Petroleum Hydrocarbon Persistence and Toxicity to Soil Dwelling Organisms in Canadian Soils

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Abstract

Petroleum hydrocarbon contamination remains a major environmental issue in Canadian soils. Site-specific risk assessments provide unique opportunities to investigate both toxicity and persistence of the contaminant, and to include the latest advances in ecotoxicology such as new test species or accounting for mixture toxicity. The main objectives of this thesis were to investigate the persistence and toxicity of a medium fraction petroleum hydrocarbon (PHC) product (lubricating oil) to soil-dwelling organisms in both standardized and Canadian field soils.

First, the toxicity of PHC-contaminated soils to a suite of standardized soil invertebrate test organisms was investigated in a standardized test soil. Acute and chronic toxicity tests were conducted on the following test organisms exposed to soil contaminated with a medium fraction PHC mixure: *Eisenia fetida, Lumbricus terrestris, Enchytraeus crypticus, Folsomia candida, Oppia nitens* and *Hypoaspis aculeifer*. Mortality and inhibition of reproduction occurred in all test species exposed to PHC-contaminated soils, with the exception of lethality to *E. crypticus*. Interspecies differences in toxic responses were reflected in unique traits. In regard to mixture toxicity, both reference models (concentration addition and independent action) provided the best fit, however, concentration addition provided superior predictions of the individual fraction effective concentrations.

Following the investigation into toxic effects of PHC-contaminated soils on soil invertebrate mortality and reproduction, the toxic effects to plants and impacts on soil invertebrates' behaviour were assessed. Two tree species (*Pinus banksiana* and *Picea glauca*) were included in testing along with the more common garden *(Lactuca sativa* and *Raphanus sativus)* and agronomic *(Elymus lanceolatus* and *Medicago sativa)* species. Behavioural responses to PHC-contaminated soils were tested in five soil invertebrate test species *(E. fetida, E. crypticus, F. candida, O. nitens and H. aculeifer)* in an avoidance test system. The key finding from this study was soil invertebrate avoidance of PHC-contaminated soil was in a similar range of toxicity values for growth endpoints of plant species sensitive to PHC-contaminated soils.

The third chapter represents a synthesis of the previous chapters as well as laboratory testing of persistence and toxicity of PHCs to soil dwelling organisms in Canadian field soils. The study explored numerous themes. The primary objective explored the impact of guidelines protective of soil-dwelling organisms on two soil invertebrates (*F. candida* and *O. nitens*) in a range of field soils. The study findings indicated that current approaches to establishing guidelines in Canada provided low levels of protection to soil invertebrates in soils lacking organic matter. The study investigated an alternative option to overcome this flaw; through the incorporation of organic carbon normalization into soil invertebrate and plant PHC toxicity data. Organic carbon normalization reduced variability in PHC toxicity data as well as provided greater protection to soil-dwelling organisms in low organic matter soils. The compilation of these findings highlights how Canadian regulations on deriving soil guidelines for PHC-contaminated soils protective of soil dwelling organisms should be revisited and consider incorporating soil organic carbon content as a modification option.

The last chapter of this thesis details one of the first studies to assess the juvenile avoidance response of soil invertebrates to contaminated soils. The differences in avoidance response to sodium chloride, phenanthrene and copper-contaminated soils in three (*E. fetida, E. crypticus, F. candida*) adult and juvenile soil invertebrates was investigated. The study had variable and inconsistent results; the juvenile's avoidance response could be more sensitive, less sensitive and the same as the adult's avoidance response, depending on the contaminant and test species. The major finding was the assumptions that juveniles are the most sensitive individuals in a population cannot be assumed for behavioral responses to contaminated soils. In addition, sodium chloride proved to be an ideal reference toxicant for avoidance tests.

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The University of Saskatchewan resides on Treart 6 Territory and Homeland of the Metis. I pay respect to the Indigenous peoples of this land and recognize I reside on this stolen land due to historical and current oppression and genocide of Indigenous people.

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List of Abbreviations

ATSM	American Society for Testing and Materials
CA	Concentation Addition
CBR	Critical body residue
CCME	Canadian Council of Ministers of the Enviroment
DNA	deoxyribonucleic acid
EC	Environment Canada
EC25 or 50	Effective concentration (25 or 50% reduction relative to control response)
EU	European Union
F1	Fraction 1 (consisting of hydrocarbons nC6 to C10)
F2	Fraction 2 (consisting of hydrocarbons >C10 to C16)
F3	Fraction 3 (consisting of hydrocarbons >C16 to C34)
F4	Fraction 4 (consisting of hydrocarbons >C34)
GC FID	Gas chromatography flame ionization detection
GC/MS	Gas chromatography mass spectrometry
HC	Hazard concentration
LC25 or 50	Lethal concentration causing mortality (25 or 50% reduction relative to control response)
IA	Independent Action
ISO	International Organization for Standardization
OECD	Organization for Economic Cooperation and Development
OM	Organic Matter
PAH	Polycyclic aromatic hydrocarbons
PHC (s)	Petroleum hydrocarbon (s)
ROS	Reactive oxygen species
SSD	Species sensitivity distribution
SSRA	Site specific risk assessment
TPH	Total petroleum hydrocarbons
US EPA	United States Environmental Protection Agency

1. Introduction

The overall goal of this research was to develop a site-specific risk assessment (SSRA) and in-situ remediation options for a range of Canadian soils with petroleum hydrocarbon (PHC) contamination. The PHC product utilized in this study was a lubricating oil, often released from equipment with hydraulics like pipeline compressor station gear or automatic gates, resulting in surficial soil contamination. This thesis mainly encompassed the development of a soil remediation guideline protective of soil dwelling organisms (soil invertebrates and plants) as well as a persistence assessment. Additionally, juvenile and adult soil organisms' behavioural responses to soils contaminated with a variety of contaminants was assessed.

Petroleum hydrocarbon products, including lubricating oils, are complex mixtures of individual aliphatic and aromatic hydrocarbons. Based on Canadian regulations classification of PHCs, lubricating oil contaminated soils are mainly composed of F2 (>C10-C16) and F3 (>C16-C34) fractions. Currently, these fractions are regulated based on single toxicity and do not account for potentially greater toxicity that may occur when the mixture of these fractions contaminate soils. Further studies on medium (F2 and F3) PHC mixture toxicity will provide a scientific basis for incorporating mixture toxicity into remediation guidelines and ecological risk assessments.

Under most land uses, the surficial remediation guidelines for F2 and F3 PHC fractions are limited by the exposure pathway protective of soil organisms. Currently, the only means to modify the protection of a soil organisms exposure pathway involves conducting a SSRA. Site-specific risk assessment for this exposure pathway relies heavily on toxicity test data on soil-dwelling organisms, sourced from literature and/or from laboratory toxicity testing. A benefit to this approach is the toxicity test data utilized reflects current trends in soil ecotoxicology literature. For instance, the guidelines for the protection of soil organisms to PHC-contaminated soils was last updated in 2008 and lacks consideration of soil invertebrates and plants from a range of environmental regions (CCME 2008). Since 2008, numerous boreal forest species have been introduced into toxicity testing literature as well as a behavioural tests are gaining popularity in both aquatic and soil toxicology (Princz et al 2010; Princz et al 2012; Owojori et al 2012).

Each manuscript in this thesis was published. Contents of Manuscript 1 were published in Environmental Toxicology and Chemistry, Manuscript 2 in the Journal of Hazardous Materials, Manuscript 3 was accepted to Science of the Total Environment and Manuscript 4 was published in Chemosphere.

The objectives of Manuscript 1 include:

- determine the toxicity of the whole PHC mixture to soil organisms from a range of habitats and with diverse taxonomy;
- explore the suitability of a trait-based approach in explaining interspecies differences in toxic responses to the PHC-contaminated soil observed; and,
- assess mixture toxicity of whole PHC mixture and estimate the individual F2 and F3 PHC toxicity to the test organisms.

The objectives of Manuscript 2 include:

- determine the toxicity of the whole PHC mixture to plant species from a range of habitats and with diverse taxonomy;
- evaluate the avoidance response of numerous soil organisms to PHC-contaminated soils;
- assess mixture toxicity of whole PHC mixture and estimate the individual F2 and F3 PHC responses of the plants and soil organisms avoidance response to PHC-contaminated soils; and,
- explore the suitability of a trait-based approach to explaining interspecies differences in plant species toxic responses to the PHC-contaminated soil.

The results from Manuscript 1 and 2 were used in combination with available literature to construct a species sensitivity distribution curve to derive a remediation guideline in accordance with Canadian regulatory guidelines. The objectives of Manuscript 3 were:

- conduct an extensive literature review for available soil invertebrate and plant toxicity data to medium PHCs (F2 and F3) mixtures, and single F2 and F3 fraction PHCs;
- normalize available data to soil organic carbon content to assess if normalization decreases variation;

- construct a species sensitivity distribution curve (SSD) and determine various hazard concentrations levels using total and carbon normalization PHC concentrations;
- assess the toxicity of the Canadian regulations hazard concentration levels from the SSD constructed with total concentrations in a range of field soils to soil invertebrates in field soils; and,
- assess persistence of whole PHC product in field soils with and without fertilizer additions.

Manuscript 4 involved assessing the avoidance response of juvenile soil invertebrate species commonly used in literature. In a population of organisms, juveniles represent a sensitive subset, more vulnerable than adults to toxic effects from contaminants. Currently, minimal literature is available about the behavioural response of juvenile soil organisms to contaminated soils. The objectives of Manuscript 4, were:

- to determine juvenile and adult soil invertebrate avoidance response of three taxonomically varying soil invertebrates to three contaminants in soil; and,
- compare the juvenile's response to that of the adults to assess juvenile avoidance response sensitivity.

2. Literature Review

2.1 Introduction

Due to extensive oil and gas activities, widespread surface and subsoil PHC contamination exists in Canada. Canadian regulations allow numerous approaches to managing PHC-contaminated soils including SSRA to develop guidelines for specific exposure pathways. This approach is advantageous in situations for exposure pathways that cannot be modified at the Tier 2 level, such as the protection of soil-dwelling organisms for F2 and F3 PHCs.

Site-specific derivation of the soil exposure pathway protective of soil dwelling organisms involves compiling toxicity test data on soil organisms and plants. Once collected, the toxicity tests measurement endpoints form a species sensivity distribution (SSD) curve. Posthuma et al (2002) defines an SSD as "a statistical distribution estimated from a sample of toxicity data and visualized as a cumulative distribution function". The result from constructing an SSD is a toxicant concentration protective of a select community of species reflected by the exposure pathway; i.e. soil dwelling organisms. Species sensitivity distributions incorporate concepts of the probability and magnitudes of effects as well as capturing that species differ in sensitivity (Posthuma and Suter 2011). In Canada, the data points for an SSD include the 25% percentile causing adverse effects relative to the control. For instance, the concentration that caused 25% mortality in a soil invertebrate species. Following this, ranking of data points as a function of concentration occurs and different points of the SSD curve represent the resulting guideline, which Canadian regulations dictates depends on the land use. In Canada, for industrial and commercial land uses, the remediation guideline corresponds to the 50% hazard concentration and for more sensitive land uses, like residential or agricultural, 25% is applied (CCME 2006). The major limitation surrounding use of SSDs in risk assessment is they are inherently statistical models with no ecological interactions and they fail to explain the differences in measurement endpoints or attempt to simulate soil ecology (Posthuma et al 2001). Although limitations exist, SSDs reflect the best method "that can be practically applied at low cost to many practical ecological risk assessment problems" (Posthuma and Suter 2011).

Regulations in Canada outline guidance on the test and test species selection process for constructing an SSD for site-specific derivation of guidelines protective of soil dwelling organisms (CCME 2006). The regulations (CCME 2006) state that at least ten data points from three tests are needed, with a minimum of two soil invertebrates and two plant data points. This contrasts other regulations, like in the European Union (EU), where at least 10 data points (15 preferred) as no observed effect concentration for species covering eight taxonomic groups are required for an SSD (Posthuma and Suter 2011).

Although Canadian regulations provide guidance in terms of the number of data points and type of test organisms for SSD compilation, they do not indicate requirements for the ecological relevance of test species. Aquatic ecotoxicologists debate over the test species data used for SSD construction and whether assemblages should reflect the ecosystem or geographic regions that the guideline is being derived for or whether a mix of species across varying regions (Posthuma et al 2001; Maltby et al 2005; Hose and van den Brink 2004; Smith and Cairns 1993). For instance, marine and freshwater test species have distinct SSDs, however, temperate region SSDs often include tropical species. Studies on pesticides in freshwater systems found no differences between SSDs constructed with consideration of geographical regions, even for island geographical regions such as Australian fish and arthropod species (Maltby et al 2005; Hose and van den Brink 2004). Few literature studies explore whether this trend occurs in soil ecosystems (Princz et al 2012). Canada provides a unique case to investigate this because earthworms, the most common soil ecotoxicology test species, are absent and considered invasive to northern boreal forest regions of Canada.

When creating a guideline to protect soil organisms, the data used in its derivation should reflect the receptors we wish to protect. Soil organisms contribute to numerous soil functions, including decomposition of organic matter, and improvement of soil structure and water retention. Jansch et al (2005) proposed four important considerations when selecting tests and species for development of soil quality guidelines that reflect functioning and biological trophic levels. These included the use of primary producers, like plants, species that fragment different sizes of organic matter and improve soil structure, like earthworms, and a mix of prey-predator species

(Jansch et al 2005). Van Gestel et al (1997) suggested the following general criteria for the test and species selection when deriving soil ecotoxicology guidelines:

- feasibility or cost-effectiveness;
- acceptability including the standardization, reproducibility and statistical validity;
- ecological relevance, trophic level and sensitivity;
- representation of different life histories, taxonomic groups and routes of exposure; and,
- the range of responses from an organism and that the responses are relevant for the protection of the ecosystem.

2.2 Petroleum Hydrocarbon Toxicity and Degradation

Petroleum hydrocarbon products include a diverse mixture of individual PHCs. Aliphatic PHCs consist of straight chains with a range of carbon lengths, from a few up to 50 (CCME 2008). Aliphatic PHCs produce toxic effects via non-polar narcosis, or baseline toxicity; a common mechanism of toxic action associated with lipophilic contaminants (Cowgill and Williams 1989; Sverdrup et al 2001). Narcotic contaminants do not interact specifically with receptors in the organisms, rather, they accumulate within cell membranes leading to interferences with cell membrane functioning and processes. Since the cellular membrane lipids are the target for narcotics, the organism lipid content influences mortality (Sverdrup et al 2002c). Lassiter (1990) proposed the "survival of the fattest" concept; species with high lipid contents withstand higher internal concentrations of narcotic contaminants in acute tests (Geyer et al 1994). An internal threshold of approximately 3 mmol/kg was proposed to be the level likely to cause mortality (CCME 2008; Baas et al 2015). In addition to aliphatics, PHC products also include aromatic PHCs whose mechanisms of toxic action differs from aliphatics.

Unlike straight chain aliphatics, aromatic PHCs form single or multipe rings, associated with a different mechanism of toxic action. Multiple aromatic PHCs, such as polyaromatic hydrocarbons (PAHs), undergo biotransformation by Phase 1 and 2 enzymes including cytochrome P450 (CYP) oxygenases and glutathione-S-transferases (Baird et al 2005). The majority of aromatic PHCs undergo detoxification, however, under certain conditions toxic effects arise. Aromatic hydrocarbons are bioactivated by CYP enzymes, forming reactive

compounds with the ability to form covalent bonds with target molecules like DNA, proteins or lipids, causing adverse effects. Bay region PAHs, like benzo(a)pyrene form DNA adducts, causing mutations and potential cancer formation. Oxidative stress results from an imbalance in reactive oxygen species (ROS) within an organism, causing cell death. Reactive oxygen species include superoxide, peroxide, hydroxyl radical and singlet oxygen. The ROS are produced in response to PAH metabolites like phenanthrene and fluoranthene or as a general response to internal stress from accumulation of aliphatic PHCs in lipids (Kaur et al 2017; Liu et al 2009). The ROS can also be released from narcotic lipid damage to membranes, specifically, damage to peroxisome membranes that store ROS (Kaur et al 2017; Chen et al 2013). Under stressed conditions, an imbalance between bioactivated metabolites and detoxification compounds, like glutathione, may result in toxic effects.

The toxicity of PAHs to soil invertebrates ranges, largely depending on the type of PAHs. Across most test species, large chain PAHs like benzo(a)pyrene produce low levels of toxicity (Owojori and Siciliano 2012) and low internal concentrations (Owojori and Siciliano 2012; Sverdup et al 2007), indicating low exposure levels, likely due to their high affinity for organic matter and low water solubility. Polycyclic aromatic hydrocarbons with fewer chains and hgher water solubility, like pyrene and phenanthrene, result in greater toxic effects across most soil invertebrate test species (Sverdup et al 2001; Sverdrup et al 2002a; Sverdrup et al 2002b; Sverdrup et al 2002c; Sverdrup et al 2007; Princz et al 2010). Stroomberg et al (2004) found numerous metabolites in the hepatopancreas of three soil invertebrate test species (*E. andrei*, *F. candida*, *P. scaber*) exposed to pyrene including 1-hydroxypyrene, pyrene-1-glucoside and pyrene-1-sulfate. However, the same study (Stroomberg et al 2004) found low phase 2 conjugation metabolite (pyrene-1-glucuronide) from glucuronidation in the three-test species. Similarly, Nota et al (2009) reported up-regulation of numerous Phase 1 and 2 xenobiotic biotransformation enzymes followed *F. candida* exposure to phenanthrene-contaminated soils. Although toxicity and the log k_{ow} of narcotic organic chemicals are often positively correlated in aquatic toxicity studies, Sverdrup et al (2002c) found this trend did not occur for many PAHs in soils.

Petroleum hydrocarbon-contaminated soils impact plants through additional mechanisms. Kaur et al (2017) categorized the effects into three categories: oxidative stress, water, oxygen and

nutrient limitations, and penetrative damage. The formation of hydrophobic films around the seed and root surfaces prevents necessary interactions at these surfaces such as water or gas exchange (Adam and Duncan 2002; Chaineau et al 1997; Merckl et al 2005). Internal damage to seeds occur in PHC-contaminated soils when PHC migrate into the seed and embryonic tissue, inhibiting growth and development (Kaur et al 2017).

Although PHC-contaminated soils produce toxic effects in many soil-dwelling organisms, PHCs degrade in surficial soils, via abiotic and biotic losses (Fine et al 1997). Following the initial release of PHC into the soil, the primary abiotic loss pathway for medium PHC mixtures is through volatilization (Fine et al 1997; Wang et al 2006). Biotic losses of PHC in soils occur via microbial breakdown of PHCs (Chang et al 2010). To ensure sustained biodegradation and prevent stagnation, a common bioremediation practice involves supplementing PHC-contaminated surficial soils with nutrients to support microbial activity. Numerous studies highlight how nitrogen and phosphorus increase microbial degradation of PHC in soils contaminated with medium PHCs (Walworth and Reynolds 1995; Chang et al 2010; Walworth et al 2007; Azubuike et al 2016; Gkorezis et al 2016; Kim et al 2018). The degree and rate of abiotic and biotic of PHC in soils is also influenced by environmental factors affecting the contaminant bioavailability, "the quantity of a contaminant which is freely available to cross the cellular membrane of an organisms from the surrounding medium", and bioaccessibility, "the quantity of contaminant potentially available to cross an organisms cellular membrane from the surrounding medium" (Gkorezis et al 2016; Semple et al 2017; Dandie et al 2010; Fine et al 1997; Chemlal et al 2013; De La et al 2006; Gkorezis et al 2016; El-Sheshtawy and Ahmed 2017). Both bioavailability and bioaccessibility of PHCs in soil is reduced when PHCs are highly sorbed to soil materials with a high affinity for PHCs such as clay and organic matter (Fine et al 1997; Cabrerizo et al 2011; El-Sheshtawy and Ahmed 2017; Fine et al 1997; Alexander 2000). Thus, the persistence, and duration of toxic effects a soil-dwelling organism may experience, is influenced by soil properties.

2.3 Toxicity Test Selection

Construction of SSDs for protection of soil dwelling organisms involves toxicity testing on soil invertebrates and plant species and/or use of toxicity test data from literature (CCME 2006; van Gestel et al 1997). The most common soil invertebrate toxicity tests assess the survival and reproduction of a population of individuals in contaminated soils. Mortality tests determine the survival of a population of individuals, expressed as the lethal concentration (LC) causing death in a specified percent, i.e. 50%, of test organisms relative to the control (EC 2014). The effects of contaminated soils on soil organisms' ability to reproduce are often expressed as an effective concentration (EC_{xx}) causing a specified percent reduction in juvenile production relative to the control. For larger soil invertebrates like earthworms, growth reductions based on mass loss in adults or juveniles represents an additional test endpoint. Most soil invertebrate toxicity tests require 28 days for assessing both mortality and reproduction, while some test species require shorter or longer durations (EC 2004).

Behavioural alterations in an organism can provide indications of sublethal effects. Changes in behaviours of organisms from exposure to contaminated in their habitat are important to species survival because behavioural changes alter important functions such as energy expenditures or the ability of an organism to obtain food or a mate. Examples of behavioural responses studied in ecotoxicology include avoidance or escape, balance, burrowing, feeding, courtship, memory learning and nesting (Hellou et al 2011). In soil ecotoxicology literature, the most common behavioral test is the avoidance response of organisms in a dual test system.

Soil avoidance tests represent a type of behavioural assay where a population of individuals face dual options, to reside in contaminated soil or in uncontaminated soil. Unlike mortality and reproduction tests, the organisms do not undergo forced exposure to contaminated soils, a scenario reflective and informative of field conditions (Aruajo et al 2014). The avoidance of contaminated zones may result in local population declines; a similar effect on populations as mortality (Aruajo et al 2014). Alternatively, mortality may even occur following avoidance behaviour. For instance, if the concentration when an avoidance response occurs is greater than the concentration that causes mortality and reproductive impairment, the organisms experience stress which may result in reduced number of individuals in the population surrounding the

contaminated soils, with less individuals available for recolonization. However, in certain situations, non-avoidance or attraction to contaminated soils occurs, corresponding to larger population reductions. For some contaminants, the avoidance response is the most sensitive test endpoint, with similar values to reproduction (Owojori and Reinecke 2009; Owojori et al 2011; Owojori et al 2014). Other advantages to conducting soil avoidance response tests include the simplicity of the tests. The dual tests involve simple test containers and quick test duration (maximum 2 days) (van Gestel 2012; Owojori et al 2014; ISO 2011).

Studies suggest avoidance behaviors to contaminated zones reflects a defense mechanism. By avoiding contamination, organisms reduce exposure and the potential onset of toxic effects (Hellou 2011; Aruajo et al 2014; Araujo et al 2016a). Thus, these authors suggest avoidance is a form of habitat selection or preference by organisms. To quote Araujo et al (2016b): "Contaminants should not be viewed solely as potential toxicants at the individual level but also as potential disturbers of habitats by making the latter, at least partially, unsuited to accommodate life". Contaminants create poor habitat by causing toxic effects as well as disrupting spatial distribution patterns (Aruajo et al 2014; Araujo et al 2016a). Perhaps most concerning is organisms that fail to avoid contaminants, like some enchytraeids to boric acid (Bicho et al 2014), as lethality and impacts on reproduction are inevitable.

The exact mechanisms triggering the avoidance responses of soil invertebrates to contaminated soils is currently not well studied for PHCs. Different categories of molecules are detected by different chemosensing organs. Aquatic ecotoxicologists attribute avoidance behavior primarily to olfactory and gustatory cells within organisms (Hellou et al 2011). Detection of contaminants by organisms is more complicated in soils where contaminants can partition to three potential phases (air, water and solids) (Bargmann and Horvitz 1991). Research on nematodes suggests volatile contaminants are detected by olfactory neurons while water-soluble contaminants, like salts and metals, are detected through gustatory neurons, however, the mechanisms are not well understood (Bargmann and Horvitz 1991; Bargmann et al 1993; Zirbes et al 2011; Sambongi et al 1999). In nematodes, olfactory chemosensing is related to the G protein-coupled receptors in olfactory epithelium cells (Bargmann and Horvitz 1993). In *Enchytreasus crypticus*, non-avoidance to boric acid was attributed to an anaesthetic effect via the gamma-aminobutyric acid

receptor (Bicho et al 2014). However, there are studies that indicate nematode chemosensing is contaminant specific. Sambongi et al (1999) found nematodes avoided cadmium and copper, but not nickel, due to multiple neural pathways in the amphid, the olfactory sensory organ of nematodes where metals detection occurs. In addition, chemosensing appears to also vary across species. While nematodes showed avoidance to cadmium and copper (Sambongi et al 1999), *F. candida* avoids copper but not cadmium (Greenslade and Vaughan 2003).

Soil ecotoxicology literature currently lacks toxicity or behavioural testing on juveniles, with the majority of standardized tests utilizing sexually mature sub-adults or adults (EC 2004). However, juveniles reflect an important demographic of populations (Heckmann et al 2005). Juvenile earthworms in agricultural soils compose up to 80% of the population (Peigne et al 2009; Schmidt et al 2003; Pelosi 2015) and juvenile oribatid mites account for approximately one-third of the total population (Norton 1994). In addition, juvenile organisms often display greater sensitivity to contaminants than adults. A meta-analysis on aquatic species in literature across several contaminants determined that for 92% of contaminants, juvenile fish were more sensitive than adults (Hutchinson et al 1998). The same trend follows in aquatic invertebrates. Fourth instars of *Chironomus tetans* were 12-27 times more resistant to copper than the younger first instars (Gauss et al 1985), and larger juveniles and adults of crustacean, *Asellus aquaticus*, were more tolerant to cadmium than smaller (Green et al 1986). The trend appears consistent in soil ecotoxicology. Egg hatching and growth of juvenile *E. crypticus* were more sensitive than adult mortality in cadmium-contaminated soil (Bicho et al 2015). Currently, minimal studies on juvenile soil invertebrate avoidance response to contaminants exist. The existing studies concern one test species, earthworms, and one contaminant group, organophosphate pesticides (Jordaan et al 2012; Hodge et al 2000). In both studies juveniles displayed non-avoidance behaviour due to lack of mobility from the inhibitionof acetylcholinesterase.

2.4 Species Selection

A battery of soil toxicity tests includes numerous test species, representing species commonly found in soils or representing species functions in the soil ecosystem. Species from both soil invertebrates and plants are required for construction of SSDs to create a guideline protective of

the soil community (CCME 2006). The main considerations when determining species selection were: ecological relevance across land uses, standardized protocols, varying trophic levels and feasibility under laboratory conditions (van Gestel 2012).

2.4.1. Soil Invertebrates

Soil invertebrates represent the biological component of soils and influence soil quality (EC 2014). Soil toxicity testing is typically conducted on meso and macrofauna including earthworms, enchytraeids, Mollusca, mites, isopods, Collembola, insects and carabid beetles (van Gestel 2012; Orgiazzi et al 2016). True soil dwellers, like Collembola, are preferred to surficial litter dwellers like beetles because they undergo higher contact with contimanat in soil (van Gestel et al 2011). The most popular and well-represented test species in literature is earthworms, especially *E. fetida or E. andrei*. Most mesofauna species graze on bacteria, decaying plant matter and fungal mycelia; however, predatory species also exist (Orgiazzi et al 2016).

2.4.1.1. Earthworms

Earthworms (Annelida: Oligochaeta) represent the most commonly studied soil invertebrate, macrofauna test species in soil ecotoxicology literature. Three functional groups of earthworms exist; epigeic who live in surficial litter; anecics who feed on surface litter but reside in soil and endogeic who solely feed on soil (Orgiazzi et al 2016). Earthworms consume large amounts of organic matter and produce simpler organic material that benefits other macro and microfauna (Lavelle 1997). Earthworms represent long-lived soil invertebrates, ranging from four to five years in *Eisenia fetida* and up to 6.5 years in *Lumbricus terrestris* (EC 2004). Earthworm burrowing activity and cast production improve soil structure, organic matter quality and infiltration of water. Dermal contact is the main route of contaminant uptake for earthworms, however, they also receive exposure orally through soil ingestion (Jager et al 2003; Vijver et al 2003). Earthworms' respiration is through their skin, which is always moist. Hence, earthworms require soils to be moist and cannot inhabit water logged, oxygen-deprived soils (EC 2004). The cuticle, or external body covering, is soft and prone to dehydration, hence, this species resides in

soils with a moderate moisture regime (Orgiazzi et al 2016). They are consumed by many animals, reflecting a route for bioaccumulation at higher trophic levels. Interestingly, earthworms have been absent from North America since glaciation and are not true soil dwellers in Canada (EC 2004; Crouau et al 1999). Due to the invasion from the south, agricultural and residential Canadian soils typically contain earthworms, with the largest populations found in southern Ontario (EC 2004).

Image 1. Earthworm belonging to the family Microchaetidae. Source: Orgiazzi et al 2016.

Earthworm standardized toxicity tests have many endpoints including survival, reproduction, growth and avoidance (van Gestel 2012). Standardized protocols exist in Canada for mortality and reproduction for three species (*E. andrei*, *E. fetida* and *Lumbricus terrestris*) (EC 2004). *Eisenia fetida* is a surface soil-dwelling earthworm while *L. terrestris* is larger and inhabits subsoil. *Lumbricus terrestris* casts remain in subsoils, a major contributor to subsoil organic matter. *Lumbricus terrestris*'s reproduction is slow, approximately every 8 to 16 months, while *E. fetida* is relatively rapid. Hence, standardization of reproduction exists for *E. fetida* but not *L. terrestris*. Earthworm mortality, reproduction and growth are sensitive to PHC-contaminated soils (Cermak et al 2010; Princz et al 2012). Avoidance of *E. fetida* to PHC- and PAH-contaminated soils was observed by Hentati et al (2013).

2.4.1.2. Enchytraeids

Enchytraeids (Annelida: Oligochaeta) are a family of soil invertebrates with global distribution in soils across numerous terrestrial habits (Jansch et al 2005) (Image 2). They inhabit top soils where they contribute to decomposition and humification, often feeding on excretions from larger soil fauna like earthworms, or on bacterial and fungal mycelia (Cortest et al 1999; Jansch et al 2005; Orgiazzi et al 2016). They are closely related to earthworms in terms of possessing soft, moist cuticles and both taxonomically annelids. Also, like earthworms, enchytraeids require close associations with soil pore water to prevent desiccation since they respire through their skin; with a low tolerance to water logged, oxygen-deprived soil conditions (Jansch et al 2005). Their routes of exposure to contaminants are primarily dermal and intestinal (Lock and Janssen 2001; Rombke 2003). Enchytraeids are generally short-lived species, with a total lifespan of about 85 days (Jansch et al 2005). Enchytraeid species reproduce sexually, or through parthenogenesis or asexually via fragmentation (Jansch et al 2005; Orgiazzi et al 2016).

Image 2. *Enchytraeus albidus* in topsoil. Source: Orgiazzi et al 2016.

Enchytraeus crypticus is a small enchytraeid species commonly used in soil ecotoxicology studies. It has more practical advantages compared to another larger enchytraeid test species, *Enchytraeus albidus*, including higher reproductive rate, shorter generation times and the shorter test duration (Castro-Ferreira et al 2012; Rombke and Moser 2002). Both Organization for Economic Cooperation and Development (OECD) and International Organization for Standardization (ISO) have standardized procedures for *E. crypticus* and *E. albidus* mortality and

reproduction tests (ISO 2004; OECD 2004). Avoidance tests with both species are also well represented in literature (Amorim et al 2005a; Amorim et al 2008b).

Enchytraeids are relatively tolerant to PHC-contaminated soils. A three-week exposure of *E. crypticus* to phenanthrene resulted in no significant effects to adult survival but resulted in a decrease of juveniles with increasing phenanthrene concentrations (Castro-Ferreira et al 2012). Older studies with crude oil, hydraulic oil and fuel oil-contaminated soils in laboratory and field conditions observed survival tolerance of adult Enchytraeids (Giere and Pfannkuche 1982; Pirhonen and Huhta 1984). Of the limited avoidance studies with Enchytraeids and PHCs, Kobeticova et al (2010) reported enchytraeids as tolerant of PAH-contaminated soils.

2.4.1.3. Collembola

Collembola represents an abundant soil invertebrate group with global distribution (EC 2014) (Image 3). The genus *Folsomia* possess a furca, allowing them to jump from predators and giving them the common name of springtail (Fountain and Hopkin 2004). The standardized species, *F. candida* occurs in Canadian soils, making them an ecological relevant species for deriving ecological soil guidelines in Canada (EC 2014; Rombke et al 1996; Rombke et al 2006; Addison 1996). Most species consume fungal hyphae and spores, bacteria, and decaying plant material (Orgiazzi et al 2016). They are also prey for predators like mites, centipedes, spider and beetles (Lee and Widden 1996; Bilde et al 2000; OECD 2009; Hopkin 2000). Collembola contribute to soil functioning in numerous ways. Some research suggests populations of *F. candida* stimulate organic matter decomposition, as their presence in laboratory microcosms resulted in higher leachate nitrate concentration (Fountain and Hopkin 2004). Reproduction, mortality and avoidance are standardized tests available for *F. candida* (ISO 2011; EC 2014).

Image 3. *Folsomia candida* age synchronized to 10 to 12 days old. Source: EC 2014.

The biology of *F. candida* is unique, with many features making it an ideal toxicity test species. *Folsomia candida* reproduces parthenogenetically, without fertilization from males (EC 2014; Jansch et al 2005). Reproduction of *F. candida* occurs 12 to 16 days after hatching and the first egg laying is most often between 21 and 22 days (EC 2014; Spahr 1981). *Folsomia candida* represents a short-lived soil invertebrate test species, with a lifespan of approximately 140 days, depending on temperature (Fountain and Hopkin 2004). *Folsomia* molts frequently, up to 45 moults in a lifetime, a key route of excretion for contaminants. In addition to cuticle moults, the lining of the mid gut is also shed and voided with faeces, a major form of contaminant excretion since they store heavy metals in their mid-gut lining (van Gestel 2002). Collembola inhabit soil pore spaces and are exposed to contaminants through respiratory, dermal and oral contact. A key feature of *F. candida* influencing contaminant exposure is their ventral tube, a thin-walled vesicle on the ventral side of the organisms involved in fluid and electrolyte exchange as well as aids in adhesion to surfaces (Fountain and Hopkin 2004; Orgiazzi et al 2016). This unique feature provides facilitated exposure to contaminants dissolved in soil pore water.

The literature suggests Collembola are sensitive to PHC-contaminated soils. Mortality and impaired reproduction occurred in Collembola species exposed to PHC products similar in composition to diesel, in both aged and unaged scenarios (Angell et al 2012; Cermak et al 2010; Princz et al 2012). Reproduction of *F. fimetaria* was impaired by aged and unaged pyrene and

phenanthrene (Sverdup et al 2001; Sverdrup et al 2002a). In terms of behaviour, Greenslade and Vaughan (2003) found *F. candida* did not avoid naphthalene in solution.

2.4.1.4. Oribatid Mites

Oribatid mites represent one of the most abundant micro-arthropods in the boreal forests around the world (Crossley and Bohnsack 1960; Walter 1985; Heneghan et al 1999; Princz et al 2010)(Image 4). Oribatid mites contribute to the breakdown of soil organic matter and are an important part of the soil food web (Jansch et al 2005). They are found in the upper horizons of forest soils where organic matter decomposition occurs and they contribute to mineralization of organic matter as well as soil formation (Johnston and Crossely 2002; Coleman et al 2004). A unique feature of oribatid mites is that their exoskeletons are hard and often fossilise (Orgiazzi et al 2016). In addition, most mites are blind, and rely heavily on physical and chemical sensing of soil pore spaces, making them ideal test species for avoidance behavior.

Image 4. The oribatid mite *Tectocepheus velatus*. Source: Orgiazzi et al 2016.

The oribatid mite *O. nitens* is a recently standardized soil toxicity test species (Prinz et al 2010; EC 2018). *Oppia nitens* life history is characterized by slow metabolism and development, long life spans, and low fecundity (Lebrun and van Straalen 1995; Norton and Behan-Pelletrier 2009). In addition to survival and reproduction tests in the literature (Princz et al 2010), avoidance tests are another available test type for *O. nitens* (Owojori et al 2011). Huguier et al (2015) proposed

the primary exposure route for *O. nitens* in standardized laboratory toxicity tests when uncontaminated food is supplied is through gaps in their legs.

Oppia nitens sensitivity to PHC- and PAH-contaminated soils is variable. Princz et al (2012) observed adult mortality in PHC-contaminated soils *O. nitens* was tolerant, however, juvenile production was sensitive. While reproduction effects were apparent from *O. nitens* exposure to phenanthrene in soils, no effects were observed for benzo(a) pyrene (Owojori and Siciliano 2012). Avoidance has also been observed in *O. nitens* when exposed to dual avoidance options with phenanthrene-contaminated soils (Owojori et al 2011).

2.4.1.5. Predatory Mites

Hypoaspis aculeifer is currently the only predatory soil invertebrate species with standardization (OECD 2008). Predatory species are important to include in toxicity test batteries as they represent a higher trophic level, with potential for biomagnification (Image 5). *Hypoaspis aculeifer* resides in the organic-rich surface layers of agricultural or forest soils, with distribution in North America and Europe. In many countries, like the Netherlands, *H. aculeifer* provides pest control as it feeds on a variety of mesofauna like Collembola, snails, fungal gnats and other mites (Jansch et al 2005). *Hypoaspis aculeifer* primarily inhabits the aerated pore spaces within the soil. *Hypoaspis aculeifer* can reproduce sexually or by parthenogenesis when males are absent (Krogh and Axelsen 1998; Lesna and Sabeis 1999; Usher and Davis 1983). Like oribatid mites, predatory mites have limited vision, and depend on physical and chemical sensing soil pore spaces (Orgiazzi et al 2016). The duration of toxicity tests for *H. aculeifer* is shorter than other test species like Collembola, with a time frame of only 14 days (OECD 2008).

Image 5. The predatory mite *Dissoloncha superbus*. Source: Orgiazzi et al 2016.

Limited literature exists on *H. aculeifer* response to PHC-contaminated soils, with existing literature reflecting PAHs. One study concluded, *H. aculeifer* exposure to benzo(a)pyrene-contaminated resulted in minimal effects to reproduction (Sverdup et al 2007). *Hypoaspis aculiefer* is suitable for dual avoidance tests (Owojori et al 2014) and displayed avoidance to phenanthrene-contaminated soils in this test system (Owojori et al 2014).

2.4.2. Plant Species Selection

As primary producers, plants play a vital role in terrestrial ecosystems. Standardized protocols for plants exist across numerous regulatory bodies such as Environment Canada (EC), OECD, ISO and American Society for Testing and Materials (ASTM) (EC 2005a; ATSM 1999; ISO 1995; OECD 1984). A total of 12 terrestrial plant species and six boreal species are standardized by Canadian regulations (EC 2005a; EC 2013). Regulatory requirements vary on the minimum number of plant tests required for SSD derivation of guidelines protective of soil dwelling organisms. A minimum of two to five species is required for ecological risk assessment with more species required for pesticide testing approval (EC 2005a; ATSM 1999; ISO 1995; OECD 1984). Furthermore, regulatory agencies recommend a 1:2 ratio of monocotyledon to dicotyledon (CCME 2006), however, many standardized boreal forest test species do not meet this classification as they are gymnosperms with no cotyledons (EC 2005a; EC 2013).

Within the Canadian context, plant species diversity ranges depending on land use. Hence, the main consideration for plant species selection in this thesis reflects species that represent the land

uses in Canada where PHC contamination may occur such as residential, commercial, parkland, agricultural and natural lands. Boreal forest species were considered representative of natural land uses as well as residential, parkland and agricultural, since many boreal forest species are planted ornamentally across all these land uses, or for shelter belts on agricultural lands. Agronomic and garden vegetable species also reflect agricultural and residential land uses. The inclusion of boreal forest species in test selection is particularly important as some native boreal forest plants exhibit greater sensitivity than non-native forest plants (Princz et al 2012). The endpoints collected from the plant species are seedling emergence and plant growth as indicated by live shoot and root length and biomass. However, for SSD construction, germination and emergence are typically omitted as they are relatively insensitive parameters to soil contamination (CCME 2008; Checkai et al 2014).

Two boreal forest tree species native to Canada's boreal forest were selected, white spruce (*Picea glauca*) and Jack pine (*Pinus banksiana*) (Image 6). Both species inhabit varying niches of the boreal forest like uplands or lowlands. They are also routinely used as ornamental species in residential areas and urban parks (EC 2013). Boreal tree species are currently underrepresented in phytotoxicity literature and represent long-lived life history strategies. Both test species displayed moderate sensitivities to PHC-contaminated soils (Prinz et al 2012).

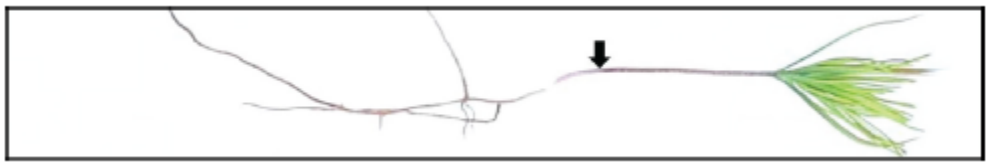

Image 6. Jack pine (*Pinus banksiana)* seedline with arrow showing transition point between shoot and root.. Source: EC 2013

The two agronomic species selected were alfalfa (*Medicago sativa*) and northern wheatgrass (*Elymus lanceolatus*) (Image 7). Alfalfa is an important forage legume, commonly grown on agricultural soils in Canada and around the world (EC 2005a). It is a perennial dicotyledonous (dicot) legume grown for livestock feed and improves soil quality with its atmospheric nitrogen-fixing abilities. Northern wheatgrass (*Elymus lanceolatus*) is a long-lived monocot grass with a

range of soil tolerances (EC 2005a). It is grown across North America for livestock forage, reclamation of disturbed areas and commonly included in sod seed mixes. Both alfalfa and northern wheatgrass are sensitive to PHC-contaminated soils (CCME 2008; Angell 2012)

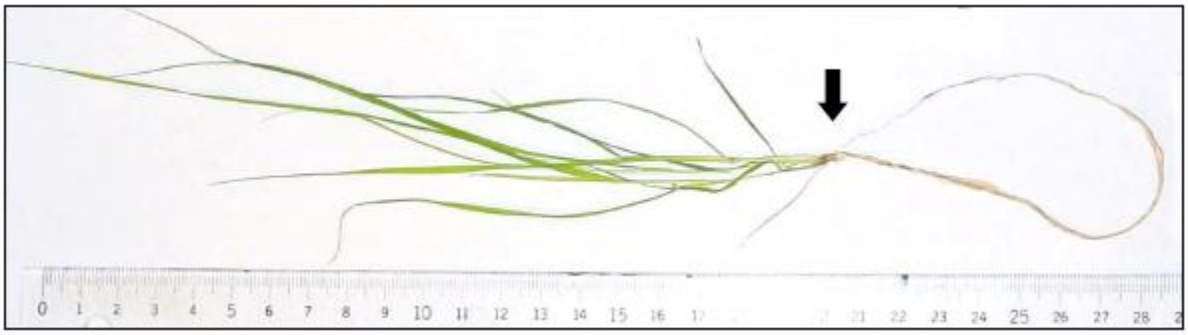

Image 7. Northern wheatgrass (*Elymus lanceolatus*) seedline with arrow showing transition point between shoot and root. Source: EC 2013

Two garden vegetable species were selected as test species, lettuce (*Lactuca sativa*) and radish (*Raphanus sativus).* Both species are standardized test species by Environment Canada (EC 2005a) and lettuce is well reflected in phytotoxicity literature. Lettuce represents an above-ground plant while radish is below ground, and both are dicotyledons. Both species are sensitive to PHC contamination, however, the majority of literature for these species is on germination rather than emergence or growth endpoints (Dorn et al 1998; Chatineau et al 1997; Efroymsom et al 2004; Banks and Schultz 2005)

2.5 Trait-Based Approach to Ecological Risk Assessment

One approach to understanding interspecies differences in responses to contaminants involves assessing organism traits. Rubach et al (2011) defined a trait as a "phenotypic or ecological character of organisms, generally measured at the individual level, but often applied as the mean state of a species". Van Straalen (1994) suggested two lines of traits are important for studying interspecies responses to contaminants; those involved with physiology and biochemistry, and those related to life history evolution. Van Straalen (1994), with amendments from Rubach et al (2011), suggested an organism's response to a contaminant was influenced by three groups of traits:

1) Traits influencing exposure; the extent an organism is exposed to a substance as well as the contaminant properties.
2) Traits related to intrinsic sensitivity like the organisms toxicokinetic and toxicodynamic responses to contaminants. Toxicokinetics reflects the absorption, distribution, biotransformation, elimination and bioaccumulation processes within the organisms. While toxicodynamic traits include those related to toxicity that occurs at the target site within the organisms and its compensation mechanisms to overcome toxic injury.
3) Traits important for population sustainability, or the ability of the population to overcome losses from contaminant injury.

Within the soil environment, the degree of exposure to a contaminant that a soil invertebrate receives varies due to a combination of both soil and contaminant properties (Janssen et al 1997; Beyer et al 1987). The soil is composed of three media; water, solids and air. The composition of these phases within a soil influenced by the soil's properties. Soil solids compromise both the mineral solids, such as clay, as well as organic materials, like organic carbon or humic acids. Contaminants partition to different soil media depending on their chemical properties, such as water solubility, Henry's law constant and octanol-water partition coefficients. For instance, water-soluble contaminants like sodium chloride mainly partition to soil pore water while a volatile organic like naphthalene will be in all three phases.

In addition to soil and contaminant properties, traits of the organism influence exposure. Traits such as habitat preference influence exposure to soil invertebrates (Rubach et al 2011; Van Straalen 1994). Food choice as a trait reveals potential exposure routes, however, since laboratory soil invertebrate toxicity tests supply uncontaminated food, ingestion is only considered an applicable exposure route for earthworms under standardized laboratory conditions. Another key trait governing the degree of exposure to soil invertebrates from contaminants in their habitat is body size. The surface area to volume ratio of organisms influences the amount of absorption, a particularly important feature of fish gills (Rubach et al 2011). Due to their large body size, earthworms dermally contact contaminants bound to soil solids as well as pore water. Whereas organisms with smaller bodies like Collembola reside mainly in pore spaces where a smaller fraction of their body is in contact with contaminated soil.

Traits associated with species intrinsic sensitivity also drive toxic responses in organisms, however, this information is less abundant for soil invertebrates (Rubach et al 2011, Van Straalen 1994). Toxicokinetic traits influencing dermal absorption and bioavailability of the toxicant to an organism include the permeability of the cuticle (or exoskeleton in mites). For soil invertebrates, the cuticle represents a biological barrier between external soil contamination and internal components (Rubach et al 2011). The permeability of the integument dictates soil habitat preferences as well as the degree of absorption. For instance, hard exoskeletons on soil invertebrates like mites reflects an adaptation to prevent desiccation and because of this feature they reside on soil pore spaces. Thus, organisms with soft cuticles that lack exoskeletons, such as Enchytraeids, require close contact with soil pore water. Furthermore, dermal absorption with soil invertebrates with exoskeletons is limited, likely occurring via the gaps in the exoskeletons (Huguier et al 2015). The intrinsic ability of organisms to biotransform contaminants varies across organisms and within families of organisms, although less well understood in soil invertebrates. For instance, the detoxification enzyme glutathione S transferases in soil organisms can be inhibited by contaminants (Oliveria et al 2015). Elimination rates are also an important trait that contribute to interspecies differences in toxic responses. From aquatic toxicity studies, some metal tolerant species report high elimination rates (Buchwalter et al 2008; Evans et al 2002), while moulting of the cuticle and gut epithelium are key excretion routes for metals in Collembola (Schmidt and Ibrahim 1994).

Traits related to toxicodynamic contribute to differences in toxic responses across species and individuals. These traits reflect the mechanism of action or defence system traits. Mechanism of action traits include receptors, transporters, enzymes or DNA interactions. The lipid content influences the accumulation of organic contaminants and resulting toxic responses across organisms (Rubach et al 2011; Hendriks et al 2001). Di Toro et al (2000) determined that the survival of organisms with higher lipid content was less sensitive to organic contaminants than lower lipid content species. Traits reflecting the defense of the organism to contaminant exposure include many detoxifying compounds like metallothionein, heat shock proteins and oxidative stress enzymes such a catalase or DNA polymerase. For instance, the activity of detoxification enzymes like catalase and superoxide dismutase influence toxic responses of soil invertebrates to heavy metal exposure (Maity et al 2008; Sahana et al 2014).

Life history traits inform an organism's life history strategy and ability to overcome population losses from contaminant exposure. Rubach et al (2011) suggested traits related to demographics as well as recolonization influence population sustainability. Life history traits are indicative of how an organism allocates its energy when faced with stressors such as exposure to toxicants. Often an organism will choose to invest energy into either the survival of individual animals, or survival of their offspring by increasing reproduction (Van Straalen 1994). Traits reflecting these investments include life span, generation time, voltinism and the number of offspring per reproductive event. Recolonization is the ability of organisms to maintain a population in a contaminated zone following losses of its members. Although not applicable to understanding interspecies differences from laboratory studies, recolonization is important for field monitoring of soil invertebrates at contaminated sites. Traits influencing recolonization include dispersal capacity as well as the mode of reproduction. For example, Collembola possess a waxy cuticle that allows them to float on water, an adaptation not only permitting survival during flooding but also recolonization into new habitats (Hopkins 1997). A reproductive mode such as parthenogenesis or fragmentation also aids the recolonization of species into disturbed habits. Mite species often colonize by wind, water or animal dispersal (Orgiazzi et al 2016).

Interspecies sensitivities to contaminants are often influenced by phylogenetic relatedness. The sensitives observed in species may be due to the tendency of related species to resemble each other (Blomberg et al 2003). This relatedness has been noted in aquatic toxicology. Buchwalter et al (2008) suggested similar metal elimination rates across varying species was due to phylogenetic relatedness.

2.6 Mixture Toxicity

Petroleum hydrocarbons represent a diverse mixture of aliphatic and aromatic structures with a range of carbon numbers. Regulatory agencies simplify management of PHCs by grouping them based on similar physical and chemical properties. In Canada, PHC groupings are as follows: fraction one (F1) composed of carbons in the 6 to 10 range, fraction two (F2) are carbons in the greater than 10 to 16 range, fraction three (F3) includes carbons greater than 16 to 34 and

fraction four (F4) contains carbons greater than 34 carbons to 50 (CCME 2008). Although most contaminated sites are a mixture of fractions, regulations do not account for potentially greater toxicity that may occur when a mixture of these fractions contaminate soils.

Two models dominate mixture toxicity, concentration addition (CA) and independent action (IA). Concentration addition (CA) theoretically applies to contaminants with similar mechanisms of action which act additively, that is, the toxicity of the mixture is equal to the sum of its individual components or regarded as dilutions of one another (van Gestel et al 2011). Petroleum hydrocarbon mixture toxicity theoretically follows CA for aliphatic PHCs with similar carbon lengths. In aquatic toxicity of crude oil water-soluble fractions, Rial et al (2013) found concentration addition modeled mixture toxicity best. However, CA does not always appear to hold true. Faust et al (1994) concluded only 66% of binary pesticide mixtures were predictable by concentration addition. The second dominant mixture toxicity model in toxicology is independent action. Independent action (IA) applies to contaminants with different mechanisms of action and the response to the mixture is determined as the product of the individual substance dose-response curve, or the combined probability of response. Both CA and IA reference models do not consider interactive effects between single components.

Mixture toxicity models exist with consideration of additional types of interactions between individual components of the mixtures. Both CA and IA serve as reference models, however, interactions are included such as synergism, antagonism, dose level toxicity or dose ratio dependent toxicity (Jonker et al 2005). If the mixture produces a more severe response than the effect determined from the reference models, this is considered synergism. While the opposite effect, lower toxic responses from the mixture, is considered antagonism. When deviations from reference models vary between low doses and high doses of contamination, it is called dose level dependent deviation. Alternatively, if deviations from reference models depend on the ratios of individual chemicals in the mixture, this is referred to as dose ratio dependent deviation.

Challenges exist when assessing mixture toxicity of PHC products. Due to the large range in PHCs in petroleum hydrocarbon products, such as lubricating oils or diesel, it is simply unfeasible to conduct the individual tests on every component of a PHC product. Hence, why

often PHC toxicity testing is conducted on the whole mixture approach, where the entire mixture is tested on organisms rather than each individual PHC. This method is limited as it is not possible to identify which chemicals contribute to the overall effect (van Gestel et al 2011). However, some soil ecotoxicology studies attempt to model PHC mixture toxicity by grouping individual PHC components based on regulatory groupings. Cermak et al (2013) studied F2 and F3 PHCs as individual components and mixtures on earthworm survival and found that on a tissue basis, mixture toxicity was additive. However, on a soil concentration basis, the toxicity of binary combinations was less than additive. Research on mixture toxicity of medium PHC products to soil invertebrates and plants is limited.

3. Manuscript 1: Petroleum Hydrocarbon Mixture Toxicity and a Trait-Based Approach to Soil Invertebrate Species for Site-Specific Risk Assessments

3.1 Preface

The toxicity of a petroleum hydrocarbon product towards a range of soil invertebrates was determined. The organism's toxic responses assessed were adult mortality and affects to juvenile reproduction. A trait-based approach was utilized to discuss the interspecies differences in responses to soil contamination. Using extensive literature, the mixture toxicity of the PHC product was assessed.

Gainer, A, Cousins, M, Hogan, N, Siciliano, SD. 2018. Petroleum Hydrocarbon Mixture Toxicity and a Trait Based Approach to Soil Invertebrate Species for Site Specific Risk Assessments. Environmental Toxicology and Chemistry. 37(8): 2222-2234

Amy Gainer: Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing (original draft), visualization
Mark Cousins: Software (created mixture toxicity model in excel)
Natacha Hogan: Writing (review and editing), project administration, funding acquisition
Steven Siciliano: Conceptualization, resources, writing (review and editing), supervision, project administration, funding acquisition

3.2 Abstract

Although petroleum hydrocarbons (PHCs) released to the environment typically occur as mixtures, PHC remediation guidelines often reflect individual substance toxicity. It is well documented that groups of aliphatic PHCs act via the same mechanism of action, nonpolar narcosis and, theoretically, concentration addition mixture toxicity principles apply. To assess this theory, ten standardized acute and chronic soil invertebrate toxicity tests on a range of organisms (*Eisenia fetida, Lumbricus terrestris, Enchytraeus crypticus, Folsomia candida, Oppia nitens* and *Hypoaspis aculeifer*) were conducted with a refined PHC binary mixture. Reference models for concentration addition and independent action were applied to the mixture toxicity data with consideration of synergism, antagonism and dose level toxicity. Both concentration addition and independent action, without further interactions, provided the best fit with observed response to the mixture. Individual fraction effective concentration values were predicted from optimized, fitted reference models. Concentration addition provided a better estimate than independent action of individual fraction effective concentrations based on comparison with available literature and species trends observed in toxic responses to the mixture. Interspecies differences in standardized laboratory soil invertebrate species responses to PHC-contaminated soil was reflected in unique traits. Diets that included soil, large body size, permeable cuticle, low lipid content, lack of ability to molt and no maternal transfer were traits linked to a sensitive survival response to PHC-contaminated soil in laboratory tests. Traits linked to sensitive reproduction response in organisms tested were long life spans with small clutch sizes. By deriving single fraction toxicity endpoints considerate of mixtures, less resources and time is required in conducting SSRA for the protection of soil organism's exposure pathway.

3.3 Introduction

Petroleum hydrocarbon (PHC) soil contamination results from releases of crude or refined mixtures such as crude, gasoline, diesel or lubricating oils. Petroleum hydrocarbons consist of molecules with varying carbon numbers and branching giving rise to different physicochemical properties ideal for their intended use (CCME 2008; Wang et al 2006). Regulatory agencies simplify management of PHCs by grouping them based on number of aliphatic and aromatic carbons. In Canada, PHC groupings are as follows: fraction one (F1) composed of carbons in the

6 to 10 range, fraction two (F2) are carbons in the greater than 10 to 16 range, fraction three (F3) includes carbons greater than 16 to 34 and fraction four (F4) contains carbons greater than 34 carbons to 50. Soil remediation guidelines for protection of soil invertebrates and plants are derived from toxicity testing on the individual hydrocarbon fraction, for instance, just F2 distillates (CCME 2008; Cermak et al 2010). Hence, the guidelines do not account for mixture toxicity between fractions even though PHCs like diesels or lubricating oils typically contain both F2 and F3 (Wang et al 2006; CCME 2008). Furthermore, the single component toxicity testing for assessing toxicity to soil invertebrates and plants uses a constant 4:1 aliphatic to aromatic PHC ratio (CCME 2008). As many refined PHCs have varying aliphatic to aromatic ratios and aromatics bioaccumulate more readily, toxicity to soil invertebrates and plants varies with PHC products (Cermak et al 2013). Although drawbacks with the current PHC remedial guidelines exist, like lack of consideration of mixtures and variations in composition of PHC products, the current PHC management framework reflects a tier 1 approach, with necessary generalizations to manage a range of contaminated sites.

Primarily two mixture toxicity approaches model hydrocarbon toxicity: concentration addition and independent action. Concentration addition (CA) theoretically applies to contaminants with similar mechanisms of action which act additively, that is, the toxicity of the mixture is equal to the sum of its individual components or regarded as dilutions of one another (van Gestel et al 2011). Independent action (IA) applies to contaminants with different mechanisms of action and the response to the mixture is determined as the product of the individual substance dose response curves, or the combined probability of response. Both CA and IA reference models do not consider interactive effects between single components such as synergism, antagonism, dose level toxicity or dose ratio dependent toxicity (Jonker et al 2005). Although CA and IA reference models make assumptions regarding mechanisms of action, the observed toxic responses to the mixture do not always follow the theory. Within certain categories of carbon chain length, aliphatic PHC act via the same toxic mechanism of action, non-polar narcosis; thus, PHC mixture toxicity theoretically follows CA (van Gestel et al 2011). Rial et al (2013) found CA was a better option than IA to explain binary toxicity of crude oil water-soluble fractions, with no synergistic or antagonistic interactions. Studies on the toxic effects of single PHC distillates to earthworms found concentration addition mixture toxicity on an organism tissue concentration

basis but antagonism on a soil concentration basis with authors suggesting the discrepancy is due to reduced bioavailability from the presence of nonaqueous phase liquid (Cermak et al 2013; ESG 2003). The range in findings for PHC mixture toxicity is potentially due to the mathematical nature of models. Concentration addition and IA models fit observed mixture responses best in simple systems rather than more complex environments with high variations such as soils or field experiments (Backhaus and Faust 2012; van Gestel et al 2011). However, these mathematical models allow one to estimate single component toxicity response from real-world mixtures.

Organism traits explain interspecies variations in responses to toxicants. Soil invertebrate functional traits are indicative of environmental changes such as land use disturbances or changes in local conditions (da Silva et al 2016; Lindberg and Bengtsson, 2005; Ponge et al 2006; Salmon et al 2014). For example, on the landscape scale, Collembola traits such as dispersal and colonization ability were linked to the ability of populations to recover following drought disturbances (Lindberg and Bengtsson 2005; Ponge et al 2006). However, research on the relationship between soil invertebrate traits and responses to toxicants is limited. Hedde et al (2012) found a trait-based index better reflected the effects of heavy metal-contaminated soils on soil invertebrates in the field compared to community density or diversity. A field study on metal-polluted soils suggested species-specific feeding mechanism was a potential factor explaining variations in invertebrate metal body burdens (van Straalen et al 2001). The observation that oribatid mite species store heavy metals in their thickened, sclerotised cuticles indicates this trait reduces the organism's toxic response to metals (Norton and Behan-Pelletier 1991). Given their success in aquatic toxicity, a trait-based approach maybe ideal for understanding variations in toxic responses to contaminants across species (Liess and von der Ohe 2005; Schäfer et al 2007; Menezes et al 2010).

Laboratory species allow for initial exploration of traits influencing toxicity. Standardized, common and recently introduced test species, including earthworms, Collembola, enchytraeids and mites, all exhibit a range of unique traits (EC 2004; EC 2014; Princz et al 2010; OECD 2008; ISO 2003). For example, earthworms experience a different degree of exposure than mites as they have a permeable outer membrane, while the oribatid mite, *Oppia nitens*, has a hard

exoskeleton (Rombke et al 2005; Princz et al 2012). Earthworms' large body size results in contact with toxicants from both soil water and solid phase while smaller organisms will typically only contact one phase (Rombke et al 2005; de Bello et al 2010). Although the drawback to using laboratory species is they exhibit traits selected for ease of rearing in laboratory conditions, the controlled laboratory environment allows for direct toxicity testing and these organisms are well represented in soil ecotoxicology literature. Thus, standardized soil laboratory toxicity species allow for initial exploration of traits influencing toxicity that can be validated with future field studies.

The objectives of this study were to: (1) determine whether PHC-contaminated soils mixture toxicity follow CA or IA across numerous species and endpoints, (2) determine the validity of single component toxicity endpoints estimated from mixture toxicity models, and (3) determine if interspecies variations in toxicity can be explained by organisms traits.

3.4 Materials and Methods

3.4.1. Soil preparation

Organization for the Economic Cooperation and Development (OECD) artificial soils were utilized for all test organisms according to Environment Canada (EC 2004), composed of 70% silica sand, 20% kaolin clay and 10% 2 mm sieved, air dried *Sphagnum* sp. peat moss, calcium carbonate and distilled water. Soils were wetted to 70% water holding capacity. Prior to test start and at test completion, soil pH was determined and adjusted with calcium carbonate to be within the acceptable range of 6 to 7.5. If needed, pH was adjusted with calcium carbonate to be within the acceptable range. Soil pH was determined by shaking soils with 0.01 M $CaCl_2$ in a 1:5 soil: solution ratio.

Artificial soils absent of peat were initially spiked with a lubricating oil and acetone as a carrier solvent, and left in a fume hood overnight to allow for dissipation of the acetone. The following day peat was added. This process was necessary to allow for removal of acetone and greater homogenization of oil amongst mineral and organic soil particles. A range of selected nominal

concentrations were utilