MTCS, or MET in earthworms. The mode of action for MET has been well characterized in humans and rodents, since it is a commonly prescribed pharmaceutical. The mode of action for TCS has been determined in bacteria since it is a common antimicrobial ingredient in consumer products. However, the target metabolic pathway is only present in bacteria and is not relevant in earthworms and other organisms. There is a general lack of information regarding the mode of action for MTCS, since it is only now emerging as an environmental contaminant of concern. The work was not designed to definitely prove a mode of action, but the information may identify likely targets for future investigations. It was hypothesized that significant changes in the metabolome will point towards a mode of action for the three contaminants tested.

1.10. Thesis outline

This thesis has 6 chapters, with this (General introduction and literature review) being the first.

Chapter 2 Lists metadata and summarizes methods for each experiment to comply with the Metabolomics Standards Initiative (MSI) minimum required information about an experiment. This includes information on the biological system, chemical analysis, and data processing, which varied in some respects between experiments.

Chapter 3 Assessment of acute triclosan toxicity to earthworms using 48 hr filter paper contact test. This chapter has been published in the Journal of Hazardous Materials.

Chapter 4 Comparison of toxicity between parent compound (triclosan) and its primary degradation product (methyltriclosan) using 14-day contact test in earthworm bedding.

Chapter 5 Evaluation of time-dependent metabolic effects of metformin, the most commonly prescribed diabetes drug worldwide.

Chapter 6 Summary and synthesis bringing together the most important contributions from each chapter. It includes a discussion of the results in a broader sense than in each chapter, and provides recommendations for future work.

Preface to Chapter 2

The preceding literature review described some of the available methods for sample preparation, chemical analysis, data normalization, and statistical analysis that are typically employed in metabolomics experiments. Chapter 2 compiles a summary of the methods used in the three experiments in this thesis (Chapters 3, 4, and 5), based on the reporting requirements for biological samples, chemical analysis, and data analysis published by the Metabolomics Standards Initiative (MSI). The MSI reporting standards seek to allow independent verification of results and improve data sharing by ensuring that all necessary metadata for a metabolomics experiment are reported. Chapter 2 also provides some justification for the decisions to use certain methods.

Chapter 2: Metabolomics Standards Initiative minimum reporting requirements for a metabolomics experiment

2.1. Overview

The growth of the metabolomics field over the last two decades has benefitted from the optimization of sample preparation techniques, advancements in analytical instrumentation, and development of new and improved software for data extraction, processing, and statistical analysis. Early on, especially in the medical community, it was recognized that standardization was necessary for conducting and reporting results from a metabolomics experiment to allow exchange of data and independent verification of results (Lindon et al. 2005). This accompanied similar efforts in other fields like genomics, transcriptomics, and proteomics, which have greatly contributed to collaboration, data sharing, and the development of searchable databases of such things as the human genome (Brazma et al. 2001; Taylor et al. 2007; Field et al. 2008). The Metabolomics Standards Initiative (MSI) began in 2005 within the Metabolomics Society to develop policies and guidelines for the scientific community engaging in metabolomics research (Lindon et al. 2005; Fiehn et al. 2007; Sansone et al. 2007). The MSI has published standards relating to chemical analysis (Sumner et al. 2007), data analysis (Goodacre et al. 2007), biological samples (Morrison et al. 2007), and plant metabolomics (Jenkins et al. 2004). In 2014, the journal *Metabolomics* (the official journal of the Metabolomics Society) started requiring compliance with these standards for all published manuscripts (Goodacre 2014).

To maintain a standard of quality in this thesis consistent with the expectations of the field, the metadata required for MSI compliance from each experiment comprising Chapters 3, 4, and 5 has been aggregated and summarized in Figs. 2.1, 2.2, 2.3, and 2.4 of this chapter. It also

allows comparison of the methods across all three experiments, which were improved over the course of this thesis based on advancements in sample preparation that were published and discovered after work had begun (Liebeke and Bundy 2012; Liebeke et al. 2012). The chronological order in which the experiments were conducted differs from how they are presented in this thesis. Chapters 3 and 4 both deal with triclosan and were arranged consecutively in this thesis, but the experiment with metformin (Chapter 5) was conducted between the two triclosan experiments. There are several aspects to the methodology that change chronologically and the information is presented in this chapter to reflect this, but it differs from the order in which the experiments appear in this thesis.

2.2. Information about biological samples

The earthworms in all three studies were taken from the same population maintained in the laboratory of Dr. Gordon Price throughout the project. The population was located at the Bio-Environmental Engineering Center (BEEC) and maintained by Doug Burris and Cory Roberts. The original population was obtained from a local supplier. The earthworm bedding was generated on-site using a balanced recipe of horse bedding and spent hay. The temperature was monitored and the pile was turned regularly with a Supreme Enviro Processor 400 compost grinder when temperature dropped to ambient. Once the temperature stabilized and the compost had matured over winter, it was adjusted to pH 6 by addition of lime and mechanical mixing. Earthworms appeared healthy throughout the project and were actively reproducing based on the presence of cocoons and juveniles.

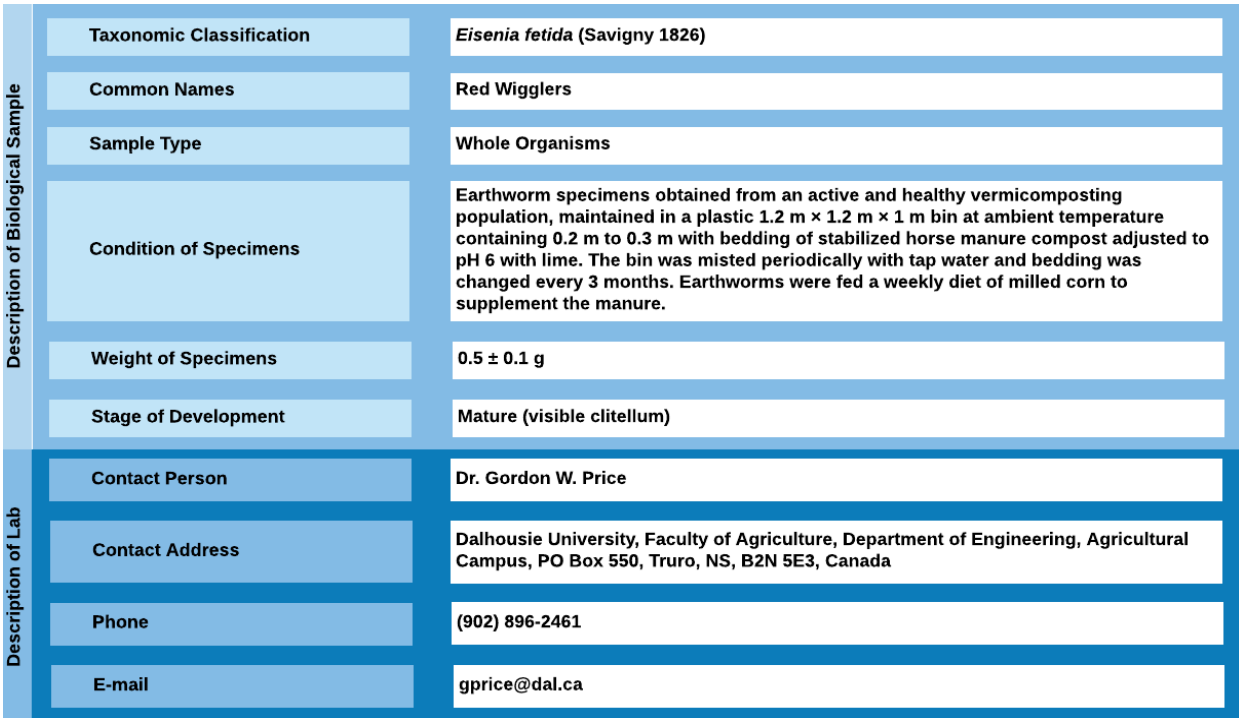

Figure 2.1. Description of biological samples involved in the exposure studies and laboratory contact information where all studies were conducted.

2.3. Information about chemical analysis

In this thesis, two different sample preparation and GC-MS analysis methods were used. Chapter 3 used a method which was adapted from a recently published literature method (McKelvie et al. 2009). It provided reliable results but had a roughly 1 hour run time and captured a limited scope of metabolites, since it was optimized to provide complementary results with NMR and used a water-based extraction that was not exhaustive. After conducting this experiment, two papers dealing with GC-MS metabolomics sample preparation were published by others (Liebeke and Bundy 2012; Liebeke et al. 2012) using a faster (~40 minutes) GC-MS method that has become a standard within the field due to the availability of mass spectral and retention index libraries based on it (Kind et al. 2009). The paper by Liebeke and Bundy (2012) tested several solvent systems and sample preparation methods, and a new method was adopted

based on their recommendations. The solvent system and derivatizing agent were changed for a wider metabolome coverage, and a heating step during extraction was added to denature any degradative enzymes present during extraction. The GC column used between all three experiments had identical characteristics, except Chapters 4 and 5 used an additional 10 m Duraguard that can be cut to extend the life of the column. Due to equipment down-time, there was no freeze-dryer available for the metformin study. Instead, samples were stored in a -80°C freezer after quenching with liquid N_2 to avoid degradation of samples during storage.

The data for Chapter 3 was generated during a single GC-MS run, but the experiments in Chapters 4 and 5 had to be broken up into several groups of samples to perform GC-MS maintenance between runs. The metformin dataset (Chapter 5) included 200 samples analyzed as groups of 40 samples randomized across five different analysis days to distribute technical variation randomly across all samples and use data normalization to remove batch effects between days. During this experiment, within-run signal drift was observed that could not be removed as a batch effect, so a different strategy was used for the next experiment. The triclosan and methyl-triclosan study had 120 samples analyzed as complete replicates of the experiment (10 groups of 12 samples) randomized over three analysis days, with four replicates (48 samples) on day 1 and three replicates (36 samples) on days 2 and 3. This allowed technical variation between replicates during the run to be quantified and removed during normalization as described in the next section.

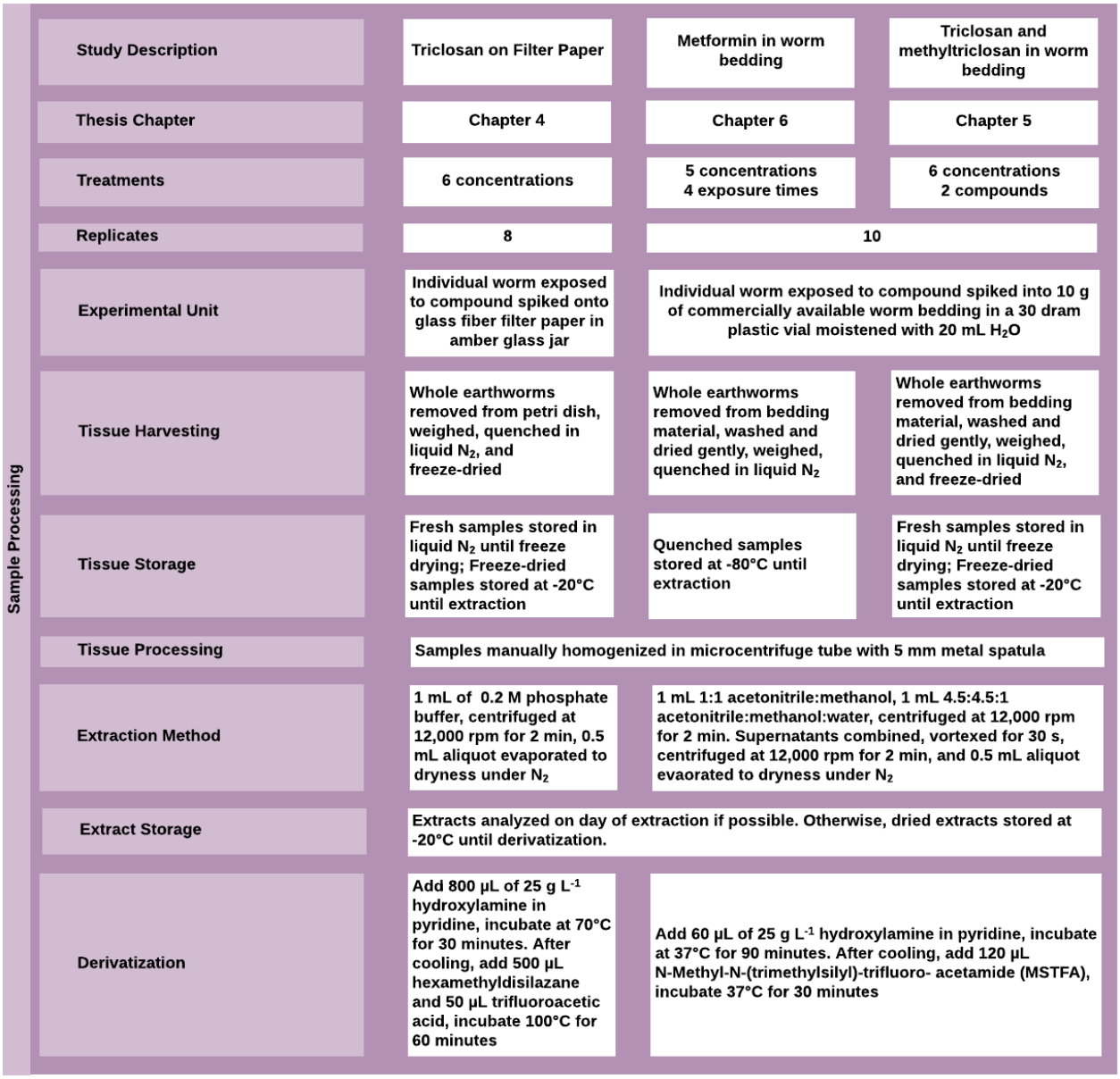

Figure 2.2. Summary of sample processing steps compared between the three earthworm exposure experiments presented in Chapters 3, 4, and 5.

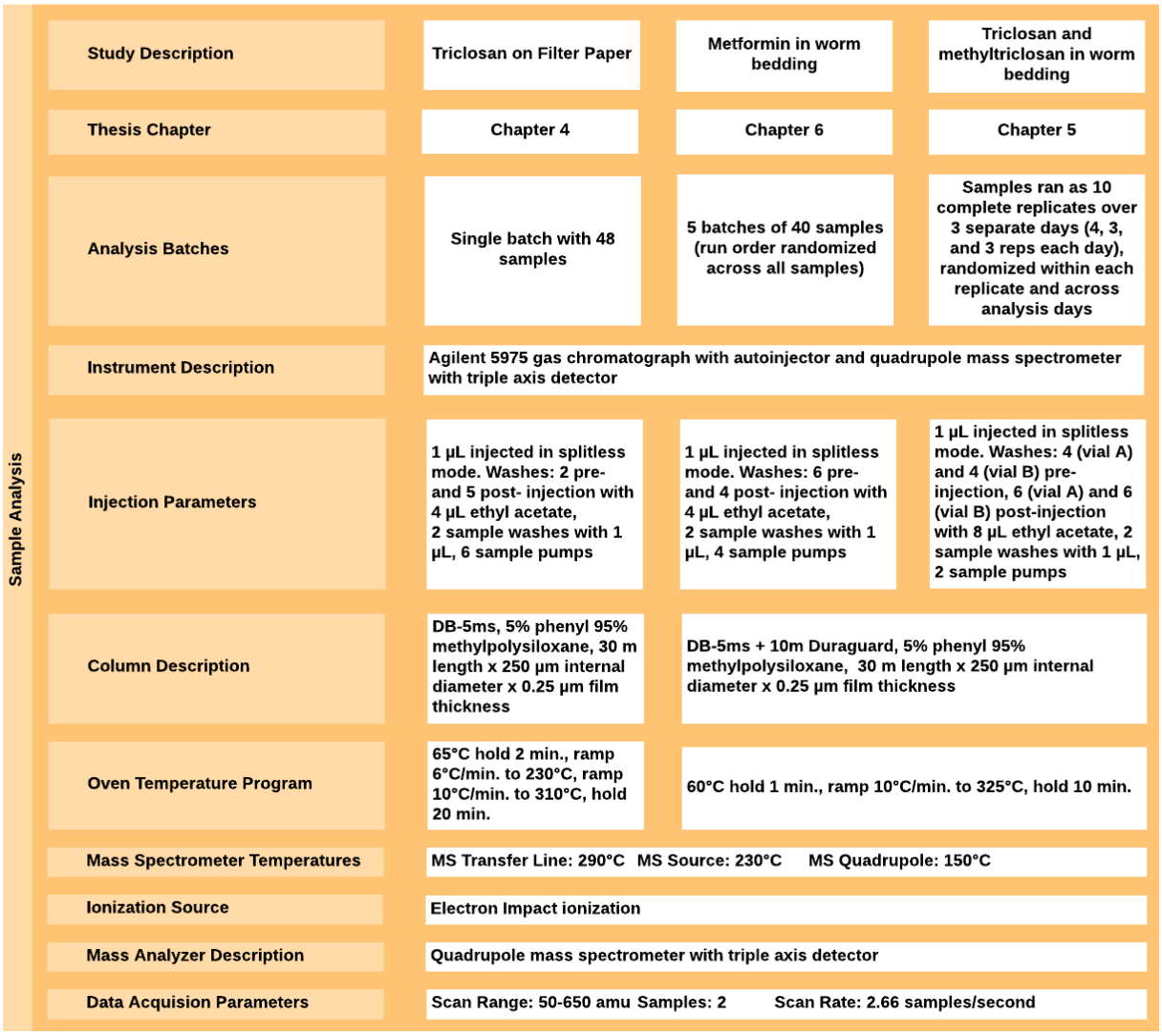

Figure 2.3. Summary of methods for sample analysis compared between the three earthworm exposure experiments presented in Chapters 3, 4, and 5.

2.4. Information about data analysis

The chromatograms were obtained in the Agilent Chemstation format, which was not compatible with the software used for data extraction and had to be converted to Network Common Data Form (*.CDF) using OpenChrom, a free chromatography-mass spectrometry data processing software (Wenig and Odermatt 2010). They were then loaded into MZMine 2 for data extraction, which includes raw data filtering, peak detection, and peak alignment (Pluskal et al.

2010). Baseline correction was used for the Chapter 3 data, but no benefit was observed for the data in Chapters 4 and 5 so it was not used. In all cases, peak detection was targeted based on m/z and retention time combinations previously established by running analytical standards of each metabolite. As shown in Chapter 1, Fig. 1.2, the retention time shifted between runs due to trimming of the GC column, which became contaminated over time. For the metformin study (Chapter 5) analyzed on five separate days, the retention time difference was too large to process all samples together due to overlapping peaks that prevented the correct peak from being identified by the program during alignment. Each analysis day was instead processed separately using narrow retention time tolerances for detection and alignment calibrated to the dataset, then each day was combined. For the triclosan and methyl-triclosan experiment (Chapter 4), the retention time differences were not large enough to prevent correct peak identification and all samples were processed together with wider retention time tolerances calibrated to all three days together.

In Chapter 3, the method used did not specify an internal standard, so the only normalization was scaling each sample to the dry tissue weight of extracted earthworm. Internal standards (leucine-d_3 and U-13C-glucose) were analyzed with each sample for Chapters 4 and 5, but normalization based on the internal standard did not improve all metabolites, and in some cases introduced unwanted errors. For this reason, internal standard normalization was not used for either dataset. The metformin experiment was randomized across all days, and assuming the measurement errors are randomly distributed throughout the batch, the mean of each group should be equal to the overall mean of all samples if there was no batch effect. To correct for this, each batch was scaled based on the ratio of the batch mean to the overall mean, reducing all samples in a high batch and increasing all samples in a low batch. This eliminated a large amount

of the batch variability in the metformin dataset, although there was still within-run signal drift as shown in Chapter 1. In the TCS-MTCS study, assuming measurement errors are randomly distributed, the mean of each replicate should be equal to the overall mean if there are no batch effects, so samples were scaled based on the mean of each replicate to the overall mean. Scaling based on tissue weight improved normalization for the metformin and TCS-MTCS datasets and was used for both datasets. For univariate statistical analysis, metabolite datasets were transformed using power transformations from the 2^{nd} to 5^{th} root and removal of outliers as necessary to achieve normality and constant variance for ANOVA and regression. Multivariate outliers were detected using scatterplot matrices in JMP to identify potentially influential samples consistently outside of the confidence region for multiple metabolite pairs. For all datasets, univariate ANOVA was performed in SAS, principal components analysis, correlation, and regression analysis were performed in Minitab or JMP, and discriminant analysis was performed in JMP. More specific details for each specific experiment are included in the Materials and Methods section of each chapter.

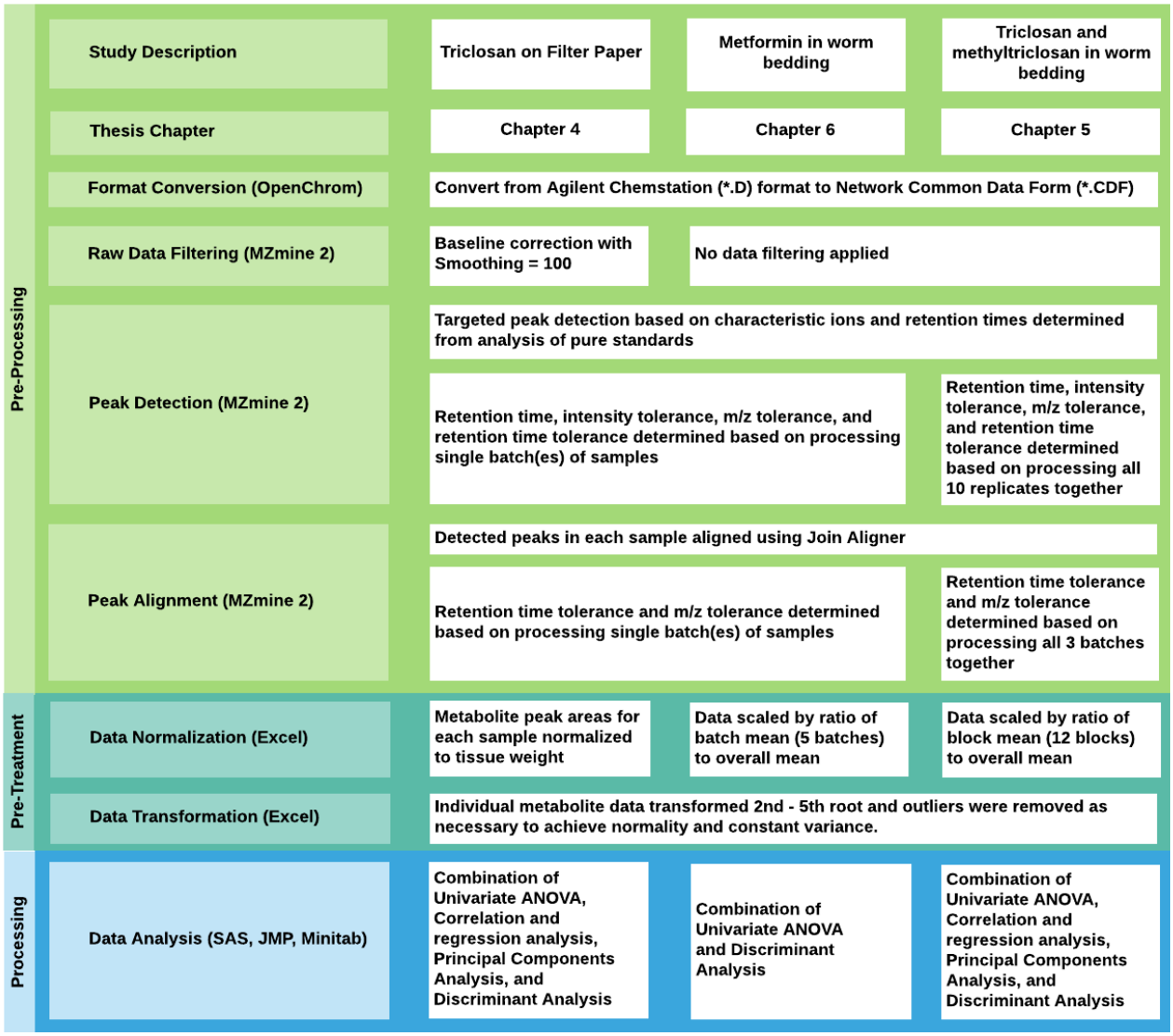

Figure 2.4. Summary of data processing and statistical analysis techniques compared between the three earthworm exposure experiments presented in Chapters 4, 5, and 6.

Preface to Chapter 3

This chapter presents results from a 48 hour filter paper earthworm acute toxicity test with triclosan, an antibacterial ingredient in personal care products and ubiquitous biosolids contaminant. Metabolomics is applied to probe deeper into the toxic response of earthworms to triclosan, and to characterize changes that occur to earthworms after death. The methodology used in this chapter is outlined in Chapter 2. This chapter has been published in the Journal of Hazardous Materials (Gillis et al. 2017, J. Haz. Mat. 323A, 203-211, IF: 6.065). The format and numbering has been modified from the published version to be consistent with this thesis, and the reference list has been combined into a single reference list at the end of this document. I am the corresponding author and first author of the manuscript, and I performed all experimental work, analyzed all data, produced all tables and figures, wrote all text in the manuscript, made all required changes to address reviewer comments, and wrote all responses to reviewer comments. Editorial comments on the original submission and on Revision 1 with regards to improving clarity were provided by G.W. Price and S.O Prasher. The work was designed by the three authors J.D. Gillis, G.W. Price, and S.O. Prasher, and was executed in the laboratory of G.W. Price. The manuscript required two revisions before it was accepted for publication.

Chapter 3: Lethal and sub-lethal effects of triclosan toxicity to the earthworm *Eisenia fetida* assessed through GC-MS metabolomics

This chapter has been published under the same title in the Journal of Hazardous Materials (Gillis et al. 2017, J. Haz. Mat. 323A, 203-211) and was available online July 7, 2016.

J.D. Gillis[1], G.W. Price[2], S.O. Prasher[1]

[1] Department of Bioresource Engineering, McGill University, Faculty of Agricultural and Environmental Sciences, Macdonald-Stewart Building MS1-027, 21111 Lakeshore Road, Sainte-Anne-de-Bellevue, Quebec, Canada, H9X 3V9

[2] Department of Engineering, Dalhousie University, Faculty of Agriculture, 39 Cox Road, PO Box 550, Truro, Nova Scotia, Canada, B2N 5E3

[*] Corresponding author: joseph.gillis@mail.mcgill.ca, 1-902-896-2461

Keywords: Metabolomics, Earthworms, Triclosan, GC-MS, MZMine 2, OpenChrom

Highlights:

1) Lethal and sub-lethal effects of triclosan toxicity were examined on earthworms

2) Biochemical evidence of tissue decomposition was observed in mortalities after 24 hr.

3) Individual metabolites were minimally affected by sub-lethal concentrations.

4) Reversed correlations between val vs. ino, man vs. ino indicated sub-lethal exposure.

3.1. Abstract

Triclosan (TCS) is a ubiquitous contaminant in municipal biosolids, which has also been detected in soils and earthworms sampled from agricultural fields amended with biosolids. The goal of this study was to evaluate the toxicity of TCS to earthworms using a metabolomics-based approach for an improved interpretation of toxicity. Toxicity of TCS was assessed using the OECD Method 207 filter paper contact test measuring the endpoints of weight loss, mortality, and ten metabolites determined by GC-MS. Eight earthworms were exposed as individual replicates to six concentrations of triclosan (0, 0.0001, 0.001, 0.01, 0.1, and 1 mg TCS cm^{-2}) on filter paper, with mortality assessed after 6, 24 and 48 hours. Mortalities were first observed at 24 hours, with 100% mortality in the 1 and 0.1 mg cm^{-2} treatments. Worms at 1 mg cm^{-2} lost most of their coelomic fluid before they could be sampled. The 48 hr LC_{50} for triclosan was estimated to be 0.006 and 0.008 mg cm^{-2} by a linear and logistic model, respectively. Based on the LC_{50}, triclosan is relatively more toxic to earthworms than a number of other emerging contaminants, but is less toxic than other chlorophenols and many pesticides. Alanine, valine, leucine, serine, phenylalanine, putrescine, spermidine, mannitol, and inositol were significantly different between treatments, although changes were most often associated with mortality rather than triclosan exposure. An increase in putrescine and decreases in amino acids, polyols, and spermidine were associated with mortality, suggesting decomposition had begun. Principal components analysis did not reveal evidence of metabolic impacts at sub-lethal concentrations. However, there were changes in the pattern of correlations between pairs of metabolite in surviving worms at both 0.0001 and 0.001 mg cm^{-2} exposure compared to the control.

3.2. Introduction

Triclosan, also called 2,4,4'-trichloro-2'-hydroxydiphenyl ether or the trade names Irgasan or Microban (CAS Reg. No. 3380-34-5), is a widely used antimicrobial compound in many household and industrial products that are disposed of in the municipal wastewater stream (Bedoux et al. 2012). Triclosan exhibits effective control against many bacteria and some fungi, blocking bacterial fatty acid synthesis through enzyme inhibition and leading to cell death (McMurry et al. 1998; Dann and Hontela 2011). Approximately 1600 cosmetic products, 13 natural health products, and 130 drug products in Canada contain triclosan (Environment Canada 2012). The recent preliminary environmental risk assessment on triclosan by Environment Canada and Health Canada determined that triclosan was bioaccumulative but not persistent, and highly toxic to a variety of aquatic organisms (Environment Canada 2012). In Canada, triclosan concentration as an ingredient must be less than 0.03% w/w in mouthwashes, and less than 0.3% w/w in topical and dentifrice agents (Health Canada 2015). Consequently, the primary routes for human exposure are through ingestion and dermal absorption, although it is rapidly excreted in urine (Wang and Tian 2015). Triclosan has been measured in Wastewater Treatment Plant (WWTP) influent and effluent water from a number of countries (Waltman et al. 2006; Ying and Kookana 2007; Ricart et al. 2010). It is among the most frequently detected compounds in biosolids across North America, measured in the range of 1000 to 39,000 ng g^{-1} in Canada and 430 to 133,000 ng g^{-1} in the United States (USEPA 2009a; Monteith et al. 2010). Triclosan has been detected in soils following the land application of biosolids (Kinney et al. 2008; Xia et al. 2010; Gillis et al. 2014), in surface and sub-surface drainage water (Lapen et al. 2008; Topp et al. 2008; Sabourin et al. 2009), and in the tissues of plants and soil biota after greenhouse or

field-based exposures (Kinney et al. 2008; Wu et al. 2010b; Karnjanapiboonwong et al. 2011; Macherius et al. 2014).

Although triclosan is frequently detected in soil, effects-based biological assays with model organisms are necessary to determine toxicity (Spurgeon et al. 2003). Under the Canadian Environmental Protection Act, "the ability of the substance to cause a reduction in metabolic functions of an organism" is included among other factors in assessing the toxicity of substances for regulatory purposes (Government of Canada 1999). Earthworms are frequently chosen due to their high abundance and biological relevance in soils, as well as the low cost and ease of maintaining laboratory populations (Spurgeon et al. 2003; Chalew and Halden 2009b; Guo et al. 2009; Dann and Hontela 2011). Typical methods of exposure can be in artificial media, natural soil, or on filter paper. Artificial media or natural soils better represent toxicity and variability in an environmentally relevant matrix, and effect concentrations can be used in combination with measured or predicted environmental concentrations to assess risk (OECD 1984; Spurgeon et al. 2003). Filter paper tests ensure earthworm contact with the toxin for determining toxicity and reduce variability due to interferences from the soil matrix, but the measured effect concentrations are not applicable to soil (OECD 1984; Spurgeon et al. 2003). Mortality, weight change, reproduction, antioxidative enzyme activities, genotoxicity, and bioaccumulation have all been used to evaluate triclosan toxicity in earthworms (Simpson and McKelvie 2009; Pannu et al. 2012; Schnug et al. 2013; Schnug et al. 2014). Mortality is a crude all-or-none endpoint to reveal toxic effects from exposure to a compound, and while weight loss and reproduction are graded endpoints yielding more accurate responses, they do not yield information on the mode of action which can be applied more generally to other organisms with similar biochemical pathways (Timbrell 2008; Lankadurai et al. 2011a).

Metabolomics, or metabolite profiling, is an emerging field of science that studies the phenotypic response (metabolite profile) of organisms exposed to a variety of stimuli (Bundy et al. 2009). Metabolomics has benefitted from rapid advances in analytical instrumentation, for which standard methodologies for sample processing and analysis, data processing, and reporting are still being developed (Fiehn et al. 2007; Morrison et al. 2007; Sumner et al. 2007; Dunn et al. 2013; Salek et al. 2013). Metabolomics is the study of small molecules and metabolites within an organism, including tissue or biofluids, with the comprehensive dataset of metabolites termed the metabolome (Viant 2008). While the underlying genetic code is the same in all cells of an organism, the metabolome will likely vary between tissues or biofluids of the same organism (Goodacre 2007; Viant 2008; Yuk et al. 2012). The microbiome of an organism has also been linked to the observed metabolic profile, and may be the primary driver of the dynamics of certain metabolites (McHardy et al. 2013). Metabolomics offers a unique approach to assessing contaminant toxicity in model organisms by revealing detailed information on changes to their physiological status. Exposure of an organism to compounds with bioactive properties causes alterations in their metabolism that may or may not be reversible, depending on the chemical's mode of action (the biochemical interaction that leads to its activity), exposure dose, exposure time, environmental variables, and the condition of the organism (Aliferis and Jabaji 2011). Metabolomics now complements traditional ecotoxicology endpoints, often yielding valuable information at sub-lethal concentrations, with the potential to elucidate the chemical mode of action (Bundy et al. 2002; Guo et al. 2009; McKelvie et al. 2009; Aliferis and Jabaji 2011; Lankadurai et al. 2011a). There is growing evidence that exposure of earthworms to certain compounds can lead to metabolic alterations within an individual that can be measured using GC-MS (Jones et al. 2008; McKelvie et al. 2009; Baylay et al. 2012; Liebeke and Bundy 2012;

Mudiam et al. 2013; Givaudan et al. 2014). Triclosan is a prevalent contaminant in biosolids destined for land application, where there is documented exposure through bioaccumulation from soil to earthworms. The objectives of this study were to determine the acute toxicity of triclosan to the earthworm (*Eisenia fetida)*, and to investigate additional lethal or sub-lethal impacts from triclosan exposure using GC-MS metabolomics.

3.3. Materials and methods

3.3.1. Earthworm acute toxicity test

Eisenia fetida earthworms were originally purchased from a local supplier and maintained on a diet of milled corn in a large plastic tub with stabilized horse bedding compost substrate (maintained at 67% moisture) that was changed every three months. Mature earthworms, defined as having a visible clitellum and weighing between 300 to 600 mg wet (OECD 1984), were randomly chosen for use in chemical exposure experiments. A 48-hour acute toxicity test was established to estimate the LC_{50} of triclosan concentration and assess impacts on the earthworm metabolic profile. Triclosan was chosen after being previously detected and quantified in agricultural soil receiving biosolids (Gillis 2011). Exposure tests were conducted in 120 mL amber glass jars with 5.5 cm diameter glass wool placed in the bottom. Solutions of triclosan in acetone were spiked onto the glass wool to achieve concentrations of 0, 1, 0.1, 0.01, 0.001, and 0.0001 mg cm^{-2} and allowed to evaporate overnight. Glass wool was moistened with 0.5 mL distilled H_2O and a single worm was placed in each jar. Earthworms were checked for mortality after 6, 24 and 48 hours by applying a gentle mechanical stimulus to the anterior end with a 5 mm metal micro-spatula. Worms not responding to stimulus before the end of the test were immediately flash-frozen in liquid nitrogen in an attempt to limit sample

degradation due to cell lysis and enzymatic activity that occurs after death (Liebeke and Bundy 2012).

3.3.2. Metabolite extraction and analysis

A method based on McKelvie et al. (2009) was used to extract and analyze a suite of metabolites in earthworms using GC-MS. Metabolites included were alanine, valine, leucine, serine, phenylalanine, lysine, putrescine, spermidine, mannitol, glucose, and myo-inositol. Metabolism was immediately quenched after sampling and earthworms were stored in liquid nitrogen until they were freeze-dried for 24 hr and stored at -18 to -20°C until analysis. Individual dried earthworms were homogenized manually in 1.5 mL centrifuge tubes using individual 5 mm micro-spatulas, and extracted using 1 mL of a 0.2 M phosphate buffer in distilled water adjusted to pH 7.4, containing 1 g L^{-1} of sodium azide as a preservative. Extraction was assisted by vortex mixing for 60 s and sonication for 15 min at ambient temperature before centrifuging at 12,000 rpm for 2 minutes. The supernatant was transferred to a new 1.5 mL centrifuge tube and centrifuged again for 2 minutes, then 500 µL of supernatant was transferred to a 2 mL GC vial. Samples were evaporated to dryness and residues freeze-dried again to remove all traces of water. Samples were then derivatized by adding 800 µL of 25 g L^{-1} hydroxylamine in pyridine, vortexed for 60 seconds, and incubated at 70°C for 30 minutes. After cooling, 500 µl of hexamethyldisilazane and 50 µL of trifluoroacetic acid was added and incubated at 100°C for 60 minutes. Samples were centrifuged again after cooling at 12,000 rpm for 2 minutes before analysis by GC-MS.

Analysis was performed on an Agilent 5975 series quadrupole GC-MS equipped with a HP-5MS capillary column (30 m × 0.25 mm × 0.5 µm). A 1 µL aliquot was injected in splitless

mode into a deactivated glass wool liner at an injection port temperature of 290°C. The injection port pressure was 8.6138 psi, with a septum purge flow of 6.1 mL min^{-1} and total flow of 57.1 mL min^{-1}. The temperature program was 65°C hold 2 min., ramp 6°C $min.^{-1}$ to 230°C, ramp 10°C $min.^{-1}$ to 310°C, hold 20 min., for a total run time of 57.5 minutes. The transfer line, ion source, and quadrupole temperatures were 300°C, 230°C, and 150°C respectively. The MS was operated in full scan mode (45 to 650 m/z) at a sampling rate of 3, with a 4.5 minute solvent delay. Metabolite identification in earthworm samples was based on comparison of the retention time and three mass fragment ions determined for analytical standards (Table 3.1).

3.3.3. Data processing and statistical methods

Agilent GC-MS chromatograms were converted into .netCDF format using OpenChrom (Wenig and Odermatt 2010). Chromatograms in .netCDF format were further processed using the MZmine 2 metabolomics software (Pluskal et al. 2010). Baseline correction was first performed on the raw data files with a slope of 10^{-5}. Peak detection was targeted using the retention times in combination with the quantifying and qualifying ions for each metabolite. Retention times varied between runs for each metabolite, so metabolites were processed individually and appropriate retention time windows and other parameters were determined for peak detection and peak alignment algorithms. Chromatograms were smoothed prior to alignment using the join aligner. Peaks were visually inspected to ensure the selected peaks had the right retention time and that there were no missing values. The final peak list was then exported as a .csv file, and metabolite abundances for the quantifying ion were normalized by tissue weight prior to statistical analysis.

The effect of exposure concentration on each metabolite was tested using the PROC MIXED procedure in SAS 9.4, totalling 11 tests. The ANOVA assumption of normal distribution

Table 3.1. Metabolite identification and quantification m/z ions, retention times, and peak identification and alignment parameters for data processing in MZMine 2.

Metabolite	Quant. Ion (m/z)	Qual. Ion 1 (m/z)	Qual. Ion 2 (m/z)	Ret. Time (min.)	Ret. Time Tolerance, ID (min.)	Ret. Time Tolerance, Align (min.)	Peak Shape Deviation (%)
Alanine	207	151	85	10.63	0.1	0.3	60
Valine	270	226	55	13.10	0.08	0.3	90
Leucine	129	284	171	15.70	0.3	0.5	90
Serine	147	240	315	16.37	0.1	0.3	60
Putrescine	167	126	154	18.50	0.3	0.4	60
Phenylalanine	205	318	104	22.18	0.1	0.3	60
Lysine	212	367	395	24.36	0.1	0.3	60
Spermidine	126	437	297	28.63	0.1	0.3	60
Mannitol	319	147	205	29.75	0.15	0.4	90
Glucose	147	319	205	30.20	0.06	0.2	60
Inositol	305	147	217	31.12	0.1	0.3	60

of the error terms was assessed using normal probability plots of the residuals and the Anderson-Darling test. Constant variance of the error terms was assessed through scatter plots of the residuals vs. fits to confirm the absence of structure in the residuals, and formally checked using Levene's test (Montgomery 2005). Square root or cube root transformations were performed on all metabolites except spermidine, and outliers were removed for alanine (n=1), leucine (n=1), serine (n=1), and putrescine (n=5), to induce normality in all response variables. Constant variance was not met for valine, leucine, serine, phenylalanine, or mannitol based on Levene's test (up to 4-fold difference in absolute deviation between least and most variable treatments within a metabolite), but the F-test is generally robust to moderate violations of this assumption for balanced designs with fixed effects (Montgomery 2005). False discoveries were controlled by the Benjamini-Hochberg procedure, with the false discovery rate controlled at 0.05 (Glickman et

al. 2014). Letter groupings were assigned by LSMEANS for metabolites identified as significant after controlling the false discovery rate.

Principal components analysis (PCA) was used for dimension reduction on the significantly altered metabolites (using complete, untransformed data) followed by ANOVA on the first four principal components. PCs were plotted according to both exposure concentration and mortality status, since PCA is unsupervised and PCs can be more strongly influenced by underlying factors rather than the experimental treatments (Steuer et al. 2007; Ren et al. 2015). To examine the effect of mortality on metabolite concentrations, earthworms were grouped according to their mortality after 48 hours and were compared using two sample t-tests assuming unequal variances and controlling the false discovery rate at 0.05.

Correlation analysis for metabolites in surviving worms at 0, 0.0001, and 0.001 μg cm^{-2}, was performed in Minitab 16 (Minitab Inc., State College, PA). Correlation maps were generated using MetaboAnalyst 3.0 (Xia et al. 2015). Significant differences between the slope and intercept for exposed worms vs. the control were tested using nested models with incremental parameters (Bates and Watts 1988) with the PROC-NLIN procedure in SAS 9.4 (SAS Institute Inc, Cary, NC). In this procedure, data for two metabolites from the control and test concentration were stacked into two data columns. A dummy variable was generated in a new column with 0 for the control and 1 for the test concentration. The expectation function in PROC NLIN was entered as follows: $y1 = (m1 + m2*x1)*y2 + (b1 + b2*x1)$, where $y1$ and $y2$ are the metabolites, $x1$ is the dummy variable, and $m2$ and $b2$ are the difference in parameters $m1$ and $b1$ respectively between the control and test concentration. Initial parameters were estimated by linear regression in Minitab 16. After fitting the model and verifying model adequacy, if $m2$ or

b2 were significantly different from zero for either parameter, then differences between the test concentration and control were deemed to be significant at a 95% confidence level.

3.4. Results and discussion

3.4.1. Earthworm weight loss and mortality

The tested concentration range of triclosan (0.0001 to 1 mg cm^{-2}) induced an earthworm mortality rate ranging from 0 to 100% (Fig. 3.1A). There were no mortalities or visible effects of exposure after six hours, but the two highest exposure concentrations led to 100% mortality after only 24 hours. Earthworms in the 0.001 mg cm^{-2} group also lost significantly less weight after 48

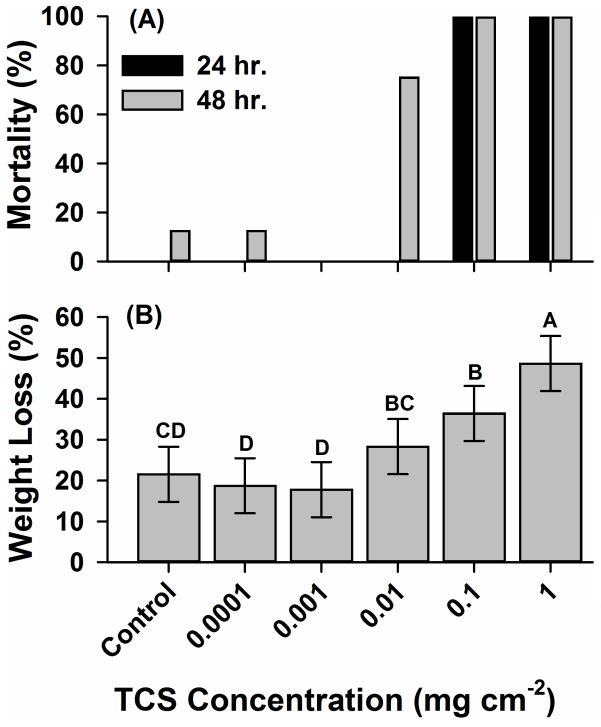

Figure 3.1. Earthworm mortality (A) and weight loss (B) following exposure to increasing concentrations of triclosan. All earthworms died within 24 hr. at concentrations of 0.1 and 1 mg cm^{-2}, and were sampled at 24 hr. to minimize degradation. Treatments with the same letter are not significantly different.

hours than worms at concentrations of 0.01 mg cm^{-2} and higher (Fig. 3.1B). Earthworms exposed to 1 mg cm^2 were severely degraded even after 24 hours and had lost a significant amount of coelomic fluid that could not be recovered, likely leading to the high weight loss. These earthworms were severely discoloured (pale flesh with red to orange fluid leakage) and tissues had lost rigidity. Earthworms at 0.1 mg cm^2 were less degraded, but still had pale discolouration with blistering or ulcerations apparent on most samples. Tissue rigidity was still present although less than living worms, and smaller amounts of coelomic fluid loss were visible.

Using a linear interpolation between the concentrations leading to 0% and 75% mortality (0.001 to 0.01 mg cm^{-2}), the lethal concentration in 50% of the exposed population (LC_{50}) was estimated to be 0.006 mg cm^{-2}. The LC_{50} calculated using a three parameter logistic model fitted to the mortality data was estimated to be 0.008 mg cm^{-2}. These estimates correspond well with the triclosan LC_{50} of 0.0039 mg cm^{-2} reported by Lin et al. (Lin et al. 2012) using the OECD acute filter paper toxicity test with *E. fetida*. While the filter paper test does not translate well into effective concentrations in soil, LC_{50} values can be used to compare relative toxicities of different chemicals tested on the same species, as long as the test conditions are equivalent. A lower LC_{50} indicates a higher toxicity, so relative toxicity was calculated as the triclosan LC_{50} divided by the LC_{50} of each compound for comparison. Phenol is approximately 180 times as toxic as triclosan, while the mono- to tetrachlorophenols are between 1800 to 6000 times as toxic, increasing with the degree of chlorination (Miyazaki et al. 2002). Based on the mean reported LC_{50} values for a selection of different classes of agricultural insecticides tested by Wang et al. (Wang et al. 2012), antibiotic, pyrethroid, carbamate, and organophosphate insecticides are approximately 4 to 7 times as toxic as triclosan. Insect growth regulator insecticides are only 0.2 times as toxic, while the neonicotinoids are on average almost 2000

times as toxic. Compared to other emerging contaminants, carbamazepine, estrone, PBDE-209, dimethyl phthalate, caffeine, and nonylphenol, are respectively <0.006, <0.006, <0.006, 0.03, 0.1, and 0.6 times as toxic as triclosan (McKelvie et al. 2011).

While triclosan is more toxic to earthworms than some emerging contaminants, it is less toxic than several commonly used pesticides. In 2012, there were over 72,000 Mg of agricultural pesticides sold in Canada (Health Canada 2012), including 100 Mg of chlorpyrifos active ingredient products which has similar earthworm LC_{50} values as triclosan (McKelvie et al. 2011). In comparison, land applying all of the biosolids generated annually in Canada, approximately 660,000 Mg dry weight of biosolids, would only contribute 4 Mg of triclosan to soil, assuming an average triclosan concentration of approximately 6 g per Mg dry biosolid (Monteith et al. 2010). It should be reinforced that the filter paper test does not reflect the toxicity in soil, where bioavailability of contaminants determines exposure dose, and where sorption and physical exclusion processes reduce bioavailability of contaminants (Alexander 2000; Chefetz 2003; Huang et al. 2003). The metabolic response of earthworms exposed to contaminants is dampened by high organic matter substrates, with the metabolic profile of exposed worms becoming more similar to the control as organic matter content increases (McKelvie et al. 2013).

3.4.2. Earthworm metabolite profiles

All metabolites exhibited generally high variability across all exposure concentrations, although metabolite abundances at 0.1 mg cm^{-2} were consistently lower than other treatments. Significant differences in metabolite concentrations were observed in at least one exposure concentration for five amino acids, one polyol, and both polyamines (Fig. 3.2). Significant alterations were observed at 1 and 0.1 μg cm^{-2} where mortality was 100% after 24 hours. Alanine

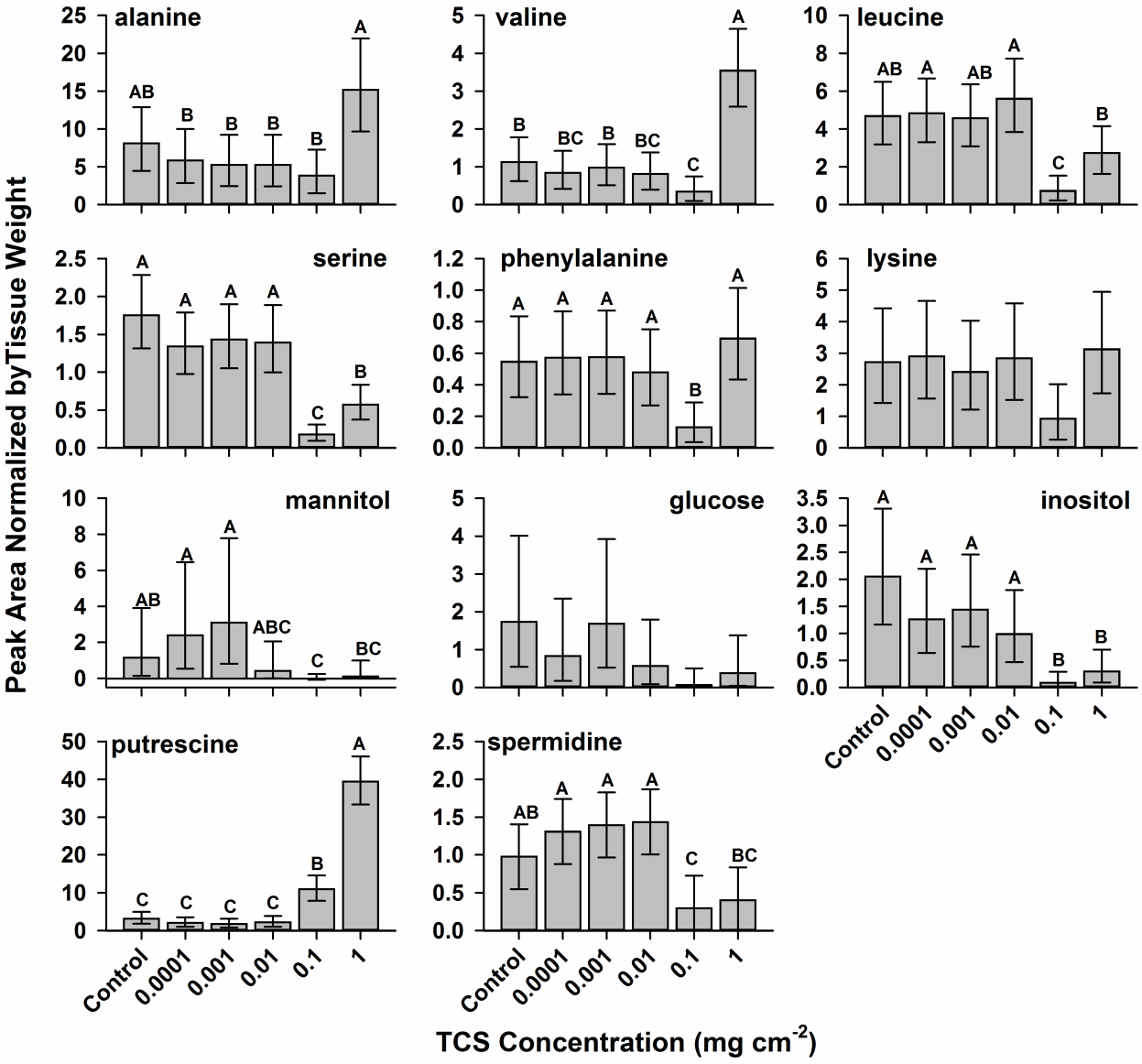

Figure 3.2. Concentrations of amino acids, polyols, and polyamines in earthworms following exposure to triclosan. Error bars show the 95% CI back-calculated from transformed values. Metabolites with letter groupings had a significant treatment effect based on ANOVA ($\alpha = 0.05$) corrected with the Benjamini-Hochberg procedure with a false detection rate of 0.05. Within each metabolite, treatments with the same letter are not significantly different.

and valine behaved similarly, both increasing at 1 mg cm^{-2} where much of the coelomic fluid had leaked. Yuk et al. (2012) showed that the earthworm metabolite profile can differ between the coelomic fluid and whole tissue, and the significant effects observed in the 1 mg cm^{-2} exposure

in our study may have been due to disproportionate representation of those metabolites in the tissue versus coelomic fluid, rather than an alteration induced by triclosan. Serine and inositol concentrations both decreased relative to the control at 0.1 and 1 mg cm^{-2}, but there were no significant differences between the control and concentrations of 0.01 mg cm^{-2} and below for either metabolite. Inositol concentrations at 1 and 0.1 mg cm^{-2} were lower than the control, and mannitol was lower than the control at 0.1 mg cm^{-2}. Changes in carbohydrate concentrations have been observed in earthworms in response to sub-lethal exposure to a number of contaminants and are reported biomarkers of toxin exposure, although the direction of change (increase or decrease) in the limited number of datasets appears to be both compound- and species-dependent (Simpson and McKelvie 2009).

The polyamines exhibited similar but opposing effects, with putrescine increasing and spermidine decreasing at the two highest exposure concentrations (with 100% mortality at 24 hours). In animals, putrescine is normally converted into spermidine in a tightly regulated pathway, which is then used in cellular processes such as growth, proliferation, stability of DNA, cell death, and for the production of other polyamines and amino acids (Heby 1981; Minois 2014). In general, all prokaryotic and eukaryotic cells are able to synthesize putrescine and spermidine (Tabor and Tabor 1976). Polyamines are thought to stimulate the production of protein and nucleic acids (Heby 1981), and as such, respond to a variety of mechanical and environmental stressors in earthworms and other invertebrates (Hamana et al. 1995). Increases in polyamines (especially putrescine) are often observed in animal tissue in the early stages of decomposition, as protein hydrolysis leads to free amino acids, which are then degraded by anaerobic microorganisms (Lakritz et al. 1975; Tamim and Doerr 2003). The marked increase in tissue putrescine concentration in earthworm mortalities, and consequent decrease in spermidine,

polyols, and some amino acids, suggests that decomposition of the earthworm tissues may have started before the worms were sampled, despite efforts to reduce sample degradation. All worms were alive six hours after exposure began, so earthworms had died less than 18 hours before sampling.

Principal components analysis using significantly altered metabolites did not reveal any effects at sub-lethal concentrations. Principal Components 1, 2, and 3 explained 39.6, 28.9, and 10.5% of the respective variability in metabolite abundances. However, as observed with univariate analysis, the only significant differences were in treatments with 100% mortality after 24 hrs (Fig. 3.3, top). The control, 0.0001, 0.001, and 0.01 mg cm^{-2} were grouped closely along both the PC 1 and PC 2 axis, although there was some separation along PC 3. Exposure concentrations of 0.1 and 1 mg cm^{-2} were separated from the rest along the PC 1 axis. There was a significant separation between 1 and 0.1 mg cm^{-2} along the PC 2 axis, perhaps reflecting the loss of coelomic fluid in the 1 mg cm^{-2} treatment. The separation along the PC 1 axis was highlighted when samples were grouped based on mortality status (Fig. 3.3, bottom). Since PCA is unstructured, metabolic effects due to mortality appear to influence PC 1 more strongly than the effects caused by triclosan exposure at the tested concentrations. Surviving worms were clearly higher along the PC 1 axis than worms that died after 24 hours (which had generally negative PC 1 scores), with earthworms dead at 48 hours clustering in between these two groups. To more closely examine mortality impacts on the metabolome, earthworm samples were grouped according to their mortality status (alive or dead) after 48 hours and compared using a t-test (Fig. 3.4). Putrescine was higher in mortalities, while mannitol, glucose, inositol, spermidine, putrescine, and serine were lower.

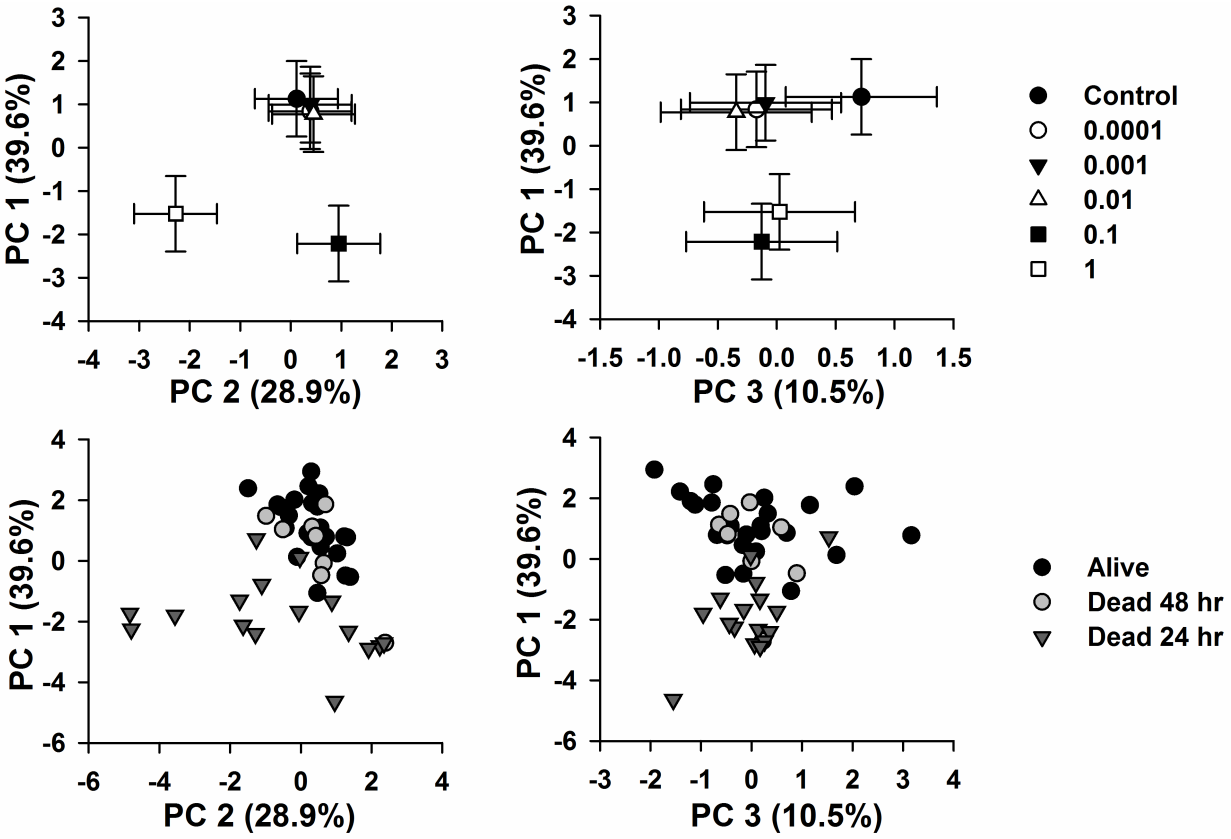

Figure 3.3. Principal components (PC) analysis scores plots of PC 1 vs. PC 2 and PC 2 vs. PC 3 grouped by triclosan exposure concentration (top) and by survival or mortality after either 24 or 48 hours (bottom). Error bars show the 95% CI.

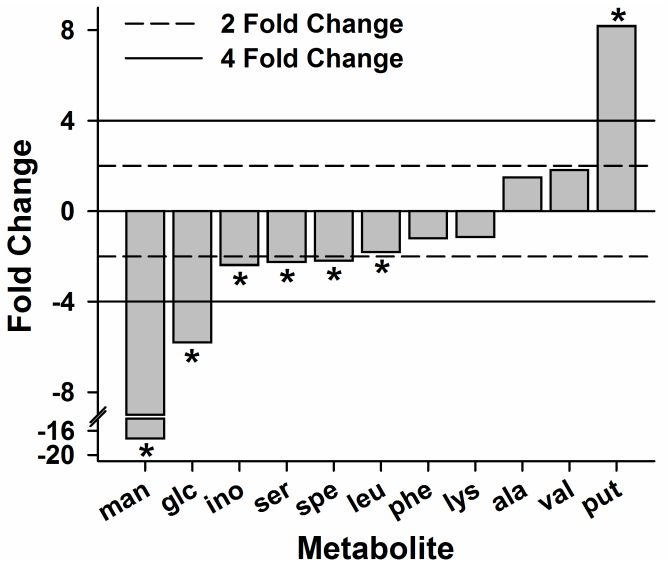

Figure 3.4. Fold change in metabolite abundance between alive and dead worms. Metabolites indicated with a * are significantly different based on a t-test ($\alpha = 0.05$) corrected with the Benjamini-Hochberg procedure with a false detection rate of 0.05.

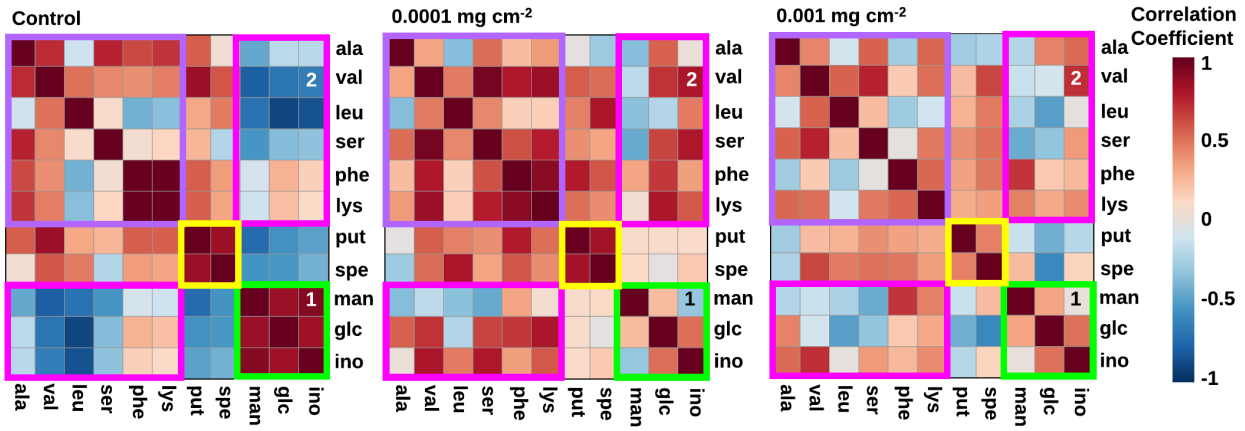

Figure 3.5. Correlation maps for metabolites in the control, 0.0001, and 0.001 mg TCS cm^{-2} treatments. Coloured boxes highlight correlations between the amino acids (purple), polyamines (yellow), carbohydrates (green), and amino acids vs. polyols (pink). The numbered squares 1 and 2 indicate metabolite pairs for which there was a large change in correlation coefficient and correspond to plots 1 and 2 in Fig. 6.

3.4.3. Correlations between metabolite pairs in surviving control vs. exposed worms

Correlation and regression analysis on metabolite profiles of surviving worms grouped by exposure concentration (Control: n = 7, 0.0001 mg cm^{-2}: n = 7, and 0.001 mg cm^{-2}: n = 8) are summarized in Figs. 3.5 and 3.6. Correlation maps are shown in Fig. 3.5, with coloured boxes

highlighting correlations within the amino acids, polyamines, and carbohydrates, and between amino acids vs carbohydrates. There were 9, 11, and 1 significantly correlated metabolites in the control, 0.0001, and 0.001 mg cm^{-2} exposure concentrations, respectively based on α=0.05. Two correlations were conserved between the control and 0.0001 mg cm^{-2}, one between 0.0001 and 0.001 mg cm^{-2}, and no correlations conserved across all three treatments. Using the Benjamini-Hochberg procedure with 55 independent tests and false positive rate of 0.05, only one metabolite pair is significantly correlated in the control, and none in either TCS concentration. The correlations are discussed further based on α=0.05, with the understanding that the potential for false discoveries is present in multiple testing scenarios.

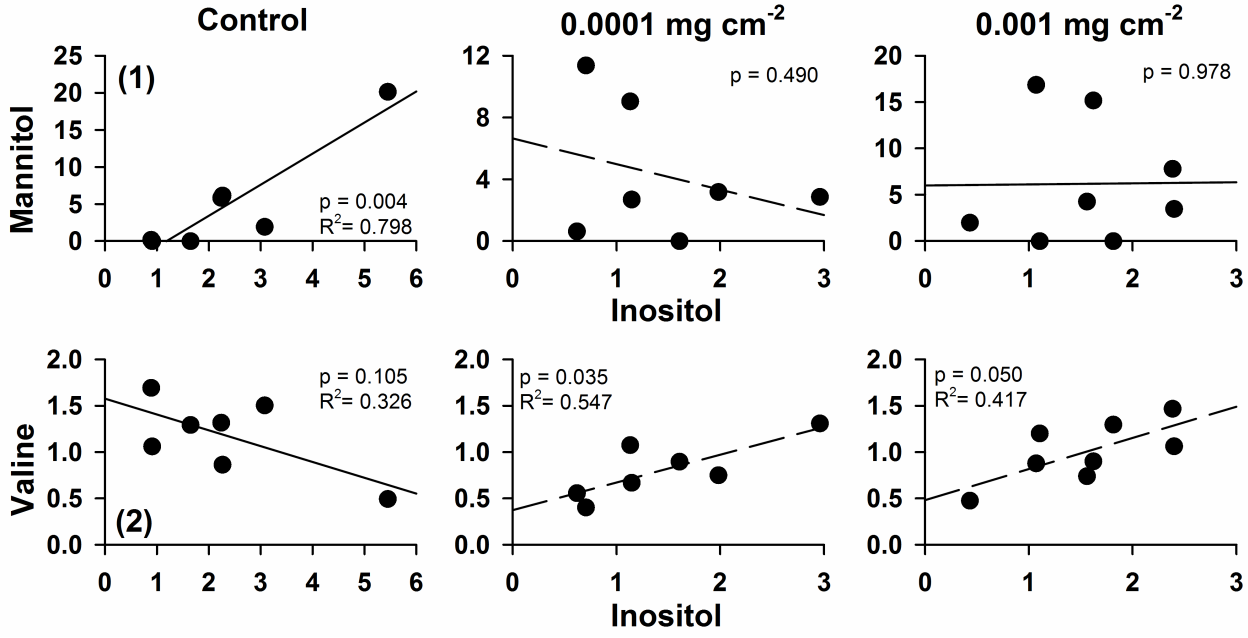

Figure 3.6. Relationships between metabolite pairs in surviving worms at the triclosan exposures indicated. Points represent individual earthworms. Plots 1 and 2 correspond to numbered squares in correlation maps (Fig. 3.5), and a dashed line indicates a significant difference in the slope and intercept of the regression line in treatments vs. the control.

Only one amino acid pair (phe vs. lys) was significantly correlated in the control group, while there were 5 and 1 correlated amino acid pairs at 0.0001 and 0.001 mg cm^{-2} respectively (Fig. 3.5, purple box). Whitfield Åslund et al. (2011a) found a large degree of correlation between amino acids measured on 24 control earthworm tissue extracts using NMR. There were five amino acids overlapping with our study, but only phe vs. lys was highly correlated in our control group. This may reflect the lower number of samples in our study (7 vs. 24) or a higher precision in NMR measurements compared to mass spectrometry-based metabolomics (Whitfield Åslund et al. 2011a). Putrescine and spermidine were strongly correlated in the control and 0.0001 mg cm^{-2} treatment, but not 0.001 mg cm^{-2} (Fig. 3.5, yellow box). There were strong positive correlations among all three carbohydrates in the control group that change to negative or no significant correlation in the exposed groups (Fig. 3.5, green box). The slope and intercept for man vs. ino were significantly different between the control and 0.0001 but not the 0.001 mg cm^{-2} treatment (Fig. 3.6). Moderate negative correlations were found between carbohydrates and a number of amino acids in the control group (pink box), which switch to positive correlations for some metabolite pairs in the two exposures. The slope and intercept for val vs. ino were significantly different from the control for both exposure concentrations (Fig. 3.6). A similar regression-based approach has been used to detect sub-lethal effects of endosulfan and DDT on earthworms by GC-MS metabolomics (McKelvie et al. 2009). The alanine to glycine ratio increased following exposure, and was identified as a potentially sensitive biomarker of DDT exposure.

An altered pattern in the distribution of correlations between metabolite pairs indicates a perturbation in the underlying physiological status of the organism (Camacho et al. 2005; Steuer 2006). The reversal of correlations (as seen for man vs. ino and val vs. ino) is characteristic of

metabolic switching and may suggest the existence of more than one steady state for the system (Steuer 2006). Since triclosan is an antimicrobial compound, potential impacts on the native earthworm microbiome may play a role in the observed changes in metabolite profiles. Targeted mechanistic studies are necessary to identify the physiological basis of any biomarkers identified in exploratory work (Johnson et al. 2016), but the relationships between man vs. ino and val vs. ino in Fig. 3.6 are potentially sensitive indicators of sub-lethal earthworm exposure to TCS.

3.5. Conclusions

Triclosan exposure to earthworms (*Eisenia fetida*) at concentrations of 0, 0.0001, 0.001, 0.01, 0.1, and 1 mg cm^{-2} led to mortality ranging from 0 to 100%, yielding an estimated LC_{50} of 0.006 and 0.008 mg cm^{-2} based on regression with a linear and three parameter logistic model respectively. There were no significant differences in the abundance of individual metabolites at any of the sub-lethal concentrations of triclosan. Lethal concentrations exhibited changes in the metabolite profile that was indicative of tissue decomposition, and which also varied between mortalities that either retained or lost their coelomic fluid prior to sampling. Principal components analysis did not reveal any additional sub-lethal effects, but confirmed the effects of decomposition and loss of coelomic fluid. There was evidence of perturbation in the physiological status based on dissimilar patterns in the pairs of significantly correlated metabolites and reversal of correlations in earthworms at both 0.0001 and 0.001 mg cm^{-2} concentrations compared to the control. This study highlights both the ability of metabolomics to improve interpretation and knowledge gained from standard ecotoxicological testing methods, and the necessity of multiple avenues of data analysis required for metabolomics datasets.

3.6. Acknowledgements

Financial support was provided by a Natural Science and Engineering Research Council (NSERC) Postgraduate Scholarship D (PGS D), the Harney Memorial Scholarship, and a McGill University Bioresource Engineering Department Graduate Excellence Award. Sources of funding had no influence on the study or decision to publish.

Preface to Chapter 4

The previous chapter examined triclosan toxicity to the earthworm on filter paper, an artificial test that provides a consistent exposure for identification of toxic effects, but does not reflect a real-world scenario. This was desirable for the initial study, but the decision was made for Chapters 5 and 6 to use earthworm bedding as the test substrate. Although triclosan is a prevalent contaminant in biosolids, it is readily degraded in soil into methyltriclosan, a more stable and potentially more toxic transformation product that is poorly understood. Chapter 4 uses metabolomics to investigate the difference in toxicity between the parent compound triclosan and its methylated transformation product at environmentally relevant concentrations.

Chapter 4: Metabolomics reveals increased toxicity in earthworms (*Eisenia fetida*) of the transformation product methyl-triclosan

J.D. Gillis[1*], G.W. Price[2], S.O. Prasher[1]

[1] Department of Bioresource Engineering, McGill University, Faculty of Agricultural and Environmental Sciences, Macdonald-Stewart Building MS1-027, 21111 Lakeshore Road, Sainte-Anne-de-Bellevue, Quebec, Canada, H9X 3V9

[2] Department of Engineering, Dalhousie University, Faculty of Agriculture, 39 Cox Road, PO Box 550, Truro, Nova Scotia, Canada, B2N 5E3

[*] Corresponding author: joseph.gillis@mail.mcgill.ca

Keywords: Metabolomics, Earthworms, Triclosan, Methyltriclosan, GC-MS

Highlights:

1) Effects of TCS and MTCS on *E. fetida* metabolome were examined

2) There were no discernible effects from TCS, only MTCS

3) MTCS affected tricarboxylic acid cycle metabolites

4) Disruption of energy metabolism is a potential mode of action for MTCS toxicity

4.1. Abstract

The effects of the antimicrobial triclosan (TCS) and its transformation product methyl-triclosan (MTCS) on the earthworm Eisenia fetida were investigated using GC-MS metabolomics. TCS is ubiquitous in sewage sludge, but a large proportion is transformed into MTCS during wastewater treatment and in soil when sewage sludge is applied to land. Our objective was to determine if earthworms exposed to ng g^{-1} to µg g^{-1} concentrations of TCS or MTCS exhibit toxic effects, and to identify the toxic mode of action of each compound. Ten individual earthworm replicates in 10 g worm bedding were exposed to 0, 0.25, 1, 4, 16, or 64 µg g^{-1} of either TCS or MTCS (120 experimental units) for 14 days. No mortalities were observed. All MTCS exposed worms had an instantaneous growth rate (IGR) over two times higher than the control during the study, but there was no effect of increasing concentration. Succinic acid was elevated relative to the control at concentrations ≥ 0.25 µg g^{-1} and glucose was elevated at 1 µg g^{-1}. There was separation from the control at all concentrations except 4 µg g^{-1} using Principal Components Analysis. Glucose, palmitic acid, and IGR contributed most strongly to the separation. Discriminant analysis with succinic acid, glucose, and IGR as variables showed a clear separation at all concentrations from the control along Canonical 1. Disruption of energy metabolism was hypothesized as a possible mode of action for MTCS.

4.2. Introduction

Triclosan (TCS) is an antimicrobial compound first introduced into consumer products in the 1970s, and has become a common biocide for use in many domestic, commercial, and health care applications (Fiss et al. 2007; Dann and Hontela 2011; Chen et al. 2015). Triclosan is frequently detected in the influent and effluent water of wastewater treatment plants (WWTPs), as well as in sewage sludge and treated biosolids (Lozano et al. 2013). Wastewater treatment removal rates for triclosan can be >95% from water, although most of the compound accumulates in the residual solids (McAvoy et al. 2002; Heidler et al. 2006; Heidler and Halden 2007; Lozano et al. 2013). Treatment processes for production of biosolids for land application from residual sewage solids can have variable removal rates (-91% to 99%) for TCS (Monteith et al. 2010). Triclosan concentrations in biosolid products across Canada are generally in the range of 0.1 – 20 $\mu g\ g^{-1}$ (Monteith et al. 2010), and approximately 50% of the over 660,000 Mg of biosolids produced annually in Canada are applied to land (Canadian Water and Wastewater Association 2012; CCME 2012). Triclosan toxicity has been demonstrated in an array of mammalian and non-mammalian species, and there is evidence of endocrine disruption (estrogenic, androgenic, and thyroid hormone activity), narcosis, mitochondrial toxicity, oxidative stress, and metabolic perturbations (Villalaín et al. 2001; Russell 2004; Lin et al. 2010; Dann and Hontela 2011; Ajao et al. 2015; Kovacevic et al. 2016; Vincent et al. 2016; Gillis et al. 2017).

Several biotic transformation products of TCS have been identified, including hydroxylation, methylation, conjugation, glucuronidation, and cleavage products (Chen et al. 2015; Ashrap et al. 2017; Tohidi and Cai 2017). Of these, the methylated transformation product methyl-triclosan (MTCS) is more persistent and bioaccumulative than TCS (Dann and Hontela

2011). Methyl-triclosan increases in concentration over time in triclosan-contaminated soils (Lozano et al. 2012) and can accumulate to equal or higher concentrations than TCS in both soil and earthworms (Macherius et al. 2014). In soils of varying textures that had TCS incorporated into the top 10 cm, decreases in soil TCS were linked to increases in MTCS, with the amount of accumulated MTCS generally lower in soils with lower soil clay content (Butler et al. 2012). There is limited information available describing the toxic mode of action of MTCS in many organisms. Methyl-triclosan was toxic in both whole organism (tadpole) and cellular (tadpole and rat) assays, whereas triclosan only affected organism assays, suggesting MTCS may have a different mode of action than TCS and may act at a cellular level (Hinther et al. 2011).

Metabolomics has become an important technique in the field of toxicology over the last two decades, using NMR and/or MS technologies to identify potential biomarkers of exposure to different toxins and to hypothesize a mode of action based on biological roles of metabolites (Bundy et al. 2004; Guo et al. 2009; Simpson and McKelvie 2009; Aliferis and Jabaji 2011; McKelvie et al. 2011; Kovacevic et al. 2016). In this study, changes to the metabolite profile in *E. fetida* caused by TCS and its methylated transformation product MTCS are assessed using targeted gas chromatography-mass spectrometry (GC-MS) metabolomics. The transformation of TCS into MTCS in sewage treatment and in soil has been well documented, but the toxicity of MTCS to soil-dwelling organisms is poorly understood. The primary objective of this study was to investigate effects on the metabolite profile of *E. fetida* exposed to TCS and MTCS at environmentally relevant concentrations, and to hypothesize the toxic mode of action based on metabolites affected by exposure.

4.3. Materials and Methods

4.3.1. Chemicals and Materials

Analytical standards of TCS and MTCS used for spiking, internal standards glucose-$^{13}C_6$ and leucine-d_3, as well as the derivatizing agents pyridine, methoxamine HCl, and N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) were purchased from Sigma Aldrich (Oakville, ON). Standards used for identification of metabolites by GC-MS were purchased from BioShop Canada Inc. (Burlington, ON). Methanol (99.9%), acetonitrile (99.9%), HPLC-grade water, and 2 mL microcentrifuge tubes with caps were purchased from Fisher Scientific (Ottawa, ON). GC vials (2 mL) with caps, and 250 µL GC vial inserts with polymer feet were purchased from Agilent Technologies (Mississauga, ON).

Table 4.1. Nutrient content of earthworm bedding used in toxicity test (n=3)

Nutrient	mean	SE
Nitrogen (g kg^{-1})	9.050	0.581
Calcium (g kg^{-1})	33.240	0.653
Potassium (g kg^{-1})	0.765	0.100
Phosphorus (g kg^{-1})	0.465	0.009
Magnesium (g kg^{-1})	1.020	0.033
Sodium (g kg^{-1})	2.438	0.238
Boron (mg kg^{-1})	<0.010	-
Copper (mg kg^{-1})	<0.005	-
Iron (mg kg^{-1})	2.838	0.118
Manganese (mg kg^{-1})	0.140	0.007
Zinc (mg kg^{-1})	0.016	0.0003

4.3.2. Maintenance of earthworm populations

The earthworms (*E. fetida*) were sampled from a population maintained in a plastic 1.2 m × 1.2 m × 1 m bin, containing 0.2 m to 0.3 m of stabilized horse bedding compost adjusted to pH 6 with lime. Commercially available Magic® worm bedding (Magic Products Inc., Amherst Junction, WI) was used for the experiments. Earthworms were fed weekly on a diet of milled corn to supplement the compost, and the bin was misted with non-chlorinated well water at regular intervals. Adult earthworms between 0.4 to 0.6 g wet weight and with a visible clitellum were sampled from the bulk population immediately prior to the experiment.

4.3.3. Experimental Design

A 2×6 factorial design with 10 replicates was established for the study. A single *E. fetida* earthworm was placed in a 100 mL vial with 10 g d.w. Magic® Worm Bedding (Magic Products Inc., Amherst Junction, WI) spiked with 0, 0.25, 1, 4, 16, or 64 μg g^{-1} d.w. of either TCS or MTCS. Nutrient content of the worm bedding is shown in Table S.1. The spiking rate of 0.25 μg g^{-1} represents an initial soil concentration resulting from a high rate of biosolids application (40 Mg ha^{-1}), incorporated into the top 20 cm of soil with a bulk density of 1500 kg m^{-3}, and concentrations in biosolids of 20 μg g^{-1} d.w (typical TCS range is 1-20 μg g^{-1}). The 1, 4, and 16 μg g^{-1} treatments reflect the typical range of TCS concentrations in biosolids. Prior to introduction of the earthworms, compounds dissolved in 1 mL of acetone were spiked into 10 g of worm bedding on aluminum dishes, stirred to mix thoroughly, and evaporated to dryness in a fume hood. The spiked worm bedding was transferred to the vial, moistened with 20 mL of distilled water, and equilibrated for 24 hr. Earthworms were collected from the maintained population, gently rinsed with distilled water and dried to remove extraneous organic matter,

weighed, and randomly assigned to an experimental vessel. The earthworms were removed after 14 days, gently rinsed to remove organic matter, weighed, and subsequently immersed in liquid N_2 to quench further metabolic activity and preserve the samples. Several authors have evacuated earthworm gut contents (depuration) prior to metabolomics analysis (Brown et al. 2008; Jones et al. 2008; Brown et al. 2009; Givaudan et al. 2014), while other authors have not (Bundy et al. 2002; Guo et al. 2009; Baylay et al. 2012; Liebeke and Bundy 2012). Earthworm gut contents were not evacuated in this study to prevent any potential starvation effects or other artifacts of depuration on the metabolome, while recognizing that the presence of gut contents may also include measurements of the microbiome metabolome or other interferences from the bedding substrate.

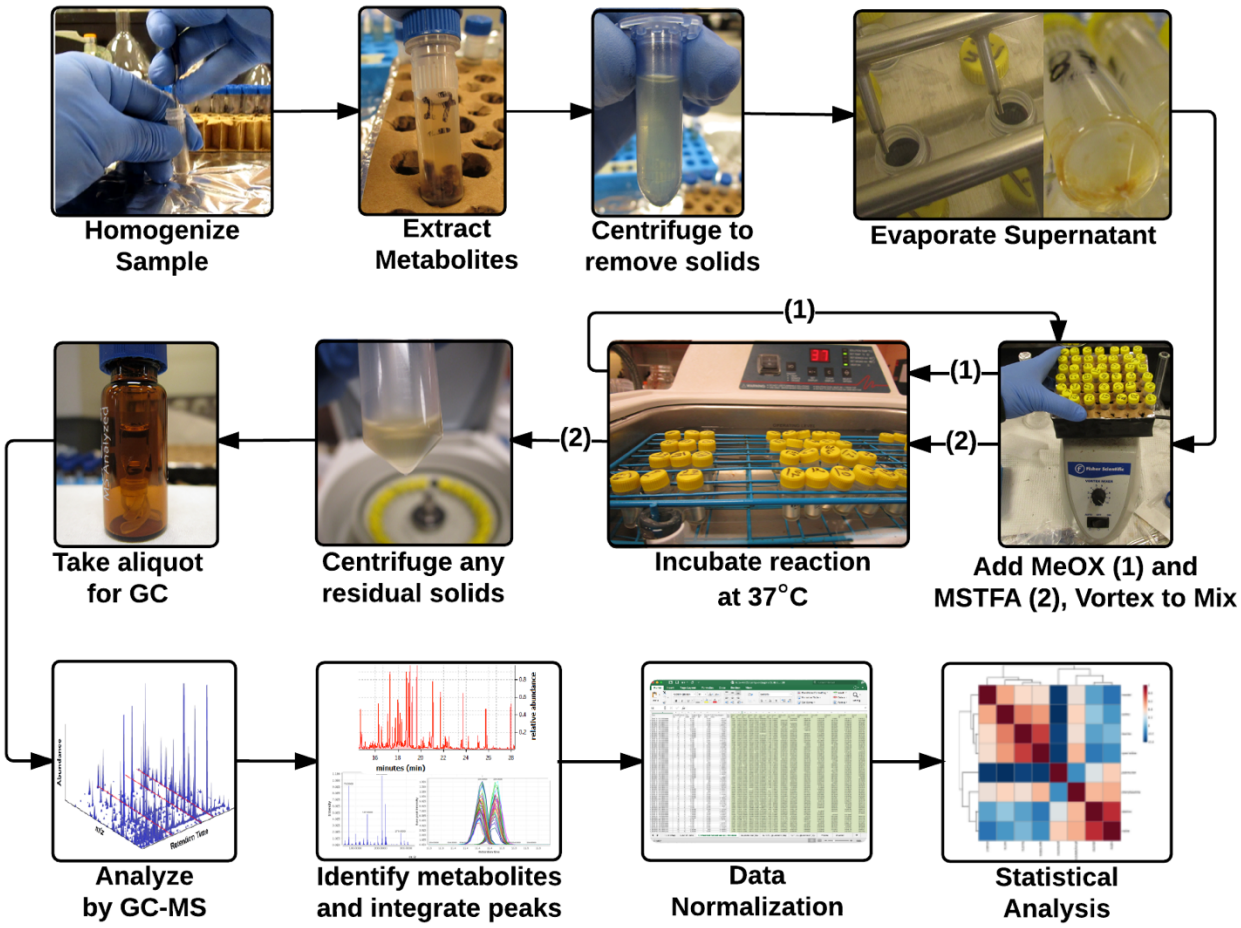

Figure 4.1. Metabolomics work flow from sample processing to data analysis

4.3.4. Sample Preparation and Analysis

Flash frozen earthworm samples were stored in liquid N_2 until freeze-drying over a 24 hr period, followed by storage at -20°C until extraction and analysis. The workflow for preparation of extracts from stored samples to the processing of data for analysis is outlined in Fig. 4.1. Sample preparation was based on methods described in Liebeke and Bundy (2012) with some modifications. Freeze-dried samples were manually broken into small particles in a 2 mL microcentrifuge tube with a 5 mm metal spatula followed by a two part extraction (1 mL of 1:1 acetonitrile:methanol followed by 1 mL of 4.5:4.5:1 acetonitrile:methanol:water) with the supernatants collected and combined in a new 2 mL centrifuge tube. Each extraction consisted of vortexing for 1 min, ultrasonic extraction for 10 min at 55°C, and centrifugation for 2 min at 12,000 × g. Combined extracts were vortexed for 1 min and centrifuged for 2 min at 12,000 × g before transferring a 500 µL aliquot to a new 2 mL tube and adding 20 µL each of 1 mM glucose-$^{13}C_6$ and leucine-d_3 internal standard prior to evaporation to dryness. A second aliquot of 100 µL was taken from each sample and added to a 30 mL glass vial to prepare a pooled quality control sample for each batch (GC-MS run) of samples. From the pooled sample for each batch, six QC samples were prepared in the same manner as real samples to monitor instrument performance within each GC-MS run. Three blanks with internal standard added were also prepared with each batch. Dried samples were methoximated by adding 60 µL of 20 mg mL^{-1} methoxamine HCl in pyridine, vortexing for 1 min., and incubating in a water bath for 90 min at 37°C. The samples were then trimethylsilylated using 120 µL of MSTFA, vortexed for 1 min, and incubated at 37°C for 20 min. The derivatized samples were vortexed for 30 s and centrifuged for 2 min at 12,000 × g. A 120 µL aliquot was transferred to a 2 mL GC vial with 250 µL micro-insert for analysis by GC-MS.

A 1 µL aliquot of the derivatized sample was analyzed on an Agilent 5795 GC-MS containing a deactivated glass wool liner followed by a 30 m × 0.25 mm i.d. × 0.25 µm film 5% phenyl 95% methylpolysiloxane DB-5 MS column with 10 m Duraguard. Samples were extracted and analyzed in 10 groups of 12 samples composed of a complete replicate of the experiment, with four reps processed on day 1 (48 samples) and three reps on days 2 and 3 (36 samples each). Samples were established in a sequence consisting of 3 blanks and 6 QC samples before the reps of experimental samples (with 1 QC sample analyzed after each rep), followed by the six QC samples and 3 blanks. Method parameters for GC-MS were based on Kind et al. (2009). The GC was operated at a constant flow of 1 mL min^{-1} of helium, with a temperature program of 60°C hold 1 min, ramp 10 °C min^{-1} to 325°C, hold 10 min, for a total run time of 37.5 min. The transfer line, ion source, and quadrupole temperatures were 290°C, 230°C, and 150°C respectively. The scan range was 50-600 amu, with a 5.9 minute solvent delay, threshold of 0, and a scan rate of 2.66 scans s^{-1} at 2 samples per scan.

4.3.5. Data Processing and Statistical Analysis

Mass spectra were converted to *.CDF format using OpenChrom (Wenig and Odermatt 2010) followed by peak detection, integration, and alignment using MZMine 2 (Pluskal et al. 2010). The sample *.CDF files have been uploaded to the MetaboLights database (Haug et al. 2013), with a study identifier number of MTBLS532 and release date of September 1, 2018. Representative total and extracted ion chromatograms are shown in Figs. 4.2 and 4.3. Parameters used for extraction of metabolite data from chromatograms in MZMine 2 are shown in Table 4.2. Analytical standards for each metabolite were analyzed using the same instrument and method prior to the experiment to determine retention times and characteristic ions used for identification

of peaks in unknown samples. All metabolites were therefore classified as Level 1 identified compounds according the Metabolomics Standards Initiative guidelines (Sumner et al. 2007).

Aligned peak lists were normalized by scaling metabolite abundances for the 12 samples within each of the 10 analysis groups based on the ratio of the group mean to the overall mean for each metabolite to remove technical variation caused by changes in instrument performance over time (e.g. decreases due to instrument contamination and increases due to ion source cleaning and column trimming). Univariate and Multivariate ANOVA, correlation analysis, Principal Components Analysis (PCA), and Discriminant Analysis (DA) were employed to assess differences between experimental treatments. Multivariate ANOVA was performed in Minitab 14 (Minitab Inc., State College, PA). Each metabolite was analyzed by ANOVA within each separate compound using PROC MIXED in SAS 9.4 (SAS Institute Inc., Cary, NC). A significance level of α=0.01 for univariate ANOVA was chosen to reduce the number of false positives due to multiple hypothesis testing (Broadhurst and Kell 2006). Normal distribution of the error terms was tested using normal probability plots of the residuals, and constant variance was assessed using scatterplots of the residuals versus fitted values. Data were transformed where necessary to achieve normality and constant variance. Correlation analysis, PCA, and DA were performed in JMP 13.2.0 (SAS Institute Inc., Cary, NC) on the transformed data. The DA model was evaluated based on the number of misclassifications (Szymańska et al. 2012). All figures were generated in Sigma Plot 12 (Systat Software Inc., San Jose, CA).

Table 4.2. Parameters for metabolite identification, peak detection, and alignment in MZMine2.

Metabolite	Abbr.	PubChem CID[1]	MW[1] (g mol^{-1})	RT (min.)	Characteristic Ions	RT Tol., det.	RT Tol., align.
Valine	val	6287	117.146	9.55	144, 218, 145	0.5	0.5
Leucine	leu	6106	131.172	10.28	158, 159, 232	0.5	0.5
Leucine-d_3 (IS)	leu-d_3	11073472	134.191	10.30	161, 162, 150, 163	0.4	0.5
Proline	pro	145742	115.130	10.68	142, 143, 216	0.4	0.5
Glycine	gly	750	75.066	10.79	174, 147, 248, 276	0.4	0.5
Succinic acid	suc	1110	118.088	10.84	147, 148, 247	0.3	0.6
Serine	ser	5951	105.092	11.47	204, 218, 147	0.4	0.5
Lactic acid	lac	612	90.077	11.80	147, 117, 191, 219	0.4	0.5
Malic acid	malic	525	134.087	13.13	147, 233, 245, 335	0.6	0.6
Phenylalanine	phen	6140	165.189	14.83	218, 192, 147	0.3	0.6
Putrescine	put	1045	88.151	16.11	174, 175, 214	0.3	1.2
Tyrosine	tyr	6057	181.188	18.21	218, 219, 280	0.6	0.6
Glucose	gluc	5793	180.155	18.31	319, 205, 147, 218	0.1	0.6
Palmitic acid	palm	985	256.424	19.25	313, 117, 129, 328	0.6	0.6
myo-Inositol	inos	892	180.155	19.70	305, 217, 147, 318	0.6	0.6
Margaric acid	marg	10465	270.450	20.19	117, 327, 132, 145	0.6	0.6
Adenosine	aden	60961	267.241	24.25	230, 236, 245, 540	0.4	0.6
Maltose	malt	6255	342.296	25.80	204, 191, 361, 271	0.5	0.6

Notes: [1] Values from PubChem Compound Search
Abbreviations: CID = Chemical Identifier; MW = Molecular Weight; RT = Retention Time; IS = Internal Standard; RT Tol., det. = Retention Time Tolerance for Peak Detection; RT Tol., align. = Retention Time Tolerance for Peak Alignment.

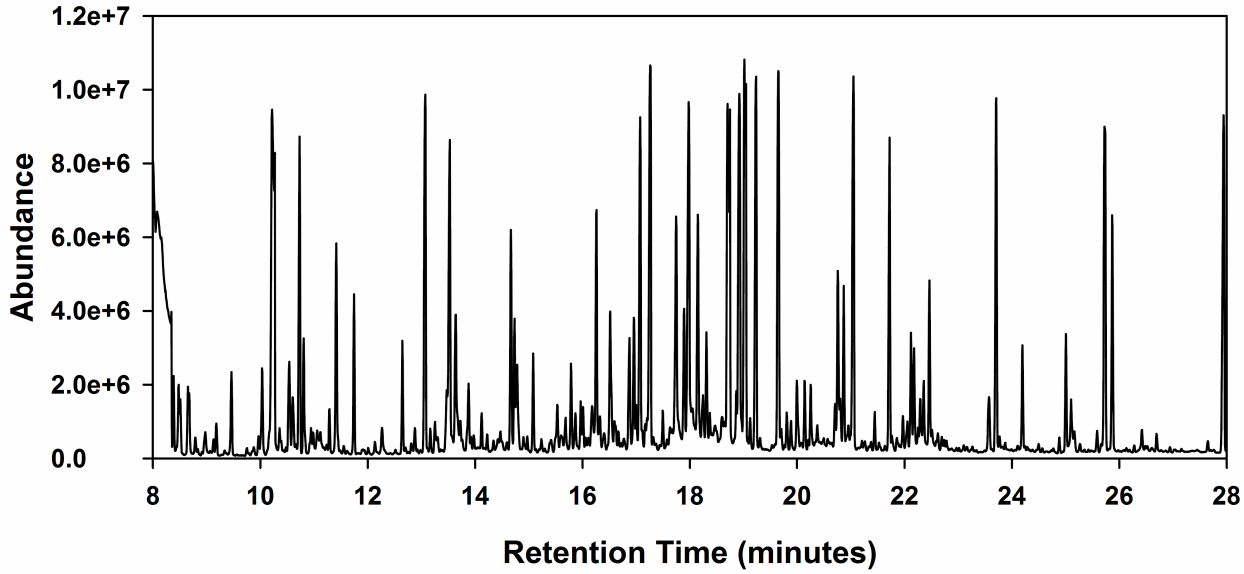

Figure 4.2. Representative total ion chromatogram from GC-MS analysis of earthworm extract.

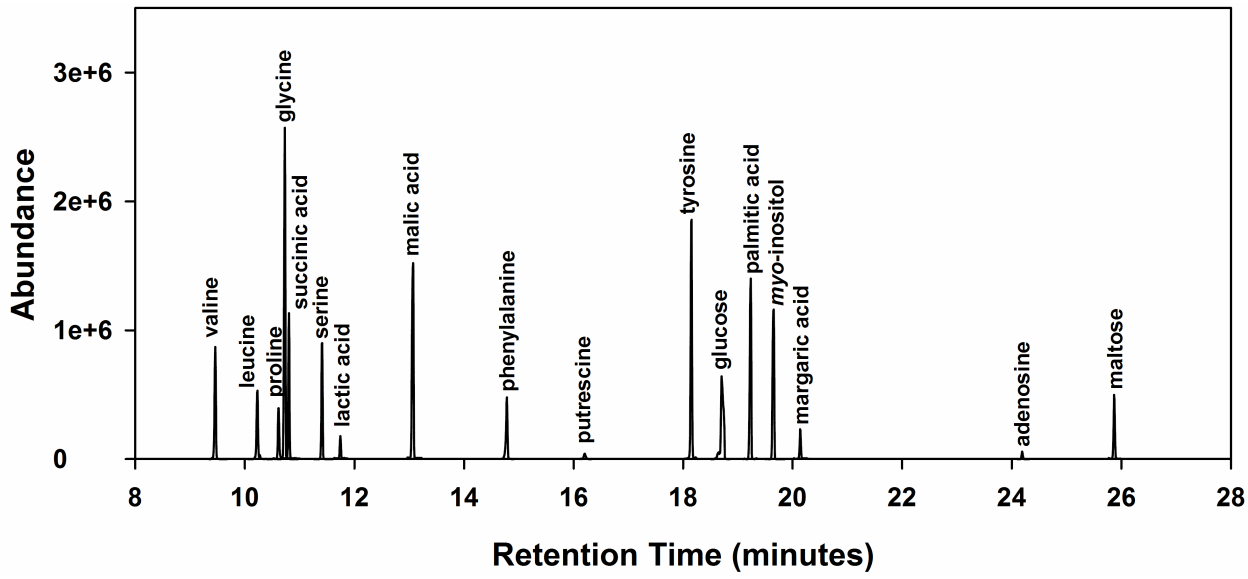

Figure 4.3. Representative extracted ion chromatogram of the target metabolites in earthworm extract analyzed by GC-MS.

4.4. Results

There were no earthworm mortalities observed over the 14-day experiment for TCS or MTCS. All MTCS exposed worms gained >2 times as much weight as the control worms during the experiment (Fig. 4.4), but there were no significant changes in worm mass for TCS. One outlier was removed from the control group, which had the highest overall weight gain (+48% vs. next closest sample at +36%) and was more than 3 times higher than the other samples in the control group. There were no significant concentration effects from TCS based on MANOVA (Wilks' lambda = 0.955) or within any individual metabolite using ANOVA (Table 4.3). A significant concentration effect was observed for MTCS based on MANOVA (Wilks' lambda = 0.011), while succinic acid and glucose differed between concentrations using ANOVA at α =

0.01. Succinic acid was elevated relative to the control at concentrations ≥ 1 μg g^{-1}, while glucose was only elevated at 1 μg g^{-1} (Fig. 4.5). Multivariate analysis using PCA for dimension reduction is shown in Fig. 4.6. Principal components (PCs) 2 and 5 differed by concentration (Fig. 4.6A), explaining 16.5% and 5.9% of the variance respectively. There was significant separation from the control for 1 μg g^{-1} along the PC 2 axis, and the PC 2 standardized coefficients were largest for glucose, succinic acid, *myo*-inositol, and malic acid (Fig. 4.6B), overlapping with the two metabolites identified through univariate analysis. Discriminant Analysis (DA) also found a significant separation between concentrations along the first two discriminant axes (Wilks' Lambda = 0.0003 and 0.0258) for MTCS samples with succinic acid, glucose, and weight change as predictors (Fig. 4.7A). The first two canonicals in DA described 59.8% and 35.9% of the variance in the dataset. There was clear separation from the control at all concentrations along canonical 1, while 1 μg g^{-1} clustered away from other treatments along canonical 2 but had high variability. There was a high degree of correlation between Canonical 1 vs. PC 2 and PC 5 ($p<0.0001$) and Canonical 2 vs. PC 2 ($p=0.0005$). Malic acid and succinic acid were significantly correlated with Canonical 1, Canonical 2, and PC 2, indicating these metabolites were closely associated with the multivariate separation that is reflected in the PCA scores.

Table 4.3. P-values for tests of significance between different exposure concentrations based on ANOVA. Significant concentration effects based on $\alpha = 0.01$ are listed in bold.

Variable	MTCS	TCS
Instantaneous Growth Rate	**0.0016**	0.4772
Valine	0.1867	0.4805
Leucine	0.3081	0.4384
Proline	0.6741	0.3320
Glycine	0.8754	0.3874
Serine	0.7726	0.8203
Tyrosine	0.4191	0.4451
Phenylalanine	0.3986	0.5494
Putrescine	0.7626	0.6742
Malic Acid	0.1461	0.8632
Lactic Acid	0.2729	0.2923
Succinic Acid	**0.0038**	0.5753
Margaric Acid	0.0412	0.5820
Palmitic Acid	0.1225	0.9545
Glucose	**0.0068**	0.9683
Maltose	0.0334	0.7034
myo-Inositol	0.5860	0.6064
Adenosine	0.8148	0.4875

Table 4.4. Percent of variance explained by each canonical and Wilks' Lambda test statistic from discriminant analysis on MTCS data.

Canonical	Percent	Cumulative Percent	Wilks' Lambda
Canonical 1	59.8	59.8	**0.0003**
Canonical 2	35.9	95.7	**0.0258**
Canonical 3	4.2	100	0.5615

Table 4.5. P-values from correlation analysis between metabolites versus the first two canonicals from discriminant analysis and the two principal components (PC 2 and PC 5) that varied significantly with concentration using MTCS data. Bold p-values are less than $\alpha = 0.01$.

Variable	**Canonical 1**	**Canonical 2**	**PC 2**	**PC 5**
Instantaneous Growth Rate	**<0.0001**	0.0451	0.3225	**<0.0001**
Valine	0.0257	0.1808	0.2734	0.9493
Leucine	0.0112	0.2207	0.2427	0.8181
Proline	0.0572	0.2729	0.3326	0.6502
Glycine	0.2888	**0.0013**	0.0410	0.9680
Serine	0.3540	0.1246	0.3876	0.4312
Tyrosine	0.1067	0.9656	0.0223	0.9511
Phenylalanine	0.0496	0.7394	**0.0089**	0.7281
Putrescine	0.2509	0.1736	0.0132	0.8176
Malic Acid	**0.0084**	**0.0002**	**<0.0001**	0.8048
Lactic Acid	0.1263	0.1258	0.4203	0.5485
Succinic Acid	**<0.0001**	**<0.0001**	**<0.0001**	0.6291
Margaric Acid	0.4714	0.5983	0.0585	**<0.0001**
Palmitic Acid	0.9609	**0.0034**	**0.0001**	0.3720
Glucose	0.9013	**<0.0001**	**<0.0001**	0.1643
Maltose	0.3888	**<0.0001**	**<0.0001**	0.1296
myo-Inositol	0.0559	0.4105	0.7998	0.7913
Adenosine	0.2657	0.0837	**<0.0001**	0.1470
Canonical 1	**<0.0001**	1	**0.0064**	**0.0005**
Canonical 2	1	**<0.0001**	**<0.0001**	0.0311
PC2	**0.0064**	**<0.0001**	**<0.0001**	1
PC5	**0.0005**	0.0311	1	**<0.0001**

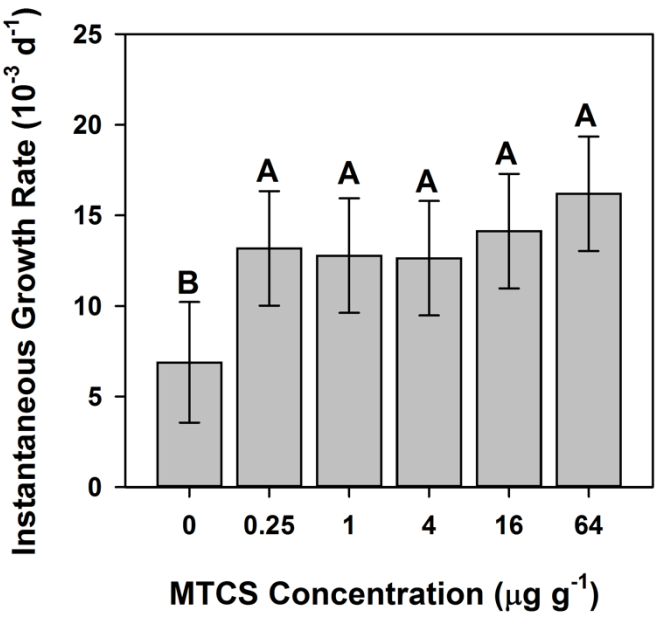

Figure 4.4. Group mean and 95% confidence interval (n=10) of earthworm weight change after 14 day exposure to MTCS.

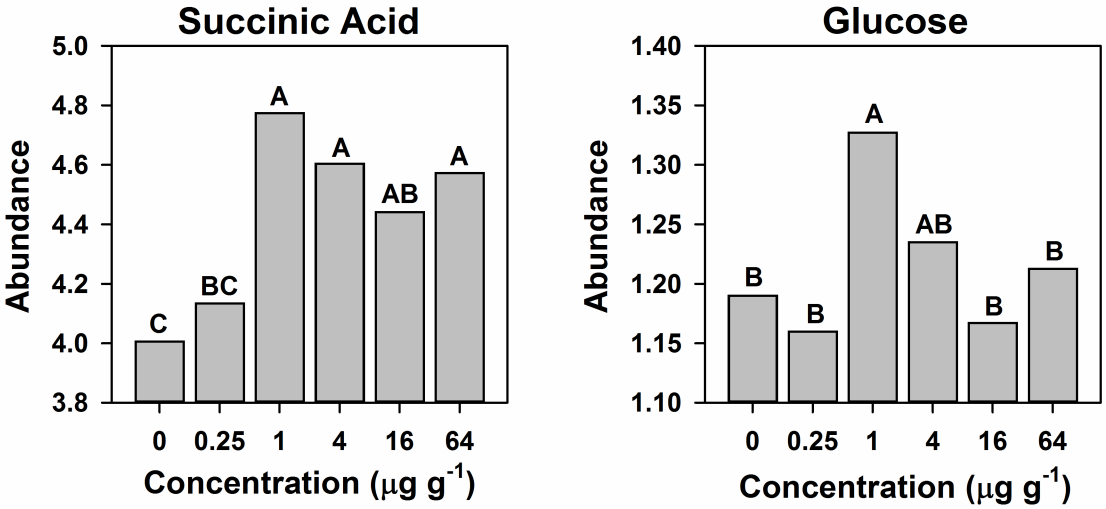

Figure 4.5. Succinic acid and glucose abundance in earthworms exposed to MTCS at the concentrations indicated. Concentrations with the same letter are not significantly different based on ANOVA ($\alpha = 0.01$).

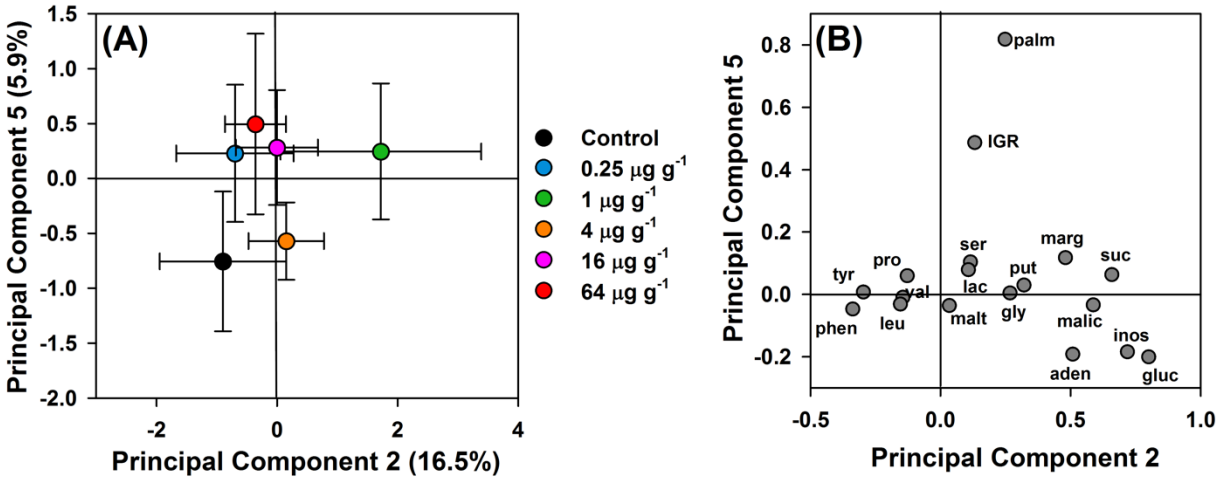

Figure 4.6. Group mean and 95% confidence interval (n=10) of Principal Components Analysis (PCA) scores for earthworms exposed to methyltriclosan (A) and scatterplot of standardized coefficients (B) for the contribution of each metabolite to the separation along PC axis 2 and 5.

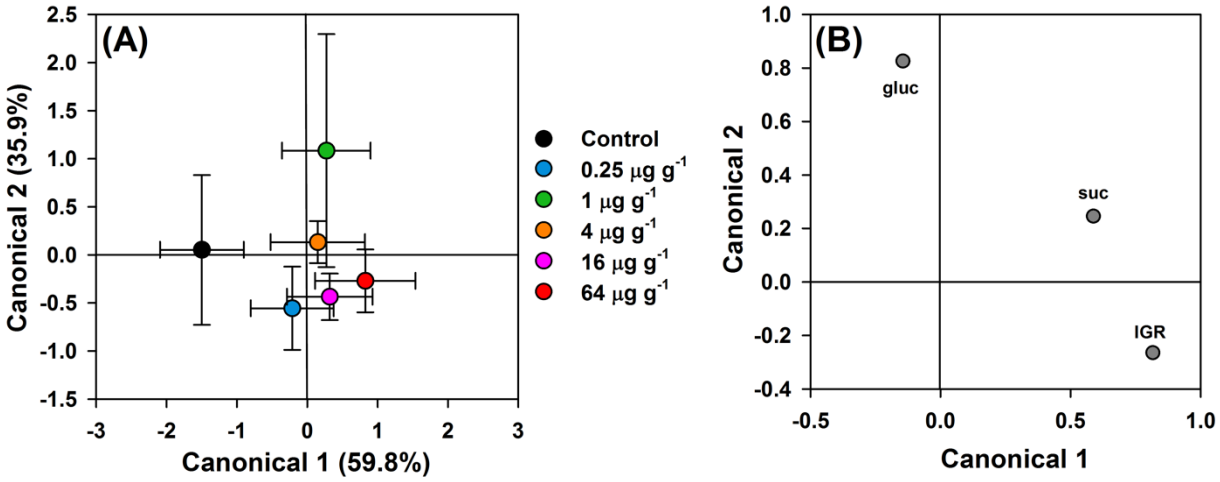

Figure 4.7. Group mean and 95% confidence interval (n=10) of Discriminant Analysis (DA) scores for earthworms exposed to methyltriclosan (A), and scatterplot of standardized canonical coefficients (B) for the contribution of glucose, succinic acid, and instantaneous growth rate (IGR) to the separation along canonical axis 1 and 2.

4.5. Discussion

The potential for contamination of soils with trace levels of organic contaminants from land application of biosolids a is a contemporary environmental issue. Several risk assessments for organic contaminants in biosolids have been published in recent years to determine if environmental concentrations of different trace organics exceed toxicity thresholds leading to adverse effects on soil organisms (Fuchsman et al. 2010; Langdon et al. 2010; McClellan and Halden 2010; Prosser and Sibley 2015; García-Santiago et al. 2016). Triclosan has been identified as a moderate to high-risk compound in various exposure pathways affecting human and environmental health, although many other frequently detected contaminants in biosolids have not been thoroughly assessed. Datasets for transformation products of known contaminants are lacking, and the risks they might pose are not as well assessed (Arnold et al. 2014; Malchi et al. 2015; Wu et al. 2015b). In our study, only the transformation product MTCS induced any measurable effects on the profile of metabolites in *E. fetida* examined, highlighting the need to assess transformation product toxicity.

We did not observe any weight loss following exposure to TCS or MTCS, and MTCS exposed worms actually gained more weight than the control. Some toxins can reduce appetite and feeding which can exhibit metabolic effects due to reduced food intake (Connor et al. 2004), but the evidence did not suggest reduced feeding for either compound in this study. There were concentration-dependent effects on succinic acid and glucose from MTCS exposure (Fig. 2), both had large standardized coefficients in PCA (Fig. 3). Although malic acid was not included as a predictor in discriminant analysis, it was strongly correlated with both canonicals. Despite succinic and malic acid being identified as biomarkers of TCS in human embryonic stem cells (Kleinstreuer et al. 2011), there were no effects of TCS on the earthworms in our study.

Increases in succinic acid have been observed in earthworms exposed to 3-trifluoromethylanaline (Warne et al. 2000) and 3-fluoro-4-nitrophenol (Bundy et al. 2001). Succinic and malic acid are intermediates in the tricarboxylic acid (TCA) cycle, where energy as ATP is generated within the mitochondria of eukaryotic cells from Acetyl-CoA produced from glucose, fatty acids, or amino acids (Nelson and Cox 2005). Altered levels of TCA cycle metabolites can be attributed to disruptions in pathways related to energy metabolism. Succinyl-CoA is converted to succinate, which is oxidized to fumarate, then hydrated to malate, the final step before oxidation to oxaloacetate to start the cycle again. Succinate dehydrogenase, which oxidizes succinate and is also involved in the electron transport chain, is the only membrane-bound TCA cycle enzyme (Nelson and Cox 2005). Therefore, its proper functioning may be sensitive to membrane instability (Villalaín et al. 2001). Methyl-triclosan was shown to be toxic in both whole organism and individual cell assays while TCS did not affect individual cells (Hinther et al. 2011), which may explain the increased toxicity of MTCS to this fundamental cellular process.

The disturbance of membrane function by accumulated pollutants in lipid tissues is called narcosis, which can lead to reduced efficiency of cellular processes and eventual death (van Wezel and Opperhuizen 1995). Many essential proteins for cellular signalling, ion channeling, electron transport systems (i.e. succinate dehydrogenase), and other processes are located in cellular membranes and are dependent on the lipid bilayer structure and function (Sandermann 1993). This effect is reversible and depends on the elimination rate of the compound (Escher et al. 2011). Narcosis in mitochondrial membranes is a potential mode of action for MTCS, which has also been reported for the parent compound TCS (Villalaín et al. 2001; Russell 2004; Vincent et al. 2016). Significant metabolite responses in *E. fetida* to MTCS exposure but not to TCS suggests MTCS is effective at a lower environmental concentration. This may reflect the

increased lipophilicity of MTCS, leading to greater protein binding or dissolution in lipids within the organism, or a reduced potential for elimination by either preventing metabolism to soluble excretion products or by evading recognition by efflux transporters of unmodified substances (Epel et al. 2008; Wu et al. 2010c; Tembe et al. 2017).

4.6. Conclusions

Triclosan exposure in the range of 0.25 to 64 μg g^{-1} did not lead to changes in the profile of metabolites measured in this study. Exposure to MTCS at ≥0.25 μg g^{-1} led to over two times faster growth than the control. MTCS exposure at ≥1 μg g^{-1} and 1 μg g^{-1} caused increases in succinic acid and glucose respectively, indicating disruptions to pathways related to energy metabolism. There was separation from the control using PCA (at 1 μg g^{-1}) and DA (at ≥0.25 μg g^{-1}). Phenylalanine, margaric acid, succinic acid, malic acid, inositol, adenosine, and glucose were significantly correlated with PC 2 (weight change and palmitic acid with PC 5), indicating that these metabolites were most closely associated with the multivariate separation reflected in PCA scores. Disruption of succinic acid metabolism through membrane destabilization in mitochondria was hypothesized as a potential mode of action for MTCS.

4.7. Acknowledgements

Financial support was provided by Canadian Water Network - Canadian Municipal Water Consortium grant (MW2013-2014), the Natural Science and Engineering Research Council (NSERC) Discovery Grant program, a NSERC Postgraduate Scholarship D (PGS D), the Harney

Memorial Scholarship, and a McGill University Bioresource Engineering Department Graduate Excellence Award. Sources of funding had no influence on the study or decision to publish.

Preface to Chapter 5

The previous chapter investigated the toxicity of a prevalent biosolids contaminant (triclosan) and its transformation product (methyltriclosan) to earthworms using metabolomics. The transformation product is many times more toxic, and appears to disrupt energy metabolism by affecting the membrane-bound citric acid cycle enzyme succinate dehydrogenase. Chapter 6 moves in a different direction and investigates the toxicity to earthworms of metformin, one of the most prescribed pharmaceuticals worldwide and an emerging contaminant detected in biosolids. As opposed to methyltriclosan, the mode of action for metformin in humans is well established since it is a commonly prescribed drug for diabetes. In this work, the objective was to confirm if a similar mode of action (reduced gluconeogenesis and increased oxidation/reduced synthesis of fatty acids) exists in earthworms. In addition, the effect of time was studied to characterize shifts in the metabolite profile with an increasing length of exposure, a factor often neglected in metabolomics studies with earthworms but which can have a large effect on the metabolome measured at a given point in time.

Chapter 5: Perturbations in the earthworm metabolite profile during a two week exposure to metformin assessed using GC-MS metabolomics

J.D. Gillis[1*], G.W. Price[2], S.O. Prasher[1]

[1] Department of Bioresource Engineering, McGill University, Faculty of Agricultural and Environmental Sciences, Macdonald-Stewart Building MS1-027, 21111 Lakeshore Road, Sainte-Anne-de-Bellevue, Quebec, Canada, H9X 3V9

[2] Department of Engineering, Dalhousie University, Faculty of Agriculture, 39 Cox Road, PO Box 550, Truro, Nova Scotia, Canada, B2N 5E3

[*] Corresponding author: joseph.gillis@mail.mcgill.ca

Keywords: Metabolomics, Earthworms, Metformin, GC-MS

Highlights:

1) Metformin exposure in soil at 160 and 640 μg g^{-1} led to weight gain relative to control

2) Time had a strong influence on the metabolite profile

3) The largest responses to metformin relative to the control were observed on day 7

4) Metabolites involved with gluconeogenesis and fatty acid metabolism were reduced

5.1. Abstract

Metformin is among the most prescribed pharmaceuticals in many countries, and as a result has shown up in biosolids derived from municipal wastewater treatment that are destined for land application. In humans taking metformin, the effects include a reduction in gluconeogenesis, increased oxidation of fatty acids, and reduced fatty acid synthesis. The effects of metformin on soil-dwelling organisms are not well understood. This study tested the effects of metformin exposure (0, 10, 40, 160, 640 μg g^{-1}) on the earthworm (*E. fetida*) at four time points (6 h, 2 d, 7 d, and 14 d) using GC-MS metabolomics to reveal sub-lethal effects and elucidate a mode of action for metformin. There were no mortalities observed, and earthworms exposed to the highest concentrations (160 and 640 μg g^{-1}) gained weight relative to the control on days 7 and 14. Significant reductions on day 7 were observed for glucose and malic acid (consistent with a reduction in gluconeogenesis), and for palmitic and margaric acid (consistent with increased oxidation and reduced synthesis of fatty acids). Metabolite levels increased to equal or greater than the control on day 14, likely caused by reduced exposure as metformin is reported to be readily transformed by microorganisms under aerobic conditions. The observations were consistent with the known effects of metformin in humans, and suggests that a similar mode of action exists in earthworms.

5.2. Introduction

Pharmaceuticals are emerging worldwide as prevalent environmental contaminants of soil and water, in large part through release into water bodies following wastewater treatment and into soil by land application of biosolids or irrigation with reclaimed wastewater (Metcalfe et al. 2004; Chefetz et al. 2008). Once present in these environmental matrices, there is potential for bioaccumulation in organisms and adverse toxic effects depending on exposure levels and species sensitivity (Hernando et al. 2006; Kinney et al. 2008; Wu et al. 2010b). Metformin is one of the most prescribed pharmaceuticals worldwide, beginning in the 1950s in Europe and 1970s in Canada for the treatment of type 2 diabetes (Pernicova and Korbonits 2014). Recently it has also shown promise in the treatment of certain cancers (Martin-Castillo et al. 2010). In 2008-2009, there were approximately 2.2 million people in Canada with type 2 diabetes and projected to top 3.3 million by 2018-2019 (Butler-Jones 2011; Government of Canada 2015). The dosage for metformin ranges from 0.5 to 2.5 g daily (Hirst et al. 2012), which is excreted unchanged with a half-life of approximately 5 hours (Gong et al. 2012). Based on the daily dosage range and projected 2018-2019 type-2 diabetes incidence, the emission of metformin to the wastewater stream could be estimated in the range of 1 to 8 Mg day^{-1} in Canada if all potential candidates were taking it.

As a result of its increasing popularity, metformin is among the most common contaminants measured in surface water bodies impacted by wastewater (Blair et al. 2013; Bradley et al. 2016). Concentrations in wastewater influent are typically in the μg L^{-1} to mg L^{-1} range, with removal rates between influent and effluent water ranging from 68-98% (Scheurer et al. 2009; Scheurer et al. 2012; Oosterhuis et al. 2013; Trautwein et al. 2014; Kleywegt et al. 2016). A large proportion of the metformin is transformed into guanylurea (Scheurer et al. 2012;

Oosterhuis et al. 2013; Trautwein et al. 2014), while a log K_{OC} ranging between 1.1-2.6 depending on the soil suggests a significant fraction of metformin is expected to be sorbed to organic matter (Scheurer et al. 2012; Mrozik and Stefańska 2014; Briones et al. 2016). Metformin has been measured in sewage sludges from several countries, generally in the 10-1000 ng g^{-1} range (USEPA 2009b; Kim et al. 2014; Gago-Ferrero et al. 2015; Klabunde 2016; Thomaidi et al. 2016). When biosolids are applied to land, residual metformin represents a potential source of contamination in soil that may impact exposed organisms.

Metformin toxicity has been measured with a variety of growth and development endpoints in aquatic organisms including fish (*Pimephales promelas*), algae (*Desmodesmus subspicatus*), plants (*Lemna minor*), and the crustacean *Daphnia magna*, with EC_{50} ranging from as low as 1 μg L^{-1} exhibiting estrogenic activity in fathead minnows while up to >320 mg L^{-1} showed no effect on algal growth (Cleuvers 2003; Niemuth and Klaper 2015; Crago et al. 2016). Dietary exposure in crickets at 1.78 mg g^{-1} food resulted in a longer life span, lower growth rate, and delayed maturation relative to the control (Hans et al. 2015). Similarly, metformin exposure as low as 25 mM in agar slowed the rate of aging and extended lifespan in *Caenorhabditis elegans* nematodes co-cultured with the bacteria *Escherechia coli*, by altering the metabolism of folate and methionine in the bacteria (Cabreiro et al. 2013). Metformin spiked in soil at 10 μg g^{-1} reduces growth of carrots and wheat seeds (Eggen et al. 2011), but not tomato, squash, beans, rapeseed, wheat, oats, barley, or potatoes (Eggen and Lillo 2012). In soil animals such as earthworms, metformin toxicity is not well understood.

Metabolomics has emerged as a powerful tool in the study of contaminant toxicology, and the effects of exposure to organic and inorganic substances on the earthworm metabolome have been demonstrated repeatedly (Simpson and McKelvie 2009). In many cases, a mode of

action for the toxin can be hypothesized based on the metabolites affected by the exposure and the biochemical pathways in which they are involved. Examples include disruption of energy metabolism by possible inhibition of the enzyme succinate dehydrogenase by phenanthrene (Lankadurai et al. 2011b) and methyltriclosan (Chapter 5), reduction of ATP synthesis and increase in fatty acid oxidation for perfluorooctanoic acid and perfluorooctane sulfonate (Lankadurai et al. 2012; Lankadurai et al. 2013a), starvation effects due to reduced food intake for pyrene (Jones et al. 2008), and oxidative stress for titanium dioxide nanoparticles (Whitfield Åslund et al. 2011b). Metabolomics has also been used to characterize time-dependent responses in the metabolite profile due to toxic stress that can differ based on the length of exposure to the toxin, although this is less common (McKelvie et al. 2010; Lankadurai et al. 2011b). In this study, our objective was to assess the toxicity of metformin to the earthworm *Eisenia fetida* using metabolomics, to evaluate metabolic perturbations in response to increasing metformin concentrations and to characterize the changes in metabolic profiles over time. Since metformin is known to affect glucose and fatty acid metabolism in other animals (Pernicova and Korbonits 2014), it was expected that changes in these metabolites would be observed in exposed earthworms.

5.3. Materials and methods

5.3.1. Experimental design

The experiment was a single factor (metformin concentration) with five levels (0, 10, 40, 160, 640 µg g^{-1}) and ten replicates repeated over time (6 h, 2 d, 7 d, and 14 d), totalling 200 experimental units. The experimental unit was a single *E. fetida* earthworm in a 100 mL plastic vial with 10 g d.w. Magic® Worm Bedding spiked at the appropriate metformin concentration.

Chemicals and materials for the experiment, maintenance of the earthworm population, and composition of the worm bedding used for testing are described in Chapter 5. Since the toxicity of metformin in earthworms was not known, the exposure concentrations in worm bedding were estimated to deliver a dose ranging from the daily therapeutic dose (5-25 mg kg^{-1} body weight) to the no observable adverse effect level in rats (200 mg kg^{-1} body weight) calculated for earthworms on a body weight basis, and assuming worms consume half their body weight in worm bedding (on dry weight basis) each day (Quaile et al. 2010; Hirst et al. 2012). Metformin was dissolved in deionized water at half of the desired soil concentrations, then 20 mL of the solution was added to the 10 g d.w. of worm bedding and equilibrated for 24 hr. Adult earthworms (0.5 g ± 0.1) were sampled from the laboratory population and randomly assigned to treatments. The earthworms were removed from the bedding after 6 h, 2 d, 7 d, and 14 d, gently rinsed to remove organic matter, weighed, and quenched in liquid N_2. Samples were stored frozen at -80°C until extraction and analysis.

5.3.2. Sample preparation and analysis

The workflow for preparation of extracts from stored samples to the processing of data for analysis is as described in Chapter 5, except that samples were stored at -80°C and not freeze-dried prior to analysis. Samples were manually ground in a 2 mL microcentrifuge tube with a 5 mm metal spatula then extracted with 1 mL of 1:1 acetonitrile:methanol followed by 1 mL of 4.5:4.5:1 acetonitrile:methanol:water. Collected supernatants were combined in a new 2 mL centrifuge tube. Samples were extracted by vortexing for 1 min, ultrasonic extraction for 10 min at 55°C, and centrifugation for 2 min at 12 × *g*. Final extracts were vortexed again for 1 min and centrifuged for 2 min at 12 × *g*. A 500 µL aliquot was transferred to a new 2 mL tube, 20 µL

each of 1 mM glucose-$^{13}C_6$ and leucine-d_3 internal standard, were added, and samples were evaporated to dryness. Four internal standard blanks were also prepared with each batch. Samples were derivatized with 60 µL of 20 mg mL^{-1} methoxamine HCl in pyridine for 90 min at 37°C, followed by 120 µL of MSTFA at 37°C for 20 min and centrifugation for 2 min at 12 × *g*. A 120 µL aliquot was transferred to a 250 µL micro-insert in a 2 mL GC vial for GC-MS analysis. Parameters for GC-MS analysis were based on Kind et al. (2009).

5.3.3. Data processing and statistical analysis

Agilent mass spectra files were converted to *.CDF format using OpenChrom (Wenig and Odermatt 2010) to be read by MZMine 2 (Pluskal et al. 2010) for peak detection, integration, and alignment. Retention time, characteristic ions, and tolerances used for data extraction are shown in Table 6.1. Aligned peak lists were exported to *.csv format for data correction based on the weight of earthworm tissue extracted. Changes in instrument performance over time caused by instrument contamination and maintenance activities (ion source cleaning and column trimming) were controlled by randomizing the analysis order of samples across the 5 separate runs, and mean-centering each run to the overall mean for each metabolite.

Earthworm weight change was expressed as the instantaneous growth rate (IGR), calculated as follows:

$$IGR = \frac{\ln (Y_T / y_t)}{T - t}$$

Where T is the final time (d), t is initial time (d), Y_T is the final mass (g), y_t is the initial mass (g), Y_T / y_t is the growth ratio, and $T - t$ is the growth interval (Whalen and Parmelee 1999). The IGR is strongly influenced by the growth interval (Whalen and Parmelee 1999), so the effect of metformin concentration on earthworm IGRs were analyzed within each time point.

Table 5.1. Parameters for metabolite identification, peak detection, and peak alignment in MZMine 2.

Metabolite	Abbr.	PubChem CID[1]	MW[1] (g mol^{-1})	RT (min.)	Characteristic Ions	RT Tol., det.	RT Tol., align.
Valine	val	6287	117.146	8.90	144, 218, 145	0.5	0.5
Leucine	leu	6106	131.172	9.60	158, 159, 232	0.5	0.5
Succinic acid	suc	1110	118.088	9.75	147, 148, 247	0.3	0.6
Proline	pro	145742	115.130	10.00	142, 143, 216	0.4	0.5
Glycine	gly	750	75.066	10.15	174, 147, 248, 276	0.4	0.5
Serine	ser	5951	105.092	10.95	204, 218, 147	0.4	0.5
Lactic acid	lac	612	90.077	11.28	147, 117, 191, 219	0.4	0.5
Malic acid	malic	525	134.087	12.61	147, 233, 245, 335	0.6	0.6
Lysine	lys	5962	146.190	17.50	174, 317, 156	0.6	0.6
Tyrosine	tyr	6057	181.188	17.65	218, 219, 280	0.6	0.6
Glucose	gluc	5793	180.156	18.31	319, 205, 147, 218	0.1	0.6
Palmitic acid	palm	985	256.424	18.70	313, 117, 129, 328	0.6	0.6
Margaric acid	marg	10465	270.450	19.15	117, 327, 132, 145	0.6	0.6

Notes: [1] Values from PubChem Compound Search
Abbreviations: CID = Chemical Identifier; MW = Molecular Weight; RT = Retention Time; IS = Internal Standard; RT Tol., det. = Retention Time Tolerance for Peak Detection; RT Tol., align. = Retention Time Tolerance for Peak Alignment.

Univariate ANOVA and Discriminant Analysis (DA) were used to analyze the metabolite data. Each metabolite was analyzed by ANOVA as a concentration×time repeated measures analysis using PROC MIXED in SAS 9.4 (SAS Institute Inc., Cary, NC). Data was transformed using square, cube, or fourth root to achieve normality and constant variance of the error terms where necessary. Normality was tested using normal probability plots of the residuals, and constant variance evaluated using scatterplots of the residuals vs. fits. To reduce the potential for false positives in multiple hypothesis testing, a lower α-level of 0.01 was used to determine significance. Linear discriminant analysis was performed in JMP 13.2.0 (SAS Institute Inc., Cary, NC) using 20 groups corresponding to the treatment combinations. The DA model

significance was assessed using Wilks' Lambda, and accuracy was evaluated using the number of misclassifications (Szymańska et al. 2012). Canonicals 1 and 2 were subsequently analyzed by ANOVA with the same design as the individual metabolites to determine if concentration or time influenced the metabolite profile in a multivariate sense as reflected in the canonical scores.

5.4. Results and discussion

5.4.1. Earthworm weight change in response to metformin

Earthworm weights pre- (Y_T) and post-exposure (y_t) did not vary by concentration or time, but there was a significant concentration by time interaction effect on the growth ratio expressed as Y_T / y_t (Table 5.2). With the exception of 640 µg g^{-1} after 6 hours, earthworms treated with metformin maintained growth similar to the control worms up until day 7, when the 160 and 640 µg g^{-1} treatments continued to gain weight and the remaining treatments lost weight (Fig. 5.1). Weight loss was most pronounced in the control. By day 14, the control, 10, and 40 µg g^{-1} treatments regained the lost weight and were similar to day 0, but the 160 and 640 µg g^{-1} treatments maintained an increase in weight of 10 to 15%. The instantaneous growth rate was heavily dependent on the growth interval (Fig. 5.2), consistent with results reported by Whalen and Parmelee (1999), so growth rates were compared within each interval (6 hr, 2 d, 7 d, 14 d). When expressed as instantaneous growth rate, earthworms in the 10 µg g^{-1} group grew significantly faster than all other exposed groups after 6 hours, but were not different from the control (Fig. 5.3). Earthworms in the 640 µg g^{-1} group grew significantly slower than the control after 6 hours. However, this was reversed by day 7 where 0 to 40 µg g^{-1} had negative growth rates and 160 to 640 µg g^{-1} were positive and significantly higher than the other treatments. After

14 days, earthworms exposed to 160 to 640 μg g^{-1} metformin maintained a higher growth rate which was not significant, while the remaining treatments had growth rates near zero.

Weight change can result from gain or loss in tissue, or an increase or decrease in feeding activity and retention of liquids or solids since gut contents were not voided prior to extraction. Weight loss can also occur if energy is limited in the diet, and stored energy in the form of glycogen is catabolized to cover maintenance energy costs in an organism (Johnston et al. 2014). An alternative mechanism involved in the reduction of blood glucose levels by metformin in humans is a decrease in glycogenolysis, the production of glucose from glycogen (de Souza Silva et al. 2010). In earthworms, glucose is absorbed from the blood stream by individual cells and stored as glycogen until needed (Prentø 1987). Glycogen content in earthworms can range from 2-5% (Prentø 1987), so it is unlikely that observed weight loss is primarily due to utilization of glycogen even if glycogen stores were entirely depleted. The slow growth rate at 6 hours in higher concentrations of metformin (Figure 5.3) may indicate an initial avoidance response to the contaminated worm bedding. The weight loss and negative growth rates in the control and low exposure treatments on day 7 (Figs. 5.1 and 5.3) may indicate that the earthworms avoided feeding on the experimental worm bedding over time and it may have been less desirable than the compost from which they originated. The weight increase in worms exposed to 160 and 640 μg g^{-1} is unexpected since metformin is frequently associated with weight loss in humans due to a reduced appetite and decreased fat storage in tissues (Malin and Kashyap 2014). However, in tadpoles exposed to metformin in combination with two lipid lowering drugs (Benzafibrate and Atorvastatin), weight gain was observed in the highest exposure of 500 μg L^{-1} relative to the control, tadpoles were more developed, and in generally better condition (Melvin et al. 2017). Blood glucose and appetite are inversely related in humans

(Mellinkoff et al. 1997), and it is possible that glucose-lowering effect of metformin led to an increased appetite in earthworms resulting in higher feeding activity than the control.

5.4.2. Metabolite responses to metformin

In general, the results show a non-linear metabolic response in earthworms to metformin, as indicated by the non-monotonic shifts in metabolite abundance over time relative to the control (Fig. 5.4). Metabolite abundances in exposed worms were consistently lower than the control on day 7 and higher on day 14, especially in the 40-640 μg g^{-1} treatments (Fig. 5.4). Leucine and tyrosine were both elevated relative to the control on day 14 in worms exposed to 10 and 40 μg g^{-1}, while tyrosine was also elevated at 640 μg g^{-1}. Malic acid showed a significant decrease relative to the control on day 7 at all metformin concentrations, while succinic acid was elevated on day 14 at 10 and 40 μg g^{-1} treatments. Palmitic and margaric acid behaved similarly, with 40, 160, and 640 μg g^{-1} treatments decreasing relative to the control on day 7, and rebounded to levels higher than the control by day 14. Glucose levels decreased sharply on day 7, which was most pronounced at 160, and 640 μg g^{-1}. In most cases, metabolite decreases on day 7 rebounded to levels equal to or higher than the control by day 14. This may indicate that the dose of metformin received by the earthworm was reduced over time since it is readily degraded in soil (up to 80% loss after 14 days) under aerobic conditions (Mrozik and Stefańska 2014). We were not able to measure metformin concentration in the earthworms, since methods for analysis of metformin in tissues by GC-MS have only recently been published and are not yet standardized (Ucakturk 2013; Goedecke et al. 2017).

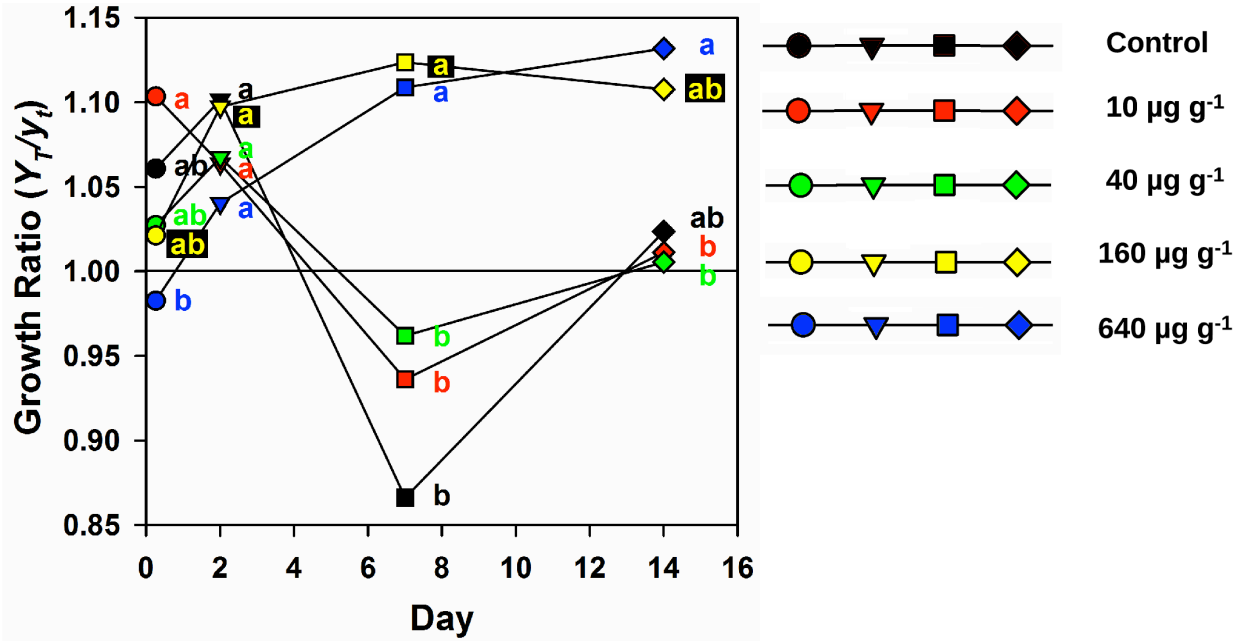

Figure 5.1. Earthworm weight change during the experiment as a percentage of the initial weight. Within each sampling time, concentrations with the same letter are not significantly different based on pairwise comparisons using LSMEANS (LSD, α=0.05).

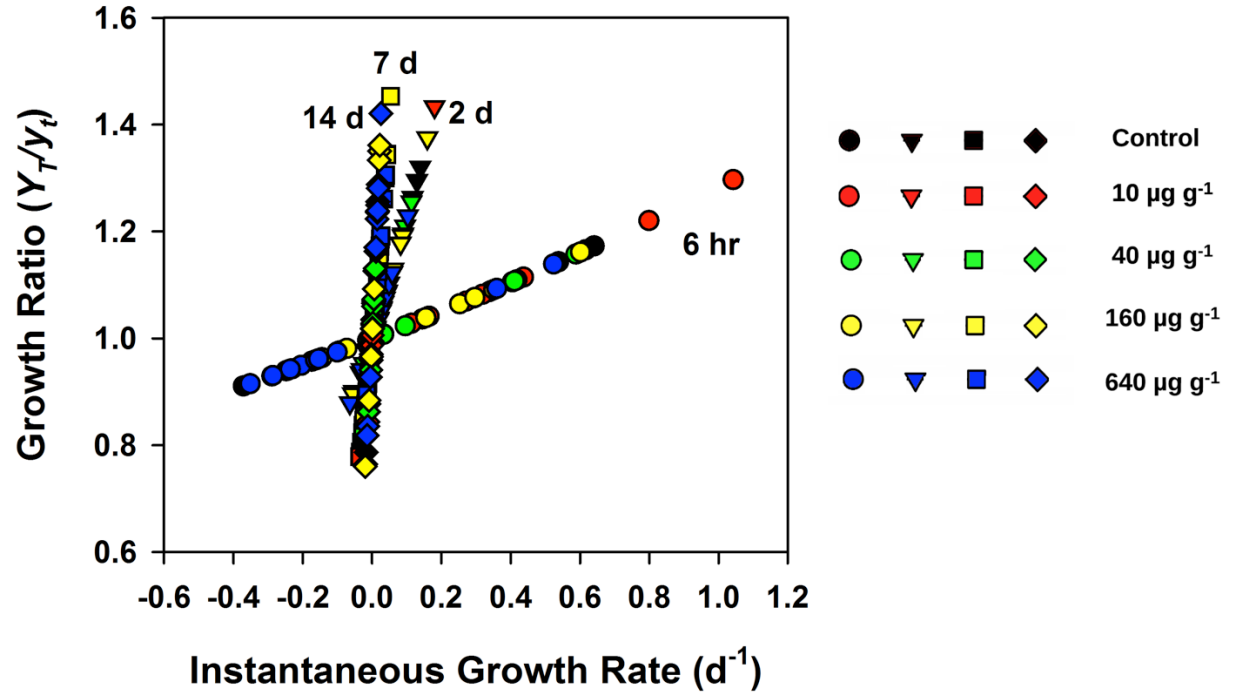

Figure 5.2. Effect of growth interval on the relationship between the instantaneous growth rate of earthworms versus the growth ratio. The relationship is strongly influenced by growth interval, so growth rates within each interval were examined separately.

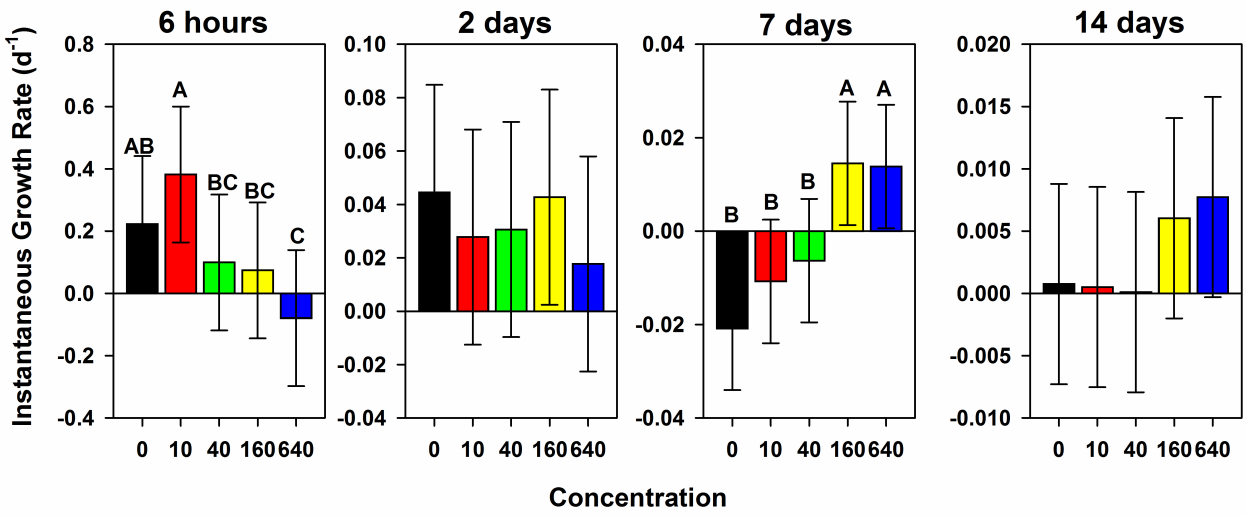

Figure 5.3. Earthworm instantaneous growth rates during the experiment, separated by growth interval. Within each growth interval, concentrations with the same letter are not significantly different based on pairwise comparisons using LSMEANS (LSD, α=0.05).

Table 5.2. P-values for metabolites and canonicals based on repeated measures ANOVA.

Significant effects based on $\alpha = 0.01$ are listed in bold.

Metabolite	conc	time	conc×time
Growth Ratio	0.0405	0.0329	**0.0014**
Valine	0.9267	0.0208	0.8218
Proline	0.9148	**0.0043**	0.9311
Leucine	0.3386	**<0.0001**	0.5165
Glycine	0.9877	0.1749	0.7104
Serine	0.9672	**0.0100**	0.7027
Lysine	0.8443	0.1732	0.1576
Tyrosine	0.3671	**<0.0001**	0.2581
Malic Acid	0.5409	0.0284	0.2358
Lactic Acid	0.9230	0.0334	0.6088
Succinic Acid	0.0682	**0.0010**	0.2016
Palmitic Acid	0.5294	0.0434	0.2468
Margaric Acid	0.4884	0.0222	0.2494
Glucose	0.1583	**<0.0001**	0.0326
Canonical 1	0.2468	**<0.0001**	0.6409
Canonical 2	0.1504	**0.0001**	0.0481

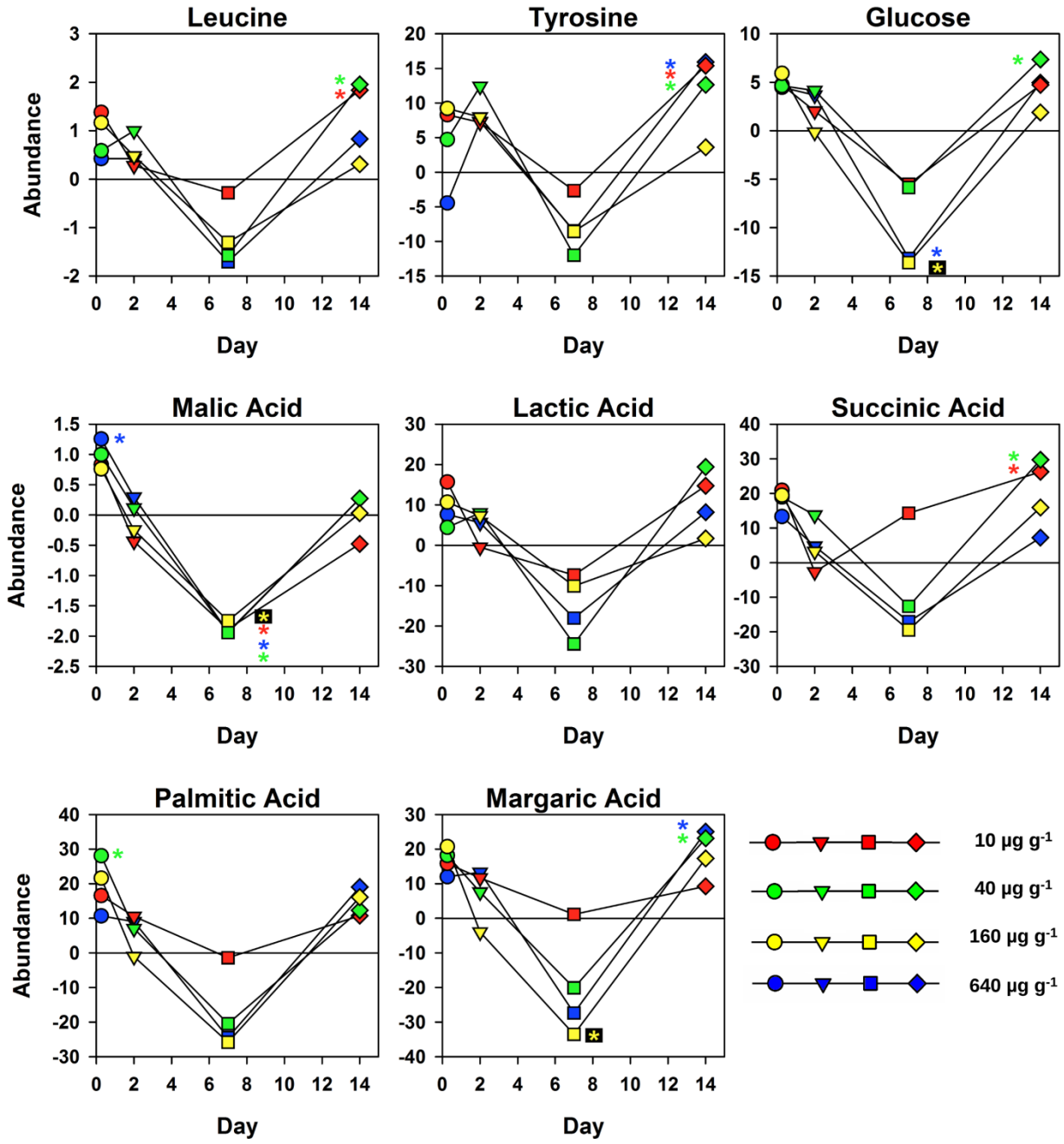

Figure 5.4. Changes over time in abundance (control subtracted) of significantly affected metabolites (α=0.01) in earthworms exposed to metformin. A * indicates a significant difference from the control at that time point based on pairwise comparisons using LSMEANS (LSD, α=0.05).

Table 5.3. Repeated measures ANOVA p-values for canonicals 1 and 2 sliced by concentration to test for differences between time points. Numbers in bold indicate p-value is ≤0.01.

Concentration (ng g^{-1})	Canonical 1	Canonical 2
0	**0.0086**	**<0.0001**
10	**0.0053**	0.1906
40	**<0.0001**	0.1229
160	**<0.0001**	0.8522
640	**0.0013**	0.5020

Table 5.4. Repeated measures ANOVA p-values for canonicals 1 and 2 sliced by time to test for differences between concentrations. Numbers in bold indicate p-value is ≤0.01.

Time (days)	Canonical 1	Canonical 2
0	0.2420	0.9942
2	0.3192	0.8938
7	0.6701	**0.0005**
14	0.6355	0.1691

Metformin is an inhibitor of mitochondrial complex I, reducing adenosine triphosphate (ATP) and increasing adenosine monophosphate (AMP) within the cell. This change in the cellular energy state causes the activation of 5'-AMP-activated protein kinase (AMPK), which coordinates a large network of biochemical pathways that sense and control the cellular energy state (Pernicova and Korbonits 2014). Metformin decreases hepatic gluconeogenesis (glucose generation from non-carbohydrate sources such as amino acids) through the activation of AMPK, which inhibits several transcription factors controlling the expression of gluconeogenic enzymes (Martin-Castillo et al. 2010; Jeon 2016). Activation of AMPK also stimulates glucose uptake and glycolysis in muscles, inhibits fatty acid synthesis, and increases the uptake and β-oxidation of fatty acids (Jeon 2016). We observed a decrease in glucose and margaric acid on day 7 in metformin exposed worms (Fig. 5.4), consistent with the mode of action of metformin.

Metformin toxicity can result from lactic acidosis due to an increase in lactate production combined with a reduced lactate metabolism or clearance of lactate from serum by gluconeogenesis (DeFronzo et al. 2016). Lactic acid levels did not differ from the control over the course of the experiment (Fig. 5.4), suggesting that toxicity due to lactic acidosis did not occur at the exposure levels tested. Malic acid levels showed a clear decrease on day 7 in exposed worms (Fig. 5.4). The TCA cycle is inhibited at several points by ATP and stimulated by ADP and AMP (Briggs 1995). This TCA cycle stimulation resulting from the ATP-lowering and ADP/AMP-increasing effects of metformin, combined with a reduced supply of fumarate from gluconeogenesis, could explain the depletion of malate relative to the control.

Discriminant analysis across all groups revealed at least one group differed from the others (Wilks' Lambda = 0.0143), so univariate ANOVA was used to investigate treatment effects within the first two canonicals (Table 5.3, Figs. 5.5 and 5.6). There was a clear trajectory over time in all groups, with the later time points generally further from the control in a negative direction within each concentration (Fig. 5.5). Glucose, lactic acid, and tyrosine had the largest standardized canonical coefficients along canonical 1, providing the largest contribution to group separation (Fig. 5.7). Glucose is the end product of gluconeogenesis (Wu 2013) which is reduced by metformin exposure, while tyrosine and lactic acid are glucogenic metabolites (Pernicova and Korbonits 2014). The multivariate trajectory (expressed as canonical scores) tracing the time course of a metabolic response to stress is often a sensitive indicator of chemical and environmental stressors in earthworms and other organisms (Malmendal et al. 2006; McKelvie et al. 2010; Lankadurai et al. 2011b). McKelvie et al. (2010) monitored the ^{1}H-NMR metabolite profile over time for earthworms exposed to sub-lethal phenanthrene in soil (250 μg g^{-1}) over 30 days and found that early and late time points could be clearly distinguished from each other

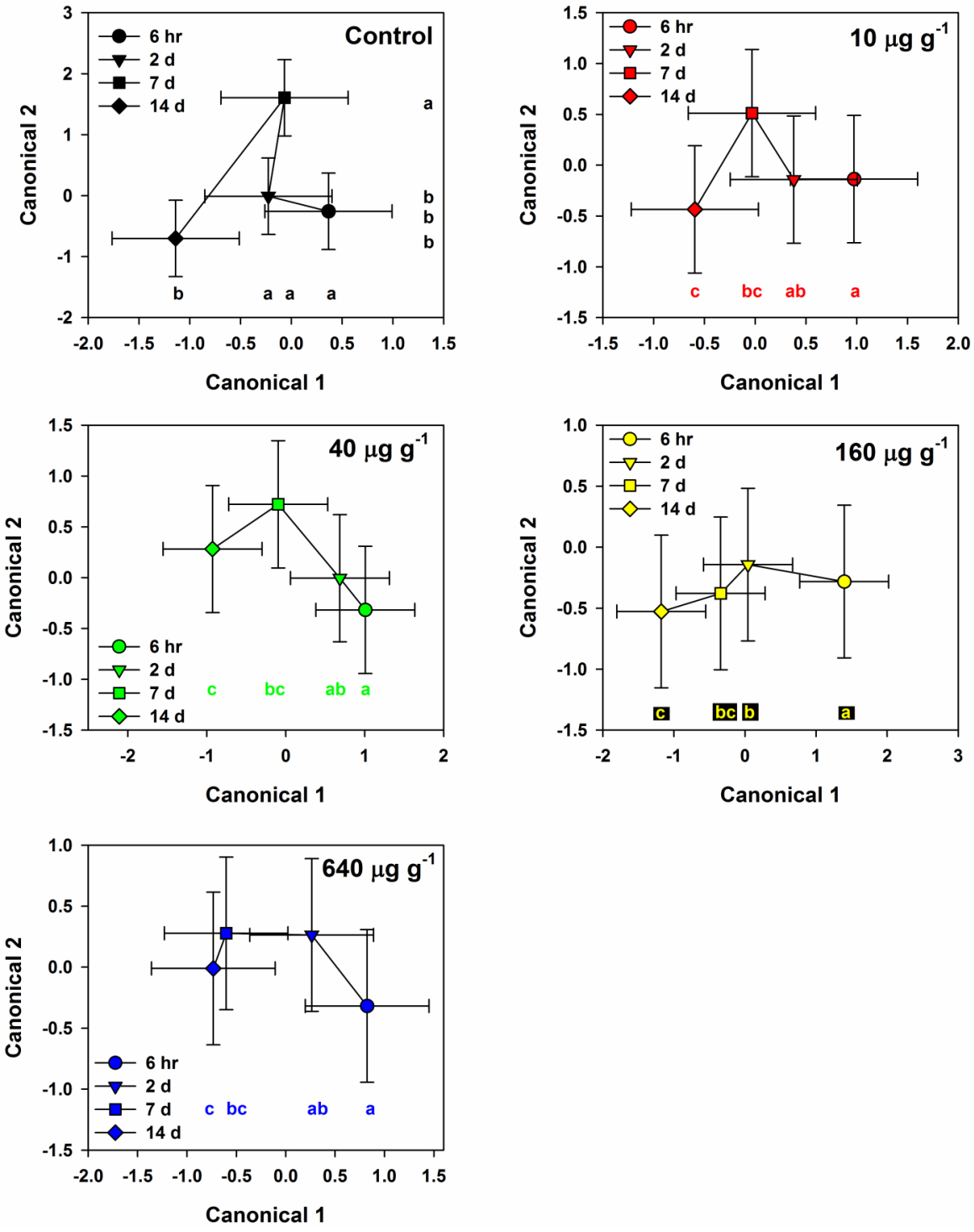

Figure 6.5. Discriminant analysis mean plots showing changes over time within each concentration. Error bars show 95% Confidence Interval of the canonical scores along canonical axis 1 and 2 at each sampling time. Letters along a canonical indicate a significant effect based on ANOVA (α=0.01). Time points with the same letter are not significantly different based on pairwise comparisons using LSMEANS (LSD, α=0.05).

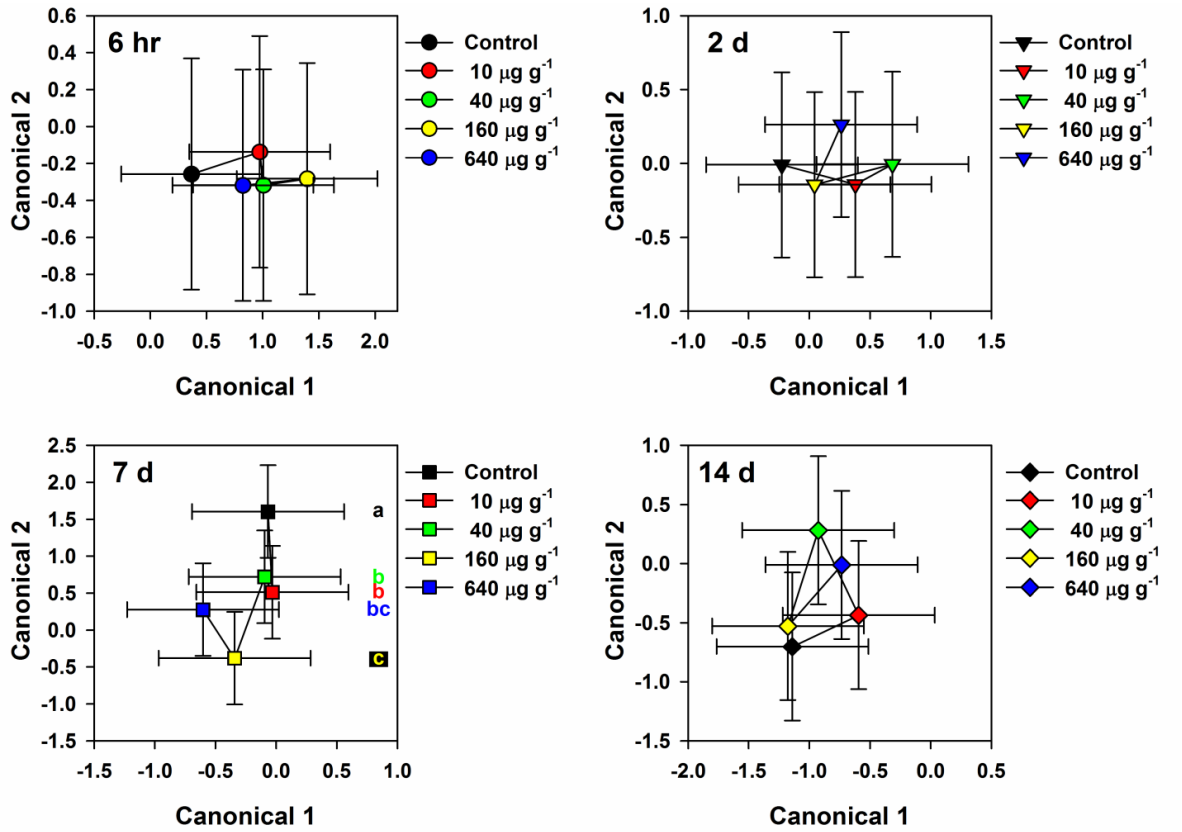

Figure 6.6. Discriminant analysis mean plots showing the effect of metformin concentration on discriminant scores for earthworms sampled over time. Error bars show 95% Confidence Interval of the canonical scores along canonical axis 1 and 2. Letters along a canonical indicate a significant effect at α=0.01. Time points with the same letter are not significantly different based on pairwise comparisons using LSMEANS (LSD, α=0.05).

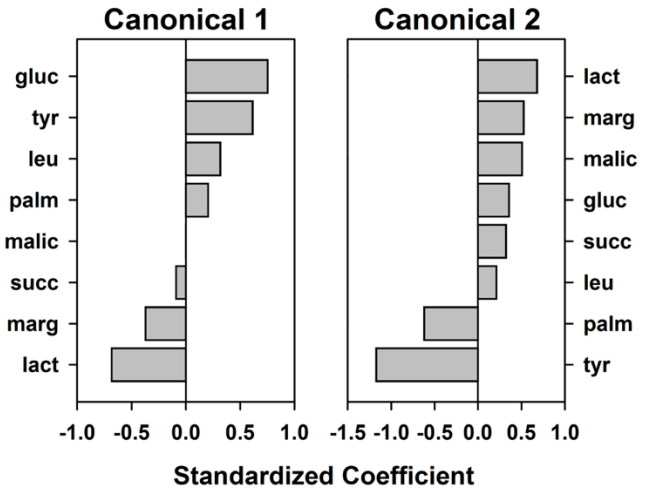

Figure 6.7. Standardized scoring coefficients for each variable in the first two canonicals from discriminant analysis.

using Principal Components Analysis (PCA). Lankadurai et al. (2011b) also found a strong dependence of the earthworm metabolite profile on the length of exposure (1-4 days) to phenanthrene (25 μg cm^{-2} on filter paper) using PCA. In their study, several metabolites fluctuated greatly within this time frame, shifting over time in their direction of change relative to control. Some metabolites had transient effects where an increase or decrease was only observed early in the exposure, while others required time to develop and differed from the control in later time points.

Since there was a strong effect of time on the metabolite profile, the dataset was sliced by time to determine if there were any concentration effects on each sampling day (Fig. 5.6). A significant difference between concentrations was only detected on day 7. There was separation from the control along canonical 2 at all exposure concentrations, showing a clear trajectory with higher concentrations generally further from the control in a negative direction (Fig. 5.6). Tyrosine, lactic acid, and palmitic acid had the largest standardized coefficients in canonical 2 (Fig. 5.7). The multivariate response takes more than 48 hours to emerge, suggesting a delayed onset of effects in earthworms and a period of acclimatization may be necessary for the system to reach a new equilibrium point. The response is also transient, and we suspect that the cause may be a reduced availability of metformin by the end of the experiment due to microbial degradation.

Metformin as low as 10 μg g^{-1} led to perturbations in some metabolites (leucine, tyrosine, malic acid, and succinic acid) and a significant difference from the control along canonical 2, suggesting that the equivalent of a human low therapeutic dose we estimated to be delivered by that treatment led to an observable effect. However, a significant reduction in glucose was only observed at concentrations of 160 μg g^{-1} and higher. The glucose-lowering mode of action of

metformin is believed to differ based on concentration (He and Wondisford 2015; Song 2016). At pharmacologic concentrations, metformin activates AMPK, but at high concentrations it inhibits complex 1 in mitochondria (He and Wondisford 2015). This may explain the low dose effects not observed at high doses. In addition, metformin has been shown to accumulate in the gut mucosa, influencing metabolism in gut microbiota and causing systemic effects on the organism, but this microbiome effect is poorly understood even in humans (Song 2016).

5.5. Conclusions

This study evaluated the effects of the antidiabetic drug and environmental contaminant metformin on the metabolite profile of the earthworm (*E. fetida*) using GC-MS metabolomics. Earthworms were exposed to metformin at 0, 10, 40, 160, 640 μg g^{-1} in worm bedding and measured at 6 h, 2 d, 7 d, and 14 d. No mortalities were observed during the experiment. After 6 hours, worms in the 640 μg g^{-1} group had the slowest growth rate which may reflect initial feeding avoidance. After 7 days, worms exposed to 160 and 640 μg g^{-1} grew significant faster than the remaining treatments, which could reflect an increased appetite due to lower blood glucose levels. Metabolites characteristic of the metformin mode of action in humans (reduction in gluconeogenesis, increased fatty acid oxidation, and reduced fatty acid synthesis) were affected by metformin exposure in the earthworm. On day 7, a decrease in glucose and malic acid was observed, pointing to a reduction in gluconeogenesis. Decreased palmitic and margaric acid on day 7 are indicative of a reduction in fatty acid synthesis and increased oxidation of fatty acids. Discriminant analysis (DA) revealed a consistent influence of time on the metabolite profile, but the only significant differences between concentrations occurred on day 7. The metabolites glucose, lactic acid, and tyrosine were large contributors to the separation between

treatments, which are all involved in gluconeogenesis. No evidence of metformin toxicity from lactic acidosis was observed at the tested concentrations. Reductions in metabolite levels on day 7 rebounded to equal or greater than the control on day 14, possibly resulting from metformin degradation over time. Overall, metformin led to physiological changes as measured by growth rate, and caused metabolic perturbations consistent with the mode of action of metformin in humans.

5.6. Acknowledgements

Financial support was provided by Canadian Water Network - Canadian Municipal Water Consortium grant (MW2013-2014), the NSERC Discovery Grant program, a Natural Science and Engineering Research Council (NSERC) Postgraduate Scholarship D (PGS D), the Harney Memorial Scholarship, and a McGill University Bioresource Engineering Department Graduate Excellence Award. Sources of funding had no influence on the study or decision to publish.

Preface to Chapter 6

This chapter summarizes the important lessons from Chapters 3, 4, and 5, with a discussion of the results in a broader sense than was provided in each chapter. Specific contributions to knowledge are listed, and it provides opportunities for improvement and recommendations for future work based in the information learned in this thesis.

Chapter 6: Contributions to knowledge and recommendations for future research

6.1. Hypotheses and outcomes

As a whole, the work tested four hypotheses, with the following outcomes:

(1) There will be metabolic effects in earthworms caused by exposure to these three contaminants, and that the effects will be concentration-dependent.

Triclosan: True using filter paper test, False using artificial soil

Methyltriclosan: True using artificial soil

Metformin: True using artificial soil

(2) The transformation product methyltriclosan will be more toxic than its parent compound triclosan.

True - Methyltriclosan caused metabolic effects at >64 times lower concentrations than triclosan, affecting growth rate, abundances of succinate and glucose, as well as multivariate representations of the data.

(3) A concentration dependent pattern in metabolite profiles will emerge as a function of time.

True – Using metformin, time had a strong effect on metabolite profile in control and exposed worms, significantly affecting multiple individual metabolites and multivariate

representations of the data. A concentration-dependent but transient response was observed after seven days.

(4) Significant changes in the metabolome will point towards a mode of action for the three contaminants tested.

Triclosan: False, only minor sub-lethal effects which did not point towards a mode of action

Methyltriclosan: True, effects were consistent with nonpolar narcosis affecting the activity of the membrane-bound succinate dehydrogenase enzyme

Metformin: True, effects were consistent with reduction of gluconeogenesis and increased oxidation of fatty acids

6.2. Contributions to knowledge

This thesis has generated one peer reviewed manuscript published in a high-quality journal (Chapter 3) and two chapters to be submitted (Chapters 4 and 5). Chapter 3 was published in the Journal of Hazardous Materials under a special issue titled "Special Issue on Emerging Contaminants in Engineered and Natural Environments". The study established a LC_{50} for TCS based on a 2-day filter paper contact test, which can be used to assess TCS toxicity relative to other substances. The study analyzed both the living and dead earthworms, identifying significant metabolic effects in survivors and decomposition products in dead worms. The analysis of decomposition products in dead worms was novel and may aid in the interpretation of future toxicology studies using metabolomics where there are fatal toxic effects.

Chapters 4 and 5 describe two different 14-day exposure toxicity experiments using earthworm bedding, to better represent the natural habitat of the earthworm species (*Eisenia fetida*) used in the experiments. Chapter 4 compares TCS to its environmental transformation product MTCS, and shows that after 14 days, the methylated product is toxic at much lower concentrations (1 μg g^{-1} for MTCS vs. >64 μg g^{-1} for TCS). No effects were detected for TCS. This is a significant finding since contaminant transformation products are often neglected in environmental monitoring and risk assessment, but are potentially more persistent and toxic than the parent compound. Based on the metabolites affected by MTCS, we hypothesized that the mode of action is related to MTCS accumulation in membranes of the mitochondria, affecting the function of succinate dehydrogenase, a membrane-bound enzyme in the TCA cycle. This provides a testable hypothesis, which will provide a better understanding of the mode of action with potential broader applications to methylated transformation products in general.

Chapter 5 describes the shifting metabolite profile in response to MET exposure across the range from estimated therapeutic dose to maximum safe dose over a 14-day period. Time played a significant role in individual metabolite levels and on the discriminant scores in canonicals 1 and 2. A significant concentration effect in the discriminant scores was only observed on day 7. This indicates that the effect has a slow onset, and is transient, which may reflect sorption or degradation processes reducing bioavailable MET. Metformin caused a reduction in glucose, malic acid, and margaric acid, which are characteristic of the MET mode of action in humans, including reduced gluconeogenesis, reduced fatty acid synthesis, and increased fatty acid oxidation caused by activation of the enzyme AMPK. This suggests the mode of action is conserved between humans and earthworms.

The major contributions from this work to the field of environmental metabolomics also include strengthening its position as an emerging tool in toxicology. The added depth of information allowed toxic effects to be investigated at the molecular level, and provided insights into the mode of action of several emerging contaminants. This work also puts a small but meaningful dent in the necessary work to assess the environmental toxicity of the more than 100,000 synthetic chemicals in use worldwide (Timbrell 2008). This work falls on researchers who must find novel ways to measure these substances, determine the extent of their occurrence and eventual fate in the environment, and establish if their presence poses a risk to the health of environmental organisms. The experiments in this thesis provide effect concentrations that can be used in environmental risk assessments for these substances in the soil compartment.

The work also had a number of limitations that need to be considered, which provide opportunities for improvement in the future. The targeted approach that was designed to provide confidence in identification and quantification came at the cost of a drastic reduction in the scope of work compared to what is achievable using the GC-MS platform. As can be seen in the chromatograms in Chapter 4, only a small fraction of the peaks in each sample were included here, and there is additional information to be gleaned from these datasets. Another factor that limits the general applicability of the work is that only a single bedding type was tested. While using a soil medium is an improvement over filter paper tests, the soil medium is known to influence the earthworm metabolic response to toxins, especially variations in soil organic matter. It would have been useful to also test a low organic matter soil such as the 80% sand OECD soil, which may have allowed triclosan to have an effect. The drawback is a doubling in the number of samples, which can quickly become unmanageable when sample processing and analysis times are considered. Dropping the number of tested concentrations to 2 plus a control

would reduce sample requirements. This work also suffered from a change in methods which prevented a direct comparison of the effects of triclosan in filter paper and soil between Chapters 3 and 4. This reduces the continuity of the thesis in some respects, but in response the scope of metabolites was increased. Furthermore, running the samples from Chapters 4 and 5 with a standardized method associated with mass spectral libraries enhanced the amount of additional information that can potentially be gained from these datasets in the future.

6.3. Recommendations for future research

Metabolomics data is most useful when placed in the context of known biochemical pathways to interpret the biological significance of the results. In targeted metabolomics, the choice of metabolites to include is a critical decision that limits the interpretation of treatment effects to this selection. However, when the expected effects are not known, it is difficult to predict which metabolites should be included. In this thesis, a shotgun approach was applied to all experiments, where metabolites were included that represent different classes of biomolecules and which have been responsive to toxic exposure in the past, but it lacked comprehensive coverage of whole biochemical pathways (e.g. TCA cycle metabolites). Future work may benefit from a more focused selection of metabolites, including more compounds within specific biochemical pathways. This information would provide corroborating evidence for an effect based on the results from upstream and downstream metabolites if they can be measured.

Even within the datasets generated in this thesis there exists additional information in the unidentified peaks that were not analyzed and interpreted within each run. There is an opportunity for additional work extracting unknown peaks from the chromatograms in each experiment, and annotating the peaks where possible with a metabolite identification based on

mass spectral library searches. This type of work is called non-targeted metabolomics where both known and unknown metabolites (identified and unidentified peaks) are used to distinguish between two or more groups of samples using multivariate analysis and visualization techniques. The automated peak extraction and mass spectral library querying necessary to process hundreds of peaks in the >360 experimental samples included in this thesis is outside of my current skillset, but it will provide a good opportunity to collaborate in the future with other scientists knowledgeable in this area. I have manually searched through the chromatographic peaks and queried the library with their mass spectrum, yielding some high percent matches, so I expect there will be new discoveries revealed if a deeper view of the data can be obtained.

There is an overall need within the field of environmental metabolomics using earthworms as the test species to generate baseline datasets and establish 'normal' ranges for metabolites across varying environmental properties such as temperature, moisture, soil organic matter, pH, conductivity, as well as earthworm species, age, developmental stage, and diet. Understanding the normal stress response in earthworms as reflected in the metabolome will improve interpretation of the kind of experiments presented in this thesis and summarized in Table 1.1. Understanding the normal variability in metabolite levels across many samples may provide context when discussing the size of a significant effect and whether the result is truly outside the expected range of observations. Ultimately, if the knowledge base for earthworms becomes comparable to medical or veterinary science, we will be able to diagnose certain conditions in earthworms based on their growth rates and metabolite biomarkers. This may require tissue or biofluid-specific metabolome analysis rather than the whole-organism extracts typical of experiments in this field.

References

Abernethyand, S. G., MacKay, D., & McCarty, L. S. (1988). "Volume fraction" correlation for narcosis in aquatic organisms: The key role of partitioning. *Environmental Toxicology and Chemistry, 7*(6), 469-481, doi:10.1002/etc.5620070607.

Ajao, C., Andersson, M. A., Teplova, V. V., Nagy, S., Gahmberg, C. G., Andersson, L. C., et al. (2015). Mitochondrial toxicity of triclosan on mammalian cells. *Toxicology Reports, 2*, 624-637, doi:http://dx.doi.org/10.1016/j.toxrep.2015.03.012.

Alexander, M. (2000). Aging, bioavailability, and overestimation of risk from environmental pollutants. *Environmental Science & Technology, 34*(20), 4259-4265.

Aliferis, K. A., & Jabaji, S. (2011). Metabolomics–A robust bioanalytical approach for the discovery of the modes-of-action of pesticides: A review. *Pesticide Biochemistry and Physiology, 100*(2), 105-117.

Alvarez, M. d. C., Donarski, J. A., Elliott, M., & Charlton, A. J. (2010). Evaluation of extraction methods for use with NMR-based metabolomics in the marine polychaete ragworm, Hediste diversicolor. [journal article]. *Metabolomics, 6*(4), 541-549, doi:10.1007/s11306-010-0222-y.

Arnold, K. E., Brown, A. R., Ankley, G. T., & Sumpter, J. P. (2014). Medicating the environment: assessing risks of pharmaceuticals to wildlife and ecosystems. *Philosophical Transactions of the Royal Society of London B: Biological Sciences, 369*(1656), 20130569.

Ashrap, P., Zheng, G., Wan, Y., Li, T., Hu, W., Li, W., et al. (2017). Discovery of a widespread metabolic pathway within and among phenolic xenobiotics. *Proceedings of the National Academy of Sciences, 114*(23), 6062-6067, doi:10.1073/pnas.1700558114.

Bates, D., & Watts, D. (1988). *Nonlinear regression analysis and its applications*. New York, USA: John Wiley and Sons.

Baylay, A., Spurgeon, D., Svendsen, C., Griffin, J., Swain, S. C., Sturzenbaum, S. R., et al. (2012). A metabolomics based test of independent action and concentration addition using the earthworm Lumbricus rubellus. *Ecotoxicology, 21*(5), 1436-1447.

Bedoux, G., Roig, B., Thomas, O., Dupont, V., & Le Bot, B. (2012). Occurrence and toxicity of antimicrobial triclosan and by-products in the environment. *Environmental Science and Pollution Research, 19*(4), 1044-1065.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological), 57*(1), 289-300.

Berkowitz, B., Dror, I., & Yaron, B. (2014). *Contaminant Geochemistry: Interactions and Transport in the Subsurface Environment*: Springer Berlin Heidelberg.

Bijlsma, S., Bobeldijk, I., Verheij, E. R., Ramaker, R., Kochhar, S., Macdonald, I. A., et al. (2006). Large-Scale Human Metabolomics Studies: A Strategy for Data (Pre-) Processing and Validation. *Analytical Chemistry, 78*(2), 567-574, doi:10.1021/ac051495j.

Blair, B. D., Crago, J. P., Hedman, C. J., & Klaper, R. D. (2013). Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. *Chemosphere, 93*(9), 2116-2123, doi:http://dx.doi.org/10.1016/j.chemosphere.2013.07.057.

Bradley, P. M., Journey, C. A., Button, D. T., Carlisle, D. M., Clark, J. M., Mahler, B. J., et al. (2016). Metformin and Other Pharmaceuticals Widespread in Wadeable Streams of the

Southeastern United States. *Environmental Science & Technology Letters, 3*(6), 243-249, doi:10.1021/acs.estlett.6b00170.

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., et al. (2001). Minimum information about a microarray experiment (MIAME)[mdash]toward standards for microarray data. [10.1038/ng1201-365]. *Nature Genetics, 29*(4), 365-371, doi:http://www.nature.com/ng/journal/v29/n4/suppinfo/ng1201-365_S1.html.

Briggs, T. (1995). Energetics and Biological Oxidation. In T. Briggs, & A. M. Chandler (Eds.), *Biochemistry* (pp. 61-79). New York, NY: Springer New York.

Bright, D. A., & Healey, N. (2003). Contaminant risks from biosolids land application. *Environmental Pollution, 126*(1), 39-49, doi:http://dx.doi.org/10.1016/S0269-7491(03)00148-9.

Briones, R. M., Sarmah, A. K., & Padhye, L. P. (2016). A global perspective on the use, occurrence, fate and effects of anti-diabetic drug metformin in natural and engineered ecosystems. *Environmental Pollution, 219*, 1007-1020, doi:http://dx.doi.org/10.1016/j.envpol.2016.07.040.

Broadhurst, D. I., & Kell, D. B. (2006). Statistical strategies for avoiding false discoveries in metabolomics and related experiments. *Metabolomics, 2*(4), 171-196, doi:10.1007/s11306-006-0037-z.

Brown, S. A., McKelvie, J. R., Simpson, A. J., & Simpson, M. J. (2010). 1H NMR metabolomics of earthworm exposure to sub-lethal concentrations of phenanthrene in soil. *Environmental Pollution, 158*(6), 2117-2123.

Brown, S. A., Simpson, A. J., & Simpson, M. J. (2008). Evaluation of sample preparation methods for nuclear magnetic resonance metabolic profiling studies with Eisenia fetida. *Environmental Toxicology and Chemistry, 27*(4), 828-836.

Brown, S. A., Simpson, A. J., & Simpson, M. J. (2009). 1H NMR metabolomics of earthworm responses to sub-lethal PAH exposure. *Environmental Chemistry, 6*(5), 432-440.

A rosier future for Montreal's sludge? (2015, December 18, 2015). *Montreal Gazette*.

Bundy, J., Sidhu, J., Rana, F., Spurgeon, D., Svendsen, C., Wren, J., et al. (2008). 'Systems toxicology' approach identifies coordinated metabolic responses to copper in a terrestrial non-model invertebrate, the earthworm Lumbricus rubellus. *BMC Biology, 6*(1), 25.

Bundy, J. G., Davey, M. P., & Viant, M. R. (2009). Environmental metabolomics: a critical review and future perspectives. *Metabolomics, 5*(1), 3-21, doi:10.1007/s11306-008-0152-0.

Bundy, J. G., Keun, H. C., Sidhu, J. K., Spurgeon, D. J., Svendsen, C., Kille, P., et al. (2007). Metabolic Profile Biomarkers of Metal Contamination in a Sentinel Terrestrial Species Are Applicable Across Multiple Sites. *Environmental Science & Technology, 41*(12), 4458-4464, doi:10.1021/es0700303.

Bundy, J. G., Lenz, E. M., Bailey, N. J., Gavaghan, C. L., Svendsen, C., Spurgeon, D., et al. (2002). Metabonomic assessment of toxicity of 4-fluoroaniline, 3,5-difluoroaniline and 2-fluoro-4-methylaniline to the earthworm Eisenia veneta (rosa): Identification of new endogenous biomarkers. *Environmental Toxicology and Chemistry, 21*(9), 1966-1972, doi:10.1002/etc.5620210926.

Bundy, J. G., Osborn, D., Weeks, J. M., Lindon, J. C., & Nicholson, J. K. (2001). An NMR-based metabonomic approach to the investigation of coelomic fluid biochemistry in earthworms under toxic stress. *FEBS Letters, 500*(1), 31-35, doi:10.1016/S0014-5793(01)02582-0.

Bundy, J. G., Spurgeon, D. J., Svendsen, C., Hankard, P. K., Weeks, J. M., Osborn, D., et al. (2004). Environmental Metabonomics: Applying Combination Biomarker Analysis in Earthworms at a Metal Contaminated Site. *Ecotoxicology, 13*(8), 797-806, doi:10.1007/s10646-003-4477-1.

Butler, E., Whelan, M. J., Sakrabani, R., & van Egmond, R. (2012). Fate of triclosan in field soils receiving sewage sludge. *Environmental Pollution, 167*, 101-109, doi:http://dx.doi.org/10.1016/j.envpol.2012.03.036.

Butler-Jones, D. (2011). Diabetes in Canada: Facts and figures from a public health perspective. In P. H. A. o. Canada (Ed.), (pp. 126): Government of Canada.

Bylesjö, M. (2015). Extracting Meaningful Information from Metabonomic Data Using Multivariate Statistics. In J. T. Bjerrum (Ed.), *Metabonomics: Methods and Protocols* (pp. 137-146). New York, NY: Springer New York.

Cabreiro, F., Au, C., Leung, K.-Y., Vergara-Irigaray, N., Cochemé, Helena M., Noori, T., et al. (2013). Metformin Retards Aging in C. elegans by Altering Microbial Folate and Methionine Metabolism. *Cell, 153*(1), 228-239, doi:https://doi.org/10.1016/j.cell.2013.02.035.

Calow, P., & Forbes, V. E. (2003). Peer Reviewed: Does Ecotoxicology Inform Ecological Risk Assessment? *Environmental Science & Technology, 37*(7), 146A-151A, doi:10.1021/es0324003.

Camacho, D., de la Fuente, A., & Mendes, P. (2005). The origin of correlations in metabolomics data. *Metabolomics, 1*(1), 53-63, doi:10.1007/s11306-005-1107-3.

Canadian Water and Wastewater Association (2012). FAQ - Biosolids. http://www.cwwa.ca/faqbiosolids_e.asp. Accessed October 18 2016.

Carter, L. J., Garman, C. D., Ryan, J., Dowle, A., Bergström, E., Thomas-Oates, J., et al. (2014). Fate and Uptake of Pharmaceuticals in Soil–Earthworm Systems. *Environmental Science & Technology, 48*(10), 5955-5963, doi:10.1021/es500567w.

CCME (2010). A Review of the Current Canadian Legislative Framework for Wastewater Biosolids. Canadian Council of Ministers of the Environment.

CCME (2012). Canada-wide approach for the management of wastewater biosolids. Canadian Council of Ministers of the Environment.

Ch, R., Singh, A. K., Pandey, P., Saxena, P. N., & Reddy Mudiam, M. K. (2015). Identifying the metabolic perturbations in earthworm induced by cypermethrin using gas chromatography-mass spectrometry based metabolomics. [Article]. *Scientific Reports, 5*, 15674, doi:10.1038/srep15674.

Chalew, T. E., & Halden, R. U. (2009a). Environmental Exposure of Aquatic and Terrestrial Biota to Triclosan and Triclocarban. *Journal - American Water Works Association, 45*(1), 4-13, doi:10.1111/j.1752-1688.2008.00284.x.

Chalew, T. E., & Halden, R. U. (2009b). Environmental exposure of aquatic and terrestrial biota to triclosan and triclocarban. *JAWRA Journal of the American Water Resources Association, 45*(1), 4-13.

Chefetz, B. (2003). Sorption of phenanthrene and atrazine by plant cuticular fractions. *Environmental Toxicology and Chemistry, 22*(10), 2492-2498, doi:10.1897/02-461.

Chefetz, B., Mualem, T., & Ben-Ari, J. (2008). Sorption and mobility of pharmaceutical compounds in soil irrigated with reclaimed wastewater. *Chemosphere, 73*(8), 1335-1343, doi:http://dx.doi.org/10.1016/j.chemosphere.2008.06.070.

Chen, J., Zhang, P., Lv, M., Guo, H., Huang, Y., Zhang, Z., et al. (2017). Influences of Normalization Method on Biomarker Discovery in Gas Chromatography–Mass Spectrometry-Based Untargeted Metabolomics: What Should Be Considered? *Analytical Chemistry, 89*(10), 5342-5348, doi:10.1021/acs.analchem.6b05152.

Chen, L., Wang, Z., Jing, Z., Wang, Z., Cao, S., & Yu, T. (2015). Accumulation and Risk of Triclosan in Surface Sediments Near the Outfalls of Municipal Wastewater Treatment Plants. *Bulletin of Environmental Contamination and Toxicology, 95*(4), 525-529, doi:10.1007/s00128-015-1630-5.

Clarke, B. O., & Smith, S. R. (2011). Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. *Environment international, 37*(1), 226-247, doi:http://dx.doi.org/10.1016/j.envint.2010.06.004.

Clarke, R. M., & Cummins, E. (2015). Evaluation of "Classic" and Emerging Contaminants Resulting from the Application of Biosolids to Agricultural Lands: A Review. *Human and Ecological Risk Assessment: An International Journal, 21*(2), 492-513.

Clasquin, M. F., Melamud, E., & Rabinowitz, J. D. (2002). LC-MS Data Processing with MAVEN: A Metabolomic Analysis and Visualization Engine. In *Current Protocols in Bioinformatics*: John Wiley & Sons, Inc.

Cleuvers, M. (2003). Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. *Toxicology Letters, 142*(3), 185-194, doi:http://dx.doi.org/10.1016/S0378-4274(03)00068-7.

Connor, S. C., Wu, W., Sweatman, B. C., Manini, J., Haselden, J. N., Crowther, D. J., et al. (2004). Effects of feeding and body weight loss on the 1H-NMR-based urine metabolic profiles of male Wistar Han Rats: Implications for biomarker discovery. *Biomarkers, 9*(2), 156-179, doi:10.1080/13547500410001720767.

Costa, I. R., Thompson, J. D., Ortega, J. M., & Prosdocimi, F. (2015). Metazoan Remaining Genes for Essential Amino Acid Biosynthesis: Sequence Conservation and Evolutionary Analyses. *Nutrients, 7*(1), 1-16, doi:10.3390/nu7010001.

Crago, J., Bui, C., Grewal, S., & Schlenk, D. (2016). Age-dependent effects in fathead minnows from the anti-diabetic drug metformin. *General and Comparative Endocrinology, 232*, 185-190, doi:http://dx.doi.org/10.1016/j.ygcen.2015.12.030.

Cunningham, V. L. (2008). Special Characteristics of Pharmaceuticals Related to Environmental Fate. In K. Kümmerer (Ed.), *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks* (pp. 23-34). Berlin, Heidelberg: Springer Berlin Heidelberg.

Dani, V. D., Simpson, A. J., & Simpson, M. J. (2018). Analysis of earthworm sublethal toxic responses to atrazine exposure using 1H nuclear magnetic resonance (NMR)-based metabolomics. *Environmental Toxicology and Chemistry, 37*(2), 473-480, doi:doi:10.1002/etc.3978.

Dann, A. B., & Hontela, A. (2011). Triclosan: environmental exposure, toxicity and mechanisms of action. *Journal of Applied Toxicology, 31*(4), 285-311, doi:10.1002/jat.1660.

De Livera, A. M., Dias, D. A., De Souza, D., Rupasinghe, T., Pyke, J., Tull, D., et al. (2012). Normalizing and Integrating Metabolomics Data. *Analytical Chemistry, 84*(24), 10768-10776, doi:10.1021/ac302748b.

de Souza Silva, F. M., Rocha Alves da Silva, M. H., Bracht, A., Eller, G. J., Constantin, R. P., & Yamamoto, N. S. (2010). Effects of metformin on glucose metabolism of perfused rat

livers. [journal article]. *Molecular and Cellular Biochemistry, 340*(1), 283-289, doi:10.1007/s11010-010-0429-2.
Dean, J., & Scott, W. (2004). Recent developments in assessing the bioavailability of persistent organic pollutants in the environment. *TrAC Trends in Analytical Chemistry, 23*(9), 609-618.
DeFronzo, R., Fleming, G. A., Chen, K., & Bicsak, T. A. (2016). Metformin-associated lactic acidosis: Current perspectives on causes and risk. *Metabolism, 65*(2), 20-29, doi:http://dx.doi.org/10.1016/j.metabol.2015.10.014.
Dettmer, K., Aronov, P. A., & Hammock, B. D. (2007). MASS SPECTROMETRY-BASED METABOLOMICS. *Mass spectrometry reviews, 26*(1), 51-78, doi:10.1002/mas.20108.
Draisma, H. H. M., Reijmers, T. H., van der Kloet, F., Bobeldijk-Pastorova, I., Spies-Faber, E., Vogels, J. T. W. E., et al. (2010). Equating, or Correction for Between-Block Effects with Application to Body Fluid LC−MS and NMR Metabolomics Data Sets. *Analytical Chemistry, 82*(3), 1039-1046, doi:10.1021/ac902346a.
Dunn, W. B., Broadhurst, D., Begley, P., Zelena, E., Francis-McIntyre, S., Anderson, N., et al. (2011). Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. [10.1038/nprot.2011.335]. *Nature Protocols, 6*(7), 1060-1083, doi:http://www.nature.com/nprot/journal/v6/n7/abs/nprot.2011.335.html#supplementary-information.
Dunn, W. B., Erban, A., Weber, R. J., Creek, D. J., Brown, M., Breitling, R., et al. (2013). Mass appeal: metabolite identification in mass spectrometry-focused untargeted metabolomics. *Metabolomics, 9*(1 Supplement), 44-66.
Duportet, X., Aggio, R. B. M., Carneiro, S., & Villas-Bôas, S. G. (2012). The biological interpretation of metabolomic data can be misled by the extraction method used. [journal article]. *Metabolomics, 8*(3), 410-421, doi:10.1007/s11306-011-0324-1.
Eggen, T., Asp, T. N., Grave, K., & Hormazabal, V. (2011). Uptake and translocation of metformin, ciprofloxacin and narasin in forage- and crop plants. *Chemosphere, 85*(1), 26-33, doi:http://dx.doi.org/10.1016/j.chemosphere.2011.06.041.
Eggen, T., & Lillo, C. (2012). Antidiabetic II Drug Metformin in Plants: Uptake and Translocation to Edible Parts of Cereals, Oily Seeds, Beans, Tomato, Squash, Carrots, and Potatoes. *Journal of Agricultural and Food Chemistry, 60*(28), 6929-6935, doi:10.1021/jf301267c.
Environment Canada (2012). Preliminary Assessment: Triclosan. Available at: http://www.ec.gc.ca/ese-ees/6EF68BEC-5620-4435-8729-9B91C57A9FD2/Triclosan_EN.pdf [Accessed October 16 2016].
Epel, D., Luckenbach, T., Stevenson, C. N., MacManus-Spencer, L. A., Hamdoun, A., Smital, et al. (2008). Efflux Transporters: Newly Appreciated Roles in Protection against Pollutants. *Environmental Science & Technology, 42*(11), 3914-3920, doi:10.1021/es087187v.
Escher, B. I., Ashauer, R., Dyer, S., Hermens, J. L. M., Lee, J.-H., Leslie, H. A., et al. (2011). Crucial role of mechanisms and modes of toxic action for understanding tissue residue toxicity and internal effect concentrations of organic chemicals. *Integrated Environmental Assessment and Management, 7*(1), 28-49, doi:10.1002/ieam.100.

Escher, B. I., & Hermens, J. L. M. (2002). Modes of Action in Ecotoxicology: Their Role in Body Burdens, Species Sensitivity, QSARs, and Mixture Effects. *Environmental Science & Technology, 36*(20), 4201-4217, doi:10.1021/es015848h.

European Commission (2003). Technical Guidance Document on Risk Assessment. In J. R. C. European Commission (Ed.).

Fiehn, O., Robertson, D., Griffin, J., van der Werf, M., Nikolau, B., Morrison, N., et al. (2007). The metabolomics standards initiative (MSI). *Metabolomics, 3*(3), 175-178.

Field, D., Garrity, G., Gray, T., Morrison, N., Selengut, J., Sterk, P., et al. (2008). The minimum information about a genome sequence (MIGS) specification. [10.1038/nbt1360]. *Nature Biotechnology, 26*(5), 541-547, doi:http://www.nature.com/nbt/journal/v26/n5/suppinfo/nbt1360_S1.html.

Fiss, E. M., Rule, K. L., & Vikesland, P. J. (2007). Formation of Chloroform and Other Chlorinated Byproducts by Chlorination of Triclosan-Containing Antibacterial Products. *Environmental Science & Technology, 41*(7), 2387-2394, doi:10.1021/es062227l.

Fuchsman, P., Lyndall, J., Bock, M., Lauren, D., Barber, T., Leigh, K., et al. (2010). Terrestrial ecological risk evaluation for triclosan in land-applied biosolids. *Integrated Environmental Assessment and Management, 6*(3), 405-418, doi:10.1897/IEAM_2009-071.1.

Gago-Ferrero, P., Borova, V., Dasenaki, M. E., & Thomaidis, N. S. (2015). Simultaneous determination of 148 pharmaceuticals and illicit drugs in sewage sludge based on ultrasound-assisted extraction and liquid chromatography–tandem mass spectrometry. [journal article]. *Analytical and Bioanalytical Chemistry, 407*(15), 4287-4297, doi:10.1007/s00216-015-8540-6.

García-Santiago, X., Franco-Uría, A., Omil, F., & Lema, J. M. (2016). Risk assessment of persistent pharmaceuticals in biosolids: Dealing with uncertainty. *Journal of Hazardous Materials, 302*, 72-81.

Gibb, J. O. T., Svendsen, C., Weeks, J. M., & Nicholson, J. K. (1997). 1H NMR spectroscopic investigations of tissue metabolite biomarker response to Cu II exposure in terrestrial invertebrates: identification of free histidine as a novel biomarker of exposure to copper in earthworms. *Biomarkers, 2*(5), 295-302, doi:10.1080/135475097231526.

Gika, H. G., Macpherson, E., Theodoridis, G. A., & Wilson, I. D. (2008). Evaluation of the repeatability of ultra-performance liquid chromatography–TOF-MS for global metabolic profiling of human urine samples. *Journal of Chromatography B, 871*(2), 299-305, doi:http://dx.doi.org/10.1016/j.jchromb.2008.05.048.

Gillis, J. D. (2011). *Monitoring Organic Contaminant Concentrations and Carbon Mineralization in Field Soils Receiving Alkaline-Stabilized Biosolids*. Dalhousie University, Halifax, N.S.

Gillis, J. D., & Price, G. W. (2016). Linking short-term soil carbon and nitrogen dynamics: Environmental and stoichiometric controls on fresh organic matter decomposition in agroecosystems. *Geoderma, 274*, 35-44, doi:http://dx.doi.org/10.1016/j.geoderma.2016.03.026.

Gillis, J. D., Price, G. W., & Prasher, S. (2017). Lethal and sub-lethal effects of triclosan toxicity to the earthworm Eisenia fetida assessed through GC–MS metabolomics. *Journal of Hazardous Materials, 323, Part A*, 203-211, doi:http://dx.doi.org/10.1016/j.jhazmat.2016.07.022.

Gillis, J. D., Price, G. W., & Stratton, G. W. (2014). Detection and degradation of organic contaminants in an agricultural soil amended with alkaline-treated biosolids. *Canadian Journal of Soil Science, 94*(5), 595-604, doi:10.4141/cjss2013-123.

Givaudan, N., Wiegand, C., Le Bot, B., Renault, D., Pallois, F., Llopis, S., et al. (2014). Acclimation of earthworms to chemicals in anthropogenic landscapes, physiological mechanisms and soil ecological implications. *Soil Biology and Biochemistry, 73*, 49-58.

Glickman, M. E., Rao, S. R., & Schultz, M. R. (2014). False discovery rate control is a recommended alternative to Bonferroni-type adjustments in health studies. *Journal of Clinical Epidemiology, 67*(8), 850-857, doi:10.1016/j.jclinepi.2014.03.012.

Goedecke, C., Fettig, I., Piechotta, C., Philipp, R., & Geissen, S. U. (2017). A novel GC-MS method for the determination and quantification of metformin in surface water. [10.1039/C6AY02606K]. *Analytical Methods, 9*(10), 1580-1584, doi:10.1039/C6AY02606K.

Gong, L., Goswami, S., Giacomini, K. M., Altman, R. B., & Klein, T. E. (2012). Metformin pathways: pharmacokinetics and pharmacodynamics. *Pharmacogenetics and genomics, 22*(11), 820-827, doi:10.1097/FPC.0b013e3283559b22.

Goodacre, R. (2007). Metabolomics of a superorganism. *The Journal of Nutrition, 137*(1), 259S-266S.

Goodacre, R. (2014). Water, water, every where, but rarely any drop to drink. [journal article]. *Metabolomics, 10*(1), 5-7, doi:10.1007/s11306-013-0618-6.

Goodacre, R., Broadhurst, D., Smilde, A. K., Kristal, B. S., Baker, J. D., Beger, R., et al. (2007). Proposed minimum reporting standards for data analysis in metabolomics. *Metabolomics, 3*(3), 231-241.

Government of Canada (1999). Canadian Environmental Protection Act. *S.C. 1999, c. 33*. Canada: Minister of Justice.

Government of Canada (2015). Type 2 diabetes. In G. o. Canada (Ed.).

Gowda, H., Ivanisevic, J., Johnson, C. H., Kurczy, M. E., Benton, H. P., Rinehart, D., et al. (2014). Interactive XCMS Online: Simplifying Advanced Metabolomic Data Processing and Subsequent Statistical Analyses. *Analytical Chemistry, 86*(14), 6931-6939, doi:10.1021/ac500734c.

Guo, Q., Sidhu, J. K., Ebbels, T. M., Rana, F., Spurgeon, D. J., Svendsen, C., et al. (2009). Validation of metabolomics for toxic mechanism of action screening with the earthworm Lumbricus rubellus. *Metabolomics, 5*(1), 72-83.

Hamana, K., Hamana, H., & Shinozawa, T. (1995). Alterations in polyamine levels of nematode, earthworm, leech and planarian during regeneration, temperature and osmotic stresses. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 111*(1), 91-97.

Han, Y., Zhu, L., Wang, J., Wang, J., Xie, H., & Zhang, S. (2014). Integrated assessment of oxidative stress and DNA damage in earthworms (Eisenia fetida) exposed to azoxystrobin. *Ecotoxicology and Environmental Safety, 107*, 214-219, doi:http://dx.doi.org/10.1016/j.ecoenv.2014.06.006.

Hans, H., Lone, A., Aksenov, V., & Rollo, C. D. (2015). Impacts of metformin and aspirin on life history features and longevity of crickets: trade-offs versus cost-free life extension? *AGE, 37*(2), 31, doi:10.1007/s11357-015-9769-x.

Hartmann, T. (2007). From waste products to ecochemicals: Fifty years research of plant secondary metabolism. *Phytochemistry, 68*(22), 2831-2846, doi:http://dx.doi.org/10.1016/j.phytochem.2007.09.017.

Haug, K., Salek, R. M., Conesa, P., Hastings, J., de Matos, P., Rijnbeek, M., et al. (2013). MetaboLights—an open-access general-purpose repository for metabolomics studies and associated meta-data. *Nucleic Acids Research, 41*(D1), D781-D786, doi:10.1093/nar/gks1004.

He, L., & Wondisford, Fredric E. (2015). Metformin Action: Concentrations Matter. *Cell Metabolism, 21*(2), 159-162, doi:https://doi.org/10.1016/j.cmet.2015.01.003.

He, Z., Wang, Y., Zhang, Y., Cheng, H., & Liu, X. (2018). Stereoselective bioaccumulation of chiral PCB 91 in earthworm and its metabolomic and lipidomic responses. *Environmental Pollution, 238*, 421-430, doi:https://doi.org/10.1016/j.envpol.2018.03.060.

Health Canada (2012). Pest Control Products Sales Report for 2012 - Executive Summary. http://www.hc-sc.gc.ca/cps-spc/pubs/pest/_corp-plan/sales-ventes/index-eng.php. Accessed November 12 2015.

Health Canada (2015). Natural Health Products Ingredients Database: Triclosan. http://webprod.hc-sc.gc.ca/nhpid-bdipsn/ingredReq.do?id=4704&lang=eng. Accessed November 7 2015.

Heby, O. (1981). Role of Polyamines in the Control of Cell Proliferation and Differentiation. *Differentiation, 19*(1-3), 1-20, doi:10.1111/j.1432-0436.1981.tb01123.x.

Heidler, J., & Halden, R. U. (2007). Mass balance assessment of triclosan removal during conventional sewage treatment. *Chemosphere, 66*(2), 362-369, doi:http://dx.doi.org/10.1016/j.chemosphere.2006.04.066.

Heidler, J., Sapkota, A., & Halden, R. U. (2006). Partitioning, Persistence, and Accumulation in Digested Sludge of the Topical Antiseptic Triclocarban during Wastewater Treatment. *Environmental Science & Technology, 40*(11), 3634-3639, doi:10.1021/es052245n.

Hernando, M. D., Mezcua, M., Fernández-Alba, A. R., & Barceló, D. (2006). Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. *Talanta, 69*(2), 334-342, doi:http://dx.doi.org/10.1016/j.talanta.2005.09.037.

Hesselsøe, M., Jensen, D., Skals, K., Olesen, T., Moldrup, P., Roslev, P., et al. (2001). Degradation of 4-Nonylphenol in Homogeneous and Nonhomogeneous Mixtures of Soil and Sewage Sludge. *Environmental Science & Technology, 35*(18), 3695-3700, doi:10.1021/es010024l.

Hickey, W. J. (2005). Microbiology and Biochemistry of Xenobiotic Compound Degradation. In D. M. Sylvia, J. J. Fuhrmann, P. G. Hartel, & D. A. Zuberer (Eds.), *Principles and Applications of Soil Microbiology* (2nd ed., pp. 640). Upper Saddle River, NJ: Pearson Education Inc.

Hiller, K., Hangebrauk, J., Jäger, C., Spura, J., Schreiber, K., & Schomburg, D. (2009). MetaboliteDetector: Comprehensive Analysis Tool for Targeted and Nontargeted GC/MS Based Metabolome Analysis. *Analytical Chemistry, 81*(9), 3429-3439, doi:10.1021/ac802689c.

Hinther, A., Bromba, C. M., Wulff, J. E., & Helbing, C. C. (2011). Effects of Triclocarban, Triclosan, and Methyl Triclosan on Thyroid Hormone Action and Stress in Frog and Mammalian Culture Systems. *Environmental Science & Technology, 45*(12), 5395-5402, doi:10.1021/es1041942.

Hirst, J. A., Farmer, A. J., Ali, R., Roberts, N. W., & Stevens, R. J. (2012). Quantifying the Effect of Metformin Treatment and Dose on Glycemic Control. [10.2337/dc11-1465]. *Diabetes Care, 35*(2), 446.

Huang, W., Peng, P. a., Yu, Z., & Fu, J. (2003). Effects of organic matter heterogeneity on sorption and desorption of organic contaminants by soils and sediments. *Applied Geochemistry, 18*(7), 955-972, doi:http://dx.doi.org/10.1016/S0883-2927(02)00205-6.

Hummel, J., Selbig, J., Walther, D., & Kopka, J. (2007). The Golm Metabolome Database: a database for GC-MS based metabolite profiling. In J. Nielsen, & M. C. Jewett (Eds.), *Metabolomics: A Powerful Tool in Systems Biology* (pp. 75-95). Berlin, Heidelberg: Springer Berlin Heidelberg.

Jager, T., van der Wal, L., Fleuren, R. H. L. J., Barendregt, A., & Hermens, J. L. M. (2005). Bioaccumulation of Organic Chemicals in Contaminated Soils: Evaluation of Bioassays with Earthworms. *Environmental Science & Technology, 39*(1), 293-298, doi:10.1021/es035317o.

Jenkins, H., Hardy, N., Beckmann, M., Draper, J., Smith, A. R., Taylor, J., et al. (2004). A proposed framework for the description of plant metabolomics experiments and their results. [10.1038/nbt1041]. *Nature Biotechnology, 22*(12), 1601-1606, doi:http://www.nature.com/nbt/journal/v22/n12/suppinfo/nbt1041_S1.html.

Jeon, S.-M. (2016). Regulation and function of AMPK in physiology and diseases. [Review]. *Experimental and Molecular Medicine, 48*, e245, doi:10.1038/emm.2016.81.

Ji, C., Wu, H., Wei, L., Zhao, J., Lu, H., & Yu, J. (2013a). Proteomic and metabolomic analysis of earthworm< i> Eisenia fetida</i> exposed to different concentrations of 2, 2′, 4, 4′-tetrabromodiphenyl ether. *Journal of Proteomics, 91*, 405-416.

Ji, C., Wu, H., Wei, L., Zhao, J., & Yu, J. (2013b). Proteomic and metabolomic analysis reveal gender-specific responses of mussel Mytilus galloprovincialis to 2,2′,4,4′-tetrabromodiphenyl ether (BDE 47). *Aquatic Toxicology, 140*, 449-457, doi:http://dx.doi.org/10.1016/j.aquatox.2013.07.009.

Johnson, C. H., Ivanisevic, J., & Siuzdak, G. (2016). Metabolomics: beyond biomarkers and towards mechanisms. [Perspectives]. *Nature Reviews Molecular Cell Biology, 17*(7), 451-459, doi:10.1038/nrm.2016.25.

Johnston, A. S. A., Hodson, M. E., Thorbek, P., Alvarez, T., & Sibly, R. M. (2014). An energy budget agent-based model of earthworm populations and its application to study the effects of pesticides. *Ecological Modelling, 280*, 5-17, doi:https://doi.org/10.1016/j.ecolmodel.2013.09.012.

Jolliffe, I. (2014). Principal Component Analysis. In *Wiley StatsRef: Statistics Reference Online*: John Wiley & Sons, Ltd.

Jones, O. A., Spurgeon, D. J., Svendsen, C., & Griffin, J. L. (2008). A metabolomics based approach to assessing the toxicity of the polyaromatic hydrocarbon pyrene to the earthworm *Lumbricus rubellus*. *Chemosphere, 71*(3), 601-609.

Kamleh, M. A., Ebbels, T. M. D., Spagou, K., Masson, P., & Want, E. J. (2012). Optimizing the Use of Quality Control Samples for Signal Drift Correction in Large-Scale Urine Metabolic Profiling Studies. *Analytical Chemistry, 84*(6), 2670-2677, doi:10.1021/ac202733q.

Kanani, H., Chrysanthopoulos, P. K., & Klapa, M. I. (2008). Standardizing GC–MS metabolomics. *Journal of Chromatography B, 871*(2), 191-201.

Karnjanapiboonwong, A., Chase, D. A., Cañas, J. E., Jackson, W. A., Maul, J. D., Morse, A. N., et al. (2011). Uptake of 17α-ethynylestradiol and triclosan in pinto bean, Phaseolus vulgaris. *Ecotoxicology and Environmental Safety, 74*(5), 1336-1342, doi:http://dx.doi.org/10.1016/j.ecoenv.2011.03.013.

Katajamaa, M., Miettinen, J., & Orešič, M. (2006). MZmine: toolbox for processing and visualization of mass spectrometry based molecular profile data. *Bioinformatics, 22*(5), 634-636, doi:10.1093/bioinformatics/btk039.

Katajamaa, M., & Orešič, M. (2005). Processing methods for differential analysis of LC/MS profile data. [journal article]. *BMC Bioinformatics, 6*(1), 179, doi:10.1186/1471-2105-6-179.

Kim, M., Guerra, P., Shah, A., Parsa, M., Alaee, M., & Smyth, S. A. (2014). Removal of pharmaceuticals and personal care products in a membrane bioreactor wastewater treatment plant. *Water Science and Technology, 69*(11), 2221-2229, doi:10.2166/wst.2014.145.

Kind, T., Wohlgemuth, G., Lee, D. Y., Lu, Y., Palazoglu, M., Shahbaz, S., et al. (2009). FiehnLib: Mass Spectral and Retention Index Libraries for Metabolomics Based on Quadrupole and Time-of-Flight Gas Chromatography/Mass Spectrometry. *Analytical Chemistry, 81*(24), 10038-10048, doi:10.1021/ac9019522.

Kinney, C. A., Furlong, E. T., Kolpin, D. W., Burkhardt, M. R., Zaugg, S. D., Werner, S. L., et al. (2008). Bioaccumulation of pharmaceuticals and other anthropogenic waste indicators in earthworms from agricultural soil amended with biosolid or swine manure. *Environmental Science & Technology, 42*(6), 1863-1870.

Kinney, C. A., Furlong, E. T., Zaugg, S. D., Burkhardt, M. R., Werner, S. L., Cahill, J. D., et al. (2006). Survey of Organic Wastewater Contaminants in Biosolids Destined for Land Application†. *Environmental Science & Technology, 40*(23), 7207-7215, doi:10.1021/es0603406.

Klabunde, C. T. (2016). *Potential impacts on groundwater quality in a fractured sedimentary bedrock aquifer from biosolids application on agricultural fields*. University of Guelph,

Klecka, W. R. (1980). *Discriminant Analysis* (Vol. 19, Quantitative Applications in the Social Sciences). Beverly Hills, CA: Sage Publications, Inc.

Kleinstreuer, N. C., Smith, A. M., West, P. R., Conard, K. R., Fontaine, B. R., Weir-Hauptman, A. M., et al. (2011). Identifying developmental toxicity pathways for a subset of ToxCast chemicals using human embryonic stem cells and metabolomics. *Toxicology and Applied Pharmacology, 257*(1), 111-121, doi:http://dx.doi.org/10.1016/j.taap.2011.08.025.

Kleywegt, S., Pileggi, V., Lam, Y. M., Elises, A., Puddicomb, A., Purba, G., et al. (2016). The contribution of pharmaceutically active compounds from healthcare facilities to a receiving sewage treatment plant in Canada. *Environmental Toxicology and Chemistry, 35*(4), 850-862, doi:10.1002/etc.3124.

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

Lai, L., Michopoulos, F., Gika, H., Theodoridis, G., Wilkinson, R. W., Odedra, R., et al. (2009). Methodological considerations in the development of HPLC-MS methods for the analysis of rodent plasma for metabonomic studies. [10.1039/B910482H]. *Molecular BioSystems, 6*(1), 108-120, doi:10.1039/B910482H.

Lakritz, L., Spinelli, A. M., & Wassermann, A. E. (1975). Determination of amines in fresh and processed pork. *Journal of Agricultural and Food Chemistry, 23*(2), 344-346, doi:10.1021/jf60198a024.

Langdon, K., Warne, M., & Kookana, R. (2010). Aquatic hazard assessment for pharmaceuticals, personal care products, and endocrine-disrupting compounds from biosolids-amended land. *Integrated Environmental Assessment and Management, 6*(4), 663-676.

Lankadurai, B. P., Furdui, V. I., Reiner, E. J., Simpson, A. J., & Simpson, M. J. (2013a). 1H NMR-Based Metabolomic Analysis of Sub-Lethal Perfluorooctane Sulfonate Exposure to the Earthworm, Eisenia fetida, in Soil. *Metabolites, 3*(3), 718-740.

Lankadurai, B. P., Nagato, E. G., Simpson, A. J., & Simpson, M. J. (2015). Analysis of Eisenia fetida earthworm responses to sub-lethal C60 nanoparticle exposure using 1H-NMR based metabolomics. *Ecotoxicology and Environmental Safety, 120*, 48-58, doi:https://doi.org/10.1016/j.ecoenv.2015.05.020.

Lankadurai, B. P., Simpson, A. J., & Simpson, M. J. (2012). 1H NMR metabolomics of Eisenia fetida responses after sub-lethal exposure to perfluorooctanoic acid and perfluorooctane sulfonate. *Environmental Chemistry, 9*(6), 502-511.

Lankadurai, B. P., Wolfe, D. M., Åslund, M. L. W., Simpson, A. J., & Simpson, M. J. (2013b). 1H NMR-based metabolomic analysis of polar and non-polar earthworm metabolites after sub-lethal exposure to phenanthrene. *Metabolomics, 9*(1), 44-56.

Lankadurai, B. P., Wolfe, D. M., Simpson, A. J., & Simpson, M. J. (2011a). 1H NMR-based metabolomic observation of a two-phased toxic mode of action in Eisenia fetida after sub-lethal phenanthrene exposure. *Environmental Chemistry, 8*(2), 105-114.

Lankadurai, B. P., Wolfe, D. M., Simpson, A. J., & Simpson, M. J. (2011b). < sup> 1</sup> H NMR-based metabolomics of time-dependent responses of< i> Eisenia fetida</i> to sub-lethal phenanthrene exposure. *Environmental Pollution, 159*(10), 2845-2851.

Lanno, R., Wells, J., Conder, J., Bradham, K., & Basta, N. (2004). The bioavailability of chemicals in soil for earthworms. *Ecotoxicology and Environmental Safety, 57*(1), 39-47, doi:http://dx.doi.org/10.1016/j.ecoenv.2003.08.014.

Lapen, D., Topp, E., Metcalfe, C., Li, H., Edwards, M., Gottschall, N., et al. (2008). Pharmaceutical and personal care products in tile drainage following land application of municipal biosolids. *Science of The Total Environment, 399*(1), 50-65.

Larsen, T., Pollierer, M. M., Holmstrup, M., D'Annibale, A., Maraldo, K., Andersen, N., et al. (2016). Substantial nutritional contribution of bacterial amino acids to earthworms and enchytraeids: A case study from organic grasslands. *Soil Biology and Biochemistry, 99*, 21-27, doi:http://dx.doi.org/10.1016/j.soilbio.2016.03.018.

Lenz, E., Weeks, J., Lindon, J., Osborn, D., & Nicholson, J. (2005). Qualitative high field 1H-NMR spectroscopy for the characterization of endogenous metabolites in earthworms with biochemical biomarker potential. *Metabolomics, 1*(2), 123-136.

Lenz, E. M., Lindon, J. C., Nicholson, J. K., Weeks, J. M., & Osborn, D. (2002). 19 F-NMR and directly coupled 19 F/ 1 H-HPLC-NMR spectroscopic investigations of the metabolism of the model ecotoxin 3-trifluoromethylaniline in the earthworm species Eisenia veneta. *Xenobiotica, 32*(6), 535-546, doi:10.1080/00498250210128666.

Liebeke, M., & Bundy, J. G. (2012). Tissue disruption and extraction methods for metabolic profiling of an invertebrate sentinel species. *Metabolomics, 8*(5), 819-830.

Liebeke, M., Dörries, K., Meyer, H., & Lalk, M. (2012). Metabolome Analysis of Gram-Positive Bacteria such as Staphylococcus aureus by GC-MS and LC-MS. In M. Kaufmann, & C.

Klinger (Eds.), *Functional Genomics: Methods and Protocols* (pp. 377-398). New York, NY: Springer New York.
Lin, D., Xie, X., Zhou, Q., & Liu, Y. (2012). Biochemical and genotoxic effect of triclosan on earthworms (Eisenia fetida) using contact and soil tests. *Environmental Toxicology, 27*(7), 385-392.
Lin, D., Zhou, Q., Xie, X., & Liu, Y. (2010). Potential biochemical and genetic toxicity of triclosan as an emerging pollutant on earthworms (Eisenia fetida). *Chemosphere, 81*(10), 1328-1333, doi:http://dx.doi.org/10.1016/j.chemosphere.2010.08.027.
Lindon, J. C., Nicholson, J. K., Holmes, E., Keun, H. C., Craig, A., Pearce, J. T. M., et al. (2005). Summary recommendations for standardization and reporting of metabolic analyses. [Computational Biology]. *Nature Biotechnology, 23*(7), 833-838, doi:10.1038/nbt0705-833.
Liu, W., Zhu, L.-S., Wang, J., Wang, J.-H., Xie, H., & Song, Y. (2009). Assessment of the Genotoxicity of Endosulfan in Earthworm and White Clover Plants Using the Comet Assay. *Archives of Environmental Contamination and Toxicology, 56*(4), 742-746, doi:10.1007/s00244-009-9309-8.
Liu, X., Yang, C., Zhang, L., Li, L., Liu, S., Yu, J., et al. (2011). Metabolic profiling of cadmium-induced effects in one pioneer intertidal halophyte Suaeda salsa by NMR-based metabolomics. *Ecotoxicology, 20*(6), 1422-1431, doi:10.1007/s10646-011-0699-9.
Livera, A. M. D., Sysi-Aho, M., Jacob, L., Gagnon-Bartsch, J. A., Castillo, S., Simpson, J. A., et al. (2015). Statistical Methods for Handling Unwanted Variation in Metabolomics Data. *Analytical Chemistry, 87*(7), 3606-3615, doi:10.1021/ac502439y.
Loos, R., Carvalho, R., António, D. C., Comero, S., Locoro, G., Tavazzi, S., et al. (2013). EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. *Water research, 47*(17), 6475-6487, doi:http://dx.doi.org/10.1016/j.watres.2013.08.024.
Lowe, C. N., & Butt, K. R. (2007). Earthworm culture, maintenance and species selection in chronic ecotoxicological studies: A critical review. *European Journal of Soil Biology, 43*, S281-S288, doi:http://dx.doi.org/10.1016/j.ejsobi.2007.08.028.
Lozano, N., Rice, C. P., Ramirez, M., & Torrents, A. (2012). Fate of Triclosan and Methyltriclosan in soil from biosolids application. *Environmental Pollution, 160*, 103-108, doi:http://dx.doi.org/10.1016/j.envpol.2011.09.020.
Lozano, N., Rice, C. P., Ramirez, M., & Torrents, A. (2013). Fate of Triclocarban, Triclosan and Methyltriclosan during wastewater and biosolids treatment processes. *Water research, 47*(13), 4519-4527, doi:http://dx.doi.org/10.1016/j.watres.2013.05.015.
Ma, W.-c., van Kleunen, A., Immerzeel, J., & de Maagd, P. G.-J. (1998). Bioaccumulation of polycyclic aromatic hydrocarbons by earthworms: Assessment of equilibrium partitioning theory in in situ studies and water experiments. *Environmental Toxicology and Chemistry, 17*(9), 1730-1737, doi:10.1002/etc.5620170913.
Macherius, A., Lapen, D. R., Reemtsma, T., Römbke, J., Topp, E., & Coors, A. (2014). Triclocarban, triclosan and its transformation product methyl triclosan in native earthworm species four years after a commercial-scale biosolids application. *Science of The Total Environment, 472*, 235-238.
Malchi, T., Maor, Y., & Chefetz, B. (2015). Comments on" Human health risk assessment of pharmaceuticals and personal care products in plant tissue due to biosolids and manure amendments, and wastewater irrigation". *Environment international, 82*, 110-112.

Malin, S. K., & Kashyap, S. R. (2014). Effects of metformin on weight loss: potential mechanisms. *Current Opinion in Endocrinology, Diabetes and Obesity, 21*(5), 323-329.
Malmendal, A., Overgaard, J., Bundy, J. G., Sørensen, J. G., Nielsen, N. C., Loeschcke, V., et al. (2006). Metabolomic profiling of heat stress: hardening and recovery of homeostasis in Drosophila. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 291*(1), R205-R212, doi:10.1152/ajpregu.00867.2005.
Martin-Castillo, B., Vazquez-Martin, A., Oliveras-Ferraros, C., & Menendez, J. A. (2010). Metformin and cancer: Doses, mechanisms and the dandelion and hormetic phenomena. *Cell Cycle, 9*(6), 1057-1064, doi:10.4161/cc.9.6.10994.
McAvoy, D. C., Schatowitz, B., Jacob, M., Hauk, A., & Eckhoff, W. S. (2002). Measurement of triclosan in wastewater treatment systems. *Environmental Toxicology and Chemistry, 21*(7), 1323-1329, doi:10.1002/etc.5620210701.
McClellan, K., & Halden, R. U. (2010). Pharmaceuticals and Personal Care Products in Archived U.S. Biosolids from the 2001 EPA National Sewage Sludge Survey. *Water research, 44*(2), 658-668, doi:10.1016/j.watres.2009.12.032.
McCormick, J. M., Es, T. V., Cooper, K. R., White, L. A., & Häggblom, M. M. (2011). Microbially Mediated O-Methylation of Bisphenol a Results in Metabolites with Increased Toxicity to the Developing Zebrafish (Danio rerio) Embryo. *Environmental Science & Technology, 45*(15), 6567-6574, doi:10.1021/es200588w.
McHardy, I. H., Goudarzi, M., Tong, M., Ruegger, P. M., Schwager, E., Weger, J. R., et al. (2013). Integrative analysis of the microbiome and metabolome of the human intestinal mucosal surface reveals exquisite inter-relationships. [journal article]. *Microbiome, 1*(1), 1-19, doi:10.1186/2049-2618-1-17.
McKelvie, J. R., Åslund, M. W., Celejewski, M. A., Simpson, A. J., & Simpson, M. J. (2013). Reduction in the earthworm metabolomic response after phenanthrene exposure in soils with high soil organic carbon content. *Environmental Pollution, 175*, 75-81.
McKelvie, J. R., Wolfe, D. M., Celejewski, M., Simpson, A. J., & Simpson, M. J. (2010). Correlations of< i> Eisenia fetida</i> metabolic responses to extractable phenanthrene concentrations through time. *Environmental Pollution, 158*(6), 2150-2157.
McKelvie, J. R., Wolfe, D. M., Celejewski, M. A., Alaee, M., Simpson, A. J., & Simpson, M. J. (2011). Metabolic responses of< i> Eisenia fetida</i> after sub-lethal exposure to organic contaminants with different toxic modes of action. *Environmental Pollution, 159*(12), 3620-3626.
McKelvie, J. R., Yuk, J., Xu, Y., Simpson, A. J., & Simpson, M. J. (2009). 1H NMR and GC/MS metabolomics of earthworm responses to sub-lethal DDT and endosulfan exposure. *Metabolomics, 5*(1), 84-94.
McMurry, L. M., Oethinger, M., & Levy, S. B. (1998). Triclosan targets lipid synthesis. [10.1038/28970]. *Nature, 394*(6693), 531-532.
Mellinkoff, S. M., Frankland, M., Boyle, D., & Greipe, M. (1997). Relationship Between Serum Amino Acid Concentration and Fluctuations in Appetite1. *Obesity Research, 5*(4), 381-384, doi:doi:10.1002/j.1550-8528.1997.tb00568.x.
Melvin, S. D., Habener, L. J., Leusch, F. D. L., & Carroll, A. R. (2017). 1H NMR-based metabolomics reveals sub-lethal toxicity of a mixture of diabetic and lipid-regulating pharmaceuticals on amphibian larvae. *Aquatic Toxicology, 184*, 123-132, doi:https://doi.org/10.1016/j.aquatox.2017.01.012.

Meng, X.-Z., Venkatesan, A. K., Ni, Y.-L., Steele, J. C., Wu, L.-L., Bignert, A., et al. (2016). Organic Contaminants in Chinese Sewage Sludge: A Meta-Analysis of the Literature of the Past 30 Years. *Environmental Science & Technology, 50*(11), 5454-5466, doi:10.1021/acs.est.5b05583.

Metcalfe, C., Miao, X.-S., Hua, W., Letcher, R., & Servos, M. (2004). Pharmaceuticals in the Canadian Environment. In K. Kümmerer (Ed.), *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks* (pp. 67-90). Berlin, Heidelberg: Springer Berlin Heidelberg.

Minois, N. (2014). Molecular Basis of the 'Anti-Aging' Effect of Spermidine and Other Natural Polyamines - A Mini-Review. *Gerontology, 60*(4), 319-326.

Miyazaki, A., Amano, T., Saito, H., & Nakano, Y. (2002). Acute toxicity of chlorophenols to earthworms using a simple paper contact method and comparison with toxicities to fresh water organisms. *Chemosphere, 47*(1), 65-69, doi:http://dx.doi.org/10.1016/S0045-6535(01)00286-7.

Monteith, H., Sterne, L., & Dong, S. (2010). Emerging substances of concern in biosolids: Concentrations and effects of treatment processes. Final report – field sampling program. Winnipeg, MB: Canadian Council of Ministers of the Environment.

Montgomery, D. C. (2005). *Design and Analysis of Experiments* (6th ed.). Hoboken, NJ: John Wiley and Sons, Inc.

Morrison, N., Bearden, D., Bundy, J., Collette, T., Currie, F., Davey, M., et al. (2007). Standard reporting requirements for biological samples in metabolomics experiments: environmental context. *Metabolomics, 3*(3), 203-210, doi:10.1007/s11306-007-0067-1.

Mrozik, W., & Stefańska, J. (2014). Adsorption and biodegradation of antidiabetic pharmaceuticals in soils. *Chemosphere, 95*, 281-288, doi:http://dx.doi.org/10.1016/j.chemosphere.2013.09.012.

Mudiam, M. K. R., Ch, R., & Saxena, P. N. (2013). Gas Chromatography-Mass Spectrometry Based Metabolomic Approach for Optimization and Toxicity Evaluation of Earthworm Sub-Lethal Responses to Carbofuran. *PLOS ONE, 8*(12), e81077.

Nagato, E. G., Simpson, A. J., & Simpson, M. J. (2016). Metabolomics reveals energetic impairments in Daphnia magna exposed to diazinon, malathion and bisphenol-A. *Aquatic Toxicology, 170*, 175-186, doi:http://dx.doi.org/10.1016/j.aquatox.2015.11.023.

Nedergaard, J., Ricquier, D., & Kozak, L. P. (2005). Uncoupling proteins: current status and therapeutic prospects. *Meeting on Uncoupling Proteins, 6*(10), 917-921, doi:10.1038/sj.embor.7400532.

Nelson, D., & Cox, M. (2005). *Lehninger Principles of Biochemistry* (4th ed.). New York: W.H. Freeman and Company.

Niemuth, N. J., & Klaper, R. D. (2015). Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish. *Chemosphere, 135*, 38-45, doi:http://dx.doi.org/10.1016/j.chemosphere.2015.03.060.

Noguera-Oviedo, K., & Aga, D. S. (2016). Lessons learned from more than two decades of research on emerging contaminants in the environment. *Journal of Hazardous Materials, 316*, 242-251, doi:http://dx.doi.org/10.1016/j.jhazmat.2016.04.058.

NSE (2010). Guidelines for land application and storage of municipal biosolids in Nova Scotia - Revised. In N. S. E. Department (Ed.), (pp. 25). Halifax, NS: Government of Nova Scotia.

OECD (1984). Test No. 207: Earthworm, Acute Toxicity Tests. *OECD Guidelines for the Testing of Chemicals*: Organisation for Economic Cooperation and Development.
OECD (2004). Test No. 222: Earthworm Reproduction Test (Eisenia fetida / Eisenia andrei). *OECD Guidelines for the Testing of Chemicals*: Organization of Economic Cooperation and Development.
Oosterhuis, M., Sacher, F., & ter Laak, T. L. (2013). Prediction of concentration levels of metformin and other high consumption pharmaceuticals in wastewater and regional surface water based on sales data. *Science of The Total Environment, 442*, 380-388, doi:http://dx.doi.org/10.1016/j.scitotenv.2012.10.046.
Pannu, M. W., O'Connor, G. A., & Toor, G. S. (2012). Toxicity and bioaccumulation of biosolids-borne triclosan in terrestrial organisms. *Environmental Toxicology and Chemistry, 31*(3), 646-653, doi:10.1002/etc.1721.
Peijnenburg, W. (2004). Chapter 9 Fate of contaminants in soil. *Developments in Soil Science, 29*, 245-280, doi:http://dx.doi.org/10.1016/S0166-2481(04)80013-8.
Pernicova, I., & Korbonits, M. (2014). Metformin[mdash]mode of action and clinical implications for diabetes and cancer. [Review]. *Nature Reviews Endocrinology, 10*(3), 143-156, doi:10.1038/nrendo.2013.256.
Pluskal, T., Castillo, S., Villar-Briones, A., & Orešič, M. (2010). MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics, 11*(1), 395.
Pokarzhevskii, A. D., Zaboyev, D. P., Ganin, G. N., & Gordienko, S. A. (1997). Amino acids in earthworms: Are earthworms ecosystemivorous? *Soil Biology and Biochemistry, 29*(3), 559-567, doi:http://dx.doi.org/10.1016/S0038-0717(96)00180-0.
Pokarzhevskij, A. D., Zaboev, D. P., Gordienko, S. A., Boháč, J., & Gusev, A. A. (1989). Biogenic turnover of matter, soil biota and problems of agroecosystem development. *Agriculture, Ecosystems & Environment, 27*(1), 281-291, doi:http://dx.doi.org/10.1016/0167-8809(89)90092-3.
Prentø, P. (1987). Blood sugar, sugar metabolism and related enzymes in the earthworm, Lumbricus terrestris L. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 86*(2), 333-341, doi:http://dx.doi.org/10.1016/0305-0491(87)90302-6.
Prosser, R., & Sibley, P. (2015). Human health risk assessment of pharmaceuticals and personal care products in plant tissue due to biosolids and manure amendments, and wastewater irrigation. *Environment international, 75*, 223-233.
Quaile, M. P., Melich, D. H., Jordan, H. L., Nold, J. B., Chism, J. P., Polli, J. W., et al. (2010). Toxicity and toxicokinetics of metformin in rats. *Toxicology and Applied Pharmacology, 243*(3), 340-347, doi:https://doi.org/10.1016/j.taap.2009.11.026.
Reinecke, S. A., & Reinecke, A. J. (2004). The Comet Assay as Biomarker of Heavy Metal Genotoxicity in Earthworms. [journal article]. *Archives of Environmental Contamination and Toxicology, 46*(2), 208-215, doi:10.1007/s00244-003-2253-0.
Ren, S., Hinzman, A., Kang, E., Szczesniak, R., & Lu, L. (2015). Computational and statistical analysis of metabolomics data. *Metabolomics, 11*(6), 1492-1513, doi:10.1007/s11306-015-0823-6.
Rencher, A. C. (2003). Multivariate Analysis of Variance. In *Methods of Multivariate Analysis* (pp. 156-247): John Wiley & Sons, Inc.

Ricart, M., Guasch, H., Alberch, M., Barceló, D., Bonnineau, C., Geiszinger, A., et al. (2010). Triclosan persistence through wastewater treatment plants and its potential toxic effects on river biofilms. *Aquatic Toxicology, 100*(4), 346-353.

Rochfort, S. J., Ezernieks, V., & Yen, A. L. (2009). NMR-based metabolomics using earthworms as potential indicators for soil health. *Metabolomics, 5*(1), 95-107.

Russell, A. (2004). Whither triclosan? *Journal of Antimicrobial Chemotherapy, 53*(5), 693-695.

Ryan, D., & Robards, K. (2006). Metabolomics: The Greatest Omics of Them All? *Analytical Chemistry, 78*(23), 7954-7958, doi:10.1021/ac0614341.

Sabourin, L., Beck, A., Duenk, P. W., Kleywegt, S., Lapen, D. R., Li, H., et al. (2009). Runoff of pharmaceuticals and personal care products following application of dewatered municipal biosolids to an agricultural field. *Science of The Total Environment, 407*(16), 4596-4604.

Salek, R. M., Steinbeck, C., Viant, M. R., Goodacre, R., & Dunn, W. B. (2013). The role of reporting standards for metabolite annotation and identification in metabolomic studies. *GigaScience, 2*(1), 13.

Sampedro, L., Jeannotte, R., & Whalen, J. K. (2006). Trophic transfer of fatty acids from gut microbiota to the earthworm Lumbricus terrestris L. *Soil Biology and Biochemistry, 38*(8), 2188-2198, doi:http://dx.doi.org/10.1016/j.soilbio.2006.02.001.

Samuelsson, L. M., Förlin, L., Karlsson, G., Adolfsson-Erici, M., & Larsson, D. G. J. (2006). Using NMR metabolomics to identify responses of an environmental estrogen in blood plasma of fish. *Aquatic Toxicology, 78*(4), 341-349, doi:http://dx.doi.org/10.1016/j.aquatox.2006.04.008.

Sandermann, H. (1993). Induction of lipid-protein mismatch by xenobiotics with general membrane targets. *Biochimica et Biophysica Acta (BBA) - Biomembranes, 1150*(2), 130-133, doi:http://dx.doi.org/10.1016/0005-2736(93)90081-A.

Sangster, T., Major, H., Plumb, R., Wilson, A. J., & Wilson, I. D. (2006). A pragmatic and readily implemented quality control strategy for HPLC-MS and GC-MS-based metabonomic analysis. [10.1039/B604498K]. *Analyst, 131*(10), 1075-1078, doi:10.1039/B604498K.

Sansone, S.-A., Fan, T. W.-M., Goodacre, R., Griffin, J. L., Hardy, N. W., Kaddurah-Daouk, R., et al. (2007). The Metabolomics Standards Initiative. [10.1038/nbt0807-846b]. *Nature Biotechnology, 25*(8), 846-848.

Scheurer, M., Michel, A., Brauch, H.-J., Ruck, W., & Sacher, F. (2012). Occurrence and fate of the antidiabetic drug metformin and its metabolite guanylurea in the environment and during drinking water treatment. *Water research, 46*(15), 4790-4802, doi:http://dx.doi.org/10.1016/j.watres.2012.06.019.

Scheurer, M., Sacher, F., & Brauch, H.-J. (2009). Occurrence of the antidiabetic drug metformin in sewage and surface waters in Germany. [10.1039/B909311G]. *Journal of Environmental Monitoring, 11*(9), 1608-1613, doi:10.1039/B909311G.

Schnug, L., Jakob, L., & Hartnik, T. (2013). The toxicity of a ternary biocide mixture to two consecutive earthworm (Eisenia fetida) generations. *Environmental Toxicology and Chemistry, 32*(4), 937-947, doi:10.1002/etc.2142.

Schnug, L., Jensen, J., Scott-Fordsmand, J. J., & Leinaas, H. P. (2014). Toxicity of three biocides to springtails and earthworms in a soil multi-species (SMS) test system. *Soil Biology and Biochemistry, 74*, 115-126, doi:http://dx.doi.org/10.1016/j.soilbio.2014.03.007.

Schock, T. B., Strickland, S., Steele, E. J., & Bearden, D. W. (2016). Effects of heat-treatment on the stability and composition of metabolomic extracts from the earthworm Eisenia fetida. [journal article]. *Metabolomics, 12*(3), 47, doi:10.1007/s11306-016-0967-z.

Schrenk, D. (2014). Toxicodynamic Tests. In F.-X. Reichl, & M. Schwenk (Eds.), *Regulatory Toxicology* (pp. 161-172). Berlin, Heidelberg: Springer Berlin Heidelberg.

Shaffer, J. P. (1995). Multiple Hypothesis Testing. *Annual Review of Psychology, 46*(1), 561-584, doi:10.1146/annurev.ps.46.020195.003021.

Shi, Y., Xu, X., Chen, J., Liang, R., Zheng, X., Shi, Y., et al. (2018). Antioxidant gene expression and metabolic responses of earthworms (Eisenia fetida) after exposure to various concentrations of hexabromocyclododecane. *Environmental Pollution, 232*, 245-251, doi:https://doi.org/10.1016/j.envpol.2017.09.039.

Simpson, M. J., & McKelvie, J. R. (2009). Environmental metabolomics: new insights into earthworm ecotoxicity and contaminant bioavailability in soil. *Analytical and Bioanalytical Chemistry, 394*(1), 137-149.

Snyder, E. H., & O'Connor, G. A. (2013). Risk assessment of land-applied biosolids-borne triclocarban (TCC). *Science of The Total Environment, 442*, 437-444, doi:http://dx.doi.org/10.1016/j.scitotenv.2012.10.007.

Song, R. (2016). Mechanism of Metformin: A Tale of Two Sites. *Diabetes Care, 39*(2), 187-189, doi:10.2337/dci15-0013.

Sotto, R. B. D., Medriano, C. D., Cho, Y., Kim, H., Chung, I.-Y., Seok, K.-S., et al. (2017). Sub-lethal pharmaceutical hazard tracking in adult zebrafish using untargeted LC–MS environmental metabolomics. *Journal of Hazardous Materials, 339*, 63-72, doi:http://dx.doi.org/10.1016/j.jhazmat.2017.06.009.

Spark, K. M., & Swift, R. S. (2002). Effect of soil composition and dissolved organic matter on pesticide sorption. *Science of The Total Environment, 298*(1), 147-161, doi:http://dx.doi.org/10.1016/S0048-9697(02)00213-9.

Sparling, D. W. (2016). Chapter 2 - Basics of Toxicity Testing. In *Ecotoxicology Essentials* (pp. 21-44). San Diego: Academic Press.

Spurgeon, D. J., Weeks, J. M., & Van Gestel, C. A. (2003). A summary of eleven years progress in earthworm ecotoxicology: The 7th international symposium on earthworm ecology· Cardiff· Wales· 2002. *Pedobiologia, 47*(5), 588-606.

Steuer, R. (2006). Review: on the analysis and interpretation of correlations in metabolomic data. *Briefings in Bioinformatics, 7*(2), 151-158.

Steuer, R., Morgenthal, K., Weckwerth, W., & Selbig, J. (2007). A gentle guide to the analysis of metabolomic data. In *Metabolomics* (pp. 105-126): Springer.

Sumner, L. W., Amberg, A., Barrett, D., Beale, M. H., Beger, R., Daykin, C. A., et al. (2007). Proposed minimum reporting standards for chemical analysis. *Metabolomics, 3*(3), 211-221.

Sysi-Aho, M., Katajamaa, M., Yetukuri, L., & Orešič, M. (2007). Normalization method for metabolomics data using optimal selection of multiple internal standards. [journal article]. *BMC Bioinformatics, 8*(1), 93, doi:10.1186/1471-2105-8-93.

Szymańska, E., Saccenti, E., Smilde, A. K., & Westerhuis, J. A. (2012). Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies. *Metabolomics, 8*(Suppl 1), 3-16, doi:10.1007/s11306-011-0330-3.

Tabor, C. W., & Tabor, H. (1976). 1,4-Diaminobutane (Putrescine), Spermidine, and Spermine. *Annual Review of Biochemistry, 45*(1), 285-306, doi:doi:10.1146/annurev.bi.45.070176.001441.

Tamim, N. M., & Doerr, J. A. (2003). Effect of Putrefaction of Poultry Carcasses Prior to Rendering on Biogenic Amine Production. *The Journal of Applied Poultry Research, 12*(4), 456-460, doi:10.1093/japr/12.4.456.

Taylor, C. F., Paton, N. W., Lilley, K. S., Binz, P.-A., Julian, R. K., Jones, A. R., et al. (2007). The minimum information about a proteomics experiment (MIAPE). [10.1038/nbt1329]. *Nature Biotechnology, 25*(8), 887-893.

Tembe, S., Ghosh, P., & Sukhi, S. (2017). Influence of methylation on the bacterial efflux pump inducing property of triclosan. *Journal of Hospital Infection*, doi:http://dx.doi.org/10.1016/j.jhin.2017.07.009.

Thomaidi, V. S., Stasinakis, A. S., Borova, V. L., & Thomaidis, N. S. (2016). Assessing the risk associated with the presence of emerging organic contaminants in sludge-amended soil: A country-level analysis. *Science of The Total Environment, 548–549*, 280-288, doi:http://dx.doi.org/10.1016/j.scitotenv.2016.01.043.

Timbrell, J. A. (2008). *Principles of Biochemical Toxicology, Fourth Edition*. New York, NY: CRC Press.

Tohidi, F., & Cai, Z. (2017). Fate and mass balance of triclosan and its degradation products: Comparison of three different types of wastewater treatments and aerobic/anaerobic sludge digestion. *Journal of Hazardous Materials, 323*, 329-340, doi:http://dx.doi.org/10.1016/j.jhazmat.2016.04.034.

Topp, E., Monteiro, S. C., Beck, A., Coelho, B. B., Boxall, A. B., Duenk, P. W., et al. (2008). Runoff of pharmaceuticals and personal care products following application of biosolids to an agricultural field. *Science of The Total Environment, 396*(1), 52-59.

Trautwein, C., Berset, J.-D., Wolschke, H., & Kümmerer, K. (2014). Occurrence of the antidiabetic drug Metformin and its ultimate transformation product Guanylurea in several compartments of the aquatic cycle. *Environment international, 70*, 203-212, doi:http://dx.doi.org/10.1016/j.envint.2014.05.008.

Ucakturk, E. (2013). The development and validation of a gas chromatography-mass spectrometry method for the determination of metformin in human plasma. [10.1039/C3AY40507A]. *Analytical Methods, 5*(18), 4723-4730, doi:10.1039/C3AY40507A.

USEPA (2009a). Targeted National Sewage Sludge Survey Overview Report. In O. o. W. U.S. Environmental Protection Agency (Ed.), (pp. 10). Washington, DC: U.S. Environmental Protection Agency.

USEPA (2009b). Targeted National Sewage Sludge Survey: Sampling and Analysis Technical Report In U. S. E. P. Agency (Ed.). Washington, DC: Office of Water.

van den Berg, R. A., Hoefsloot, H. C., Westerhuis, J. A., Smilde, A. K., & van der Werf, M. J. (2006). Centering, scaling, and transformations: improving the biological information content of metabolomics data. [journal article]. *BMC Genomics, 7*(1), 142, doi:10.1186/1471-2164-7-142.

van der Kloet, F. M., Bobeldijk, I., Verheij, E. R., & Jellema, R. H. (2009). Analytical Error Reduction Using Single Point Calibration for Accurate and Precise Metabolomic Phenotyping. *Journal of Proteome Research, 8*(11), 5132-5141, doi:10.1021/pr900499r.

van Wezel, A. P., & Opperhuizen, A. (1995). Narcosis Due to Environmental Pollutants in Aquatic Organisms: Residue-Based Toxicity, Mechanisms, and Membrane Burdens. *Critical Reviews in Toxicology, 25*(3), 255-279, doi:10.3109/10408449509089890.

Verhaar, H. J. M., van Leeuwen, C. J., & Hermens, J. L. M. (1992). Classifying environmental pollutants. *Chemosphere, 25*(4), 471-491, doi:http://dx.doi.org/10.1016/0045-6535(92)90280-5.

Verlicchi, P., & Zambello, E. (2015). Pharmaceuticals and personal care products in untreated and treated sewage sludge: Occurrence and environmental risk in the case of application on soil—A critical review. *Science of The Total Environment, 538*, 750-767.

Viant, M. R. (2008). Recent developments in environmental metabolomics. *Molecular BioSystems, 4*(10), 980-986.

Viant, M. R., Kurland, I. J., Jones, M. R., & Dunn, W. B. (2017). How close are we to complete annotation of metabolomes? *Current Opinion in Chemical Biology, 36*, 64-69, doi:http://dx.doi.org/10.1016/j.cbpa.2017.01.001.

Villalaín, J., Mateo, C. R., Aranda, F. J., Shapiro, S., & Micol, V. (2001). Membranotropic effects of the antibacterial agent triclosan. *Archives of Biochemistry and Biophysics, 390*(1), 128-136.

Vincent, I. M., Ehmann, D. E., Mills, S. D., Perros, M., & Barrett, M. P. (2016). Untargeted metabolomics to ascertain antibiotic modes of action. *Antimicrobial agents and chemotherapy, 60*(4), 2281-2291.

Wagner, N. D., Simpson, A. J., & Simpson, M. J. (2017). Metabolomic responses to sublethal contaminant exposure in neonate and adult Daphnia magna. *Environmental Toxicology and Chemistry, 36*(4), 938-946, doi:10.1002/etc.3604.

Waltman, E. L., Venables, B. J., & Waller, W. T. (2006). Triclosan in a North Texas wastewater treatment plant and the influent and effluent of an experimental constructed wetland. *Environmental Toxicology and Chemistry, 25*(2), 367-372.

Wang, C.-F., & Tian, Y. (2015). Reproductive endocrine-disrupting effects of triclosan: Population exposure, present evidence and potential mechanisms. *Environmental Pollution, 206*, 195-201.

Wang, S.-Y., Kuo, C.-H., & Tseng, Y. J. (2013). Batch Normalizer: A Fast Total Abundance Regression Calibration Method to Simultaneously Adjust Batch and Injection Order Effects in Liquid Chromatography/Time-of-Flight Mass Spectrometry-Based Metabolomics Data and Comparison with Current Calibration Methods. *Analytical Chemistry, 85*(2), 1037-1046, doi:10.1021/ac302877x.

Wang, W., Zhou, H., Lin, H., Roy, S., Shaler, T. A., Hill, L. R., et al. (2003). Quantification of Proteins and Metabolites by Mass Spectrometry without Isotopic Labeling or Spiked Standards. *Analytical Chemistry, 75*(18), 4818-4826, doi:10.1021/ac026468x.

Wang, Y., Cang, T., Zhao, X., Yu, R., Chen, L., Wu, C., et al. (2012). Comparative acute toxicity of twenty-four insecticides to earthworm, Eisenia fetida. *Ecotoxicology and Environmental Safety, 79*, 122-128.

Warne, M., Lenz, E. M., Osborn, D., Weeks, J. M., & Nicholson, J. K. (2000). An NMR-based metabonomic investigation of the toxic effects of 3-trifluoromethyl-aniline on the earthworm Eisenia veneta. *Biomarkers, 5*(1), 56-72, doi:10.1080/135475000230541.

Warne, M. A., Lenz, E. M., Osborn, D., Weeks, J. M., & Nicholson, J. K. (2001). Comparative biochemistry and short-term starvation effects on the earthworms Eisenia veneta and Lumbricus terrestris studied by 1H NMR spectroscopy and pattern recognition. *Soil*

Biology and Biochemistry, 33(9), 1171-1180, doi:http://dx.doi.org/10.1016/S0038-0717(01)00021-9.

Warrack, B. M., Hnatyshyn, S., Ott, K.-H., Reily, M. D., Sanders, M., Zhang, H., et al. (2009). Normalization strategies for metabonomic analysis of urine samples. *Journal of Chromatography B, 877*(5), 547-552, doi:http://dx.doi.org/10.1016/j.jchromb.2009.01.007.

Webber, M. D., & Lesage, S. (1989). Organic contaminants in canadian municipal sludges. *Waste Management & Research, 7*(1), 63-82, doi:http://dx.doi.org/10.1016/0734-242X(89)90009-8.

Wenig, P., & Odermatt, J. (2010). OpenChrom: a cross-platform open source software for the mass spectrometric analysis of chromatographic data. *BMC Bioinformatics, 11*(1), 405.

Whalen, J. K., & Parmelee, R. W. (1999). Growth of Aporrectodea tuberculata(Eisen) and Lumbricus terrestris L. under laboratory and field conditions. *Pedobiologia, 43*(1), 1-10.

Whitfield Åslund, M., Celejewski, M., Lankadurai, B. P., Simpson, A. J., & Simpson, M. J. (2011a). Natural variability and correlations in the metabolic profile of healthy< i> Eisenia fetida</i> earthworms observed using< sup> 1</sup> H NMR metabolomics. *Chemosphere, 83*(8), 1096-1101.

Whitfield Åslund, M., Simpson, M. J., Simpson, A. J., Zeeb, B. A., & Rutter, A. (2012). Earthworm metabolomic responses after exposure to aged PCB contaminated soils. *Ecotoxicology, 21*(7), 1947-1956.

Whitfield Åslund, M., Stephenson, G. L., Simpson, A. J., & Simpson, M. J. (2013). Comparison of earthworm responses to petroleum hydrocarbon exposure in aged field contaminated soil using traditional ecotoxicity endpoints and< sup> 1</sup> H NMR-based metabolomics. *Environmental Pollution, 182*, 263-268.

Whitfield Åslund, M. L., McShane, H., Simpson, M. J., Simpson, A. J., Whalen, J. K., Hendershot, W. H., et al. (2011b). Earthworm sublethal responses to titanium dioxide nanomaterial in soil detected by 1H NMR metabolomics. *Environmental Science & Technology, 46*(2), 1111-1118.

Whitfield Åslund, M. L., Simpson, A. J., & Simpson, M. J. (2011c). 1H NMR metabolomics of earthworm responses to polychlorinated biphenyl (PCB) exposure in soil. *Ecotoxicology, 20*(4), 836-846.

Wishart, D. S., Jewison, T., Guo, A. C., Wilson, M., Knox, C., Liu, Y., et al. (2013). HMDB 3.0—The Human Metabolome Database in 2013. *Nucleic Acids Research, 41*(Database issue), D801-D807, doi:10.1093/nar/gks1065.

Worley, B., & Powers, R. (2013). Multivariate Analysis in Metabolomics. *Current Metabolomics, 1*(1), 92-107, doi:10.2174/2213235X11301010092.

Wu, C., Spongberg, A. L., Witter, J. D., Fang, M., Ames, A., & Czajkowski, K. P. (2010a). Detection of Pharmaceuticals and Personal Care Products in Agricultural Soils Receiving Biosolids Application. *CLEAN – Soil, Air, Water, 38*(3), 230-237, doi:10.1002/clen.200900263.

Wu, C., Spongberg, A. L., Witter, J. D., Fang, M., & Czajkowski, K. P. (2010b). Uptake of pharmaceutical and personal care products by soybean plants from soils applied with biosolids and irrigated with contaminated water. *Environmental Science & Technology, 44*(16), 6157-6161.

Wu, G. (2013). *Amino Acids: Biochemistry and Nutrition*. Boca Raton, FL: CRC Press.

Wu, J.-l., Liu, J., & Cai, Z. (2010c). Determination of triclosan metabolites by using in-source fragmentation from high-performance liquid chromatography/negative atmospheric pressure chemical ionization ion trap mass spectrometry. *Rapid Communications in Mass Spectrometry, 24*(13), 1828-1834, doi:10.1002/rcm.4558.

Wu, Q., Yang, Q., Zhou, W., & Zhu, L. (2015a). Sorption characteristics and contribution of organic matter fractions for atrazine in soil. *Journal of Soils and Sediments, 15*(11), 2210-2219, doi:10.1007/s11368-015-1162-y.

Wu, X., Dodgen, L. K., Conkle, J. L., & Gan, J. (2015b). Plant uptake of pharmaceutical and personal care products from recycled water and biosolids: a review. *Science of The Total Environment, 536*, 655-666.

Wu, Y., & Li, L. (2016). Sample normalization methods in quantitative metabolomics. *Journal of Chromatography A, 1430*, 80-95, doi:http://dx.doi.org/10.1016/j.chroma.2015.12.007.

Xia, J., Sinelnikov, I. V., Han, B., & Wishart, D. S. (2015). MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucleic Acids Research, 43*(W1), W251-W257, doi:10.1093/nar/gkv380.

Xia, K., Hundal, L. S., Kumar, K., Armbrust, K., Cox, A. E., & Granato, T. C. (2010). Triclocarban, triclosan, polybrominated diphenyl ethers, and 4-nonylphenol in biosolids and in soil receiving 33-year biosolids application. *Environmental Toxicology and Chemistry, 29*(3), 597-605, doi:10.1002/etc.66.

Xing, B. (1997). The effect of the quality of soil organic matter on sorption of naphthalene. *Chemosphere, 35*(3), 633-642, doi:http://dx.doi.org/10.1016/S0045-6535(97)00125-2.

Xue, Y., Gu, X., Wang, X., Sun, C., Xu, X., Sun, J., et al. (2009). The hydroxyl radical generation and oxidative stress for the earthworm Eisenia fetida exposed to tetrabromobisphenol A. *Ecotoxicology, 18*(6), 693-699, doi:10.1007/s10646-009-0333-2.

Yang, X., Song, Y., Kai, J., & Cao, X. (2012). Enzymatic biomarkers of earthworms Eisenia fetida in response to individual and combined cadmium and pyrene. *Ecotoxicology and Environmental Safety, 86*, 162-167, doi:http://dx.doi.org/10.1016/j.ecoenv.2012.09.022.

Ying, G.-G., & Kookana, R. S. (2007). Triclosan in wastewaters and biosolids from Australian wastewater treatment plants. *Environment international, 33*(2), 199-205, doi:http://dx.doi.org/10.1016/j.envint.2006.09.008.

Yuk, J., McKelvie, J. R., Simpson, M. J., Spraul, M., & Simpson, A. J. (2010). Comparison of 1-D and 2-D NMR techniques for screening earthworm responses to sub-lethal endosulfan exposure. *Environmental Chemistry, 7*(6), 524-536.

Yuk, J., Simpson, M. J., & Simpson, A. J. (2011). 1-D and 2-D NMR metabolomics of earthworm responses to sub-lethal trifluralin and endosulfan exposure. *Environmental Chemistry, 8*(3), 281-294.

Yuk, J., Simpson, M. J., & Simpson, A. J. (2012). Coelomic fluid: a complimentary biological medium to assess sub-lethal endosulfan exposure using 1H NMR-based earthworm metabolomics. *Ecotoxicology, 21*(5), 1301-1313.

Yuk, J., Simpson, M. J., & Simpson, A. J. (2013). 1-D and 2-D NMR-based metabolomics of earthworms exposed to endosulfan and endosulfan sulfate in soil. *Environmental Pollution, 175*, 35-44.

Zhang, L., Duan, X., He, N., Chen, X., Shi, J., Li, W., et al. (2017). Exposure to lethal levels of benzo[a]pyrene or cadmium trigger distinct protein expression patterns in earthworms (Eisenia fetida). *Science of The Total Environment, 595*, 733-742, doi:http://dx.doi.org/10.1016/j.scitotenv.2017.04.003.

Zhang, L., Liu, X., You, L., Zhou, D., Wu, H., Li, L., et al. (2011). Metabolic responses in gills of Manila clam Ruditapes philippinarum exposed to copper using NMR-based metabolomics. *Marine Environmental Research, 72*(1), 33-39, doi:http://dx.doi.org/10.1016/j.marenvres.2011.04.002.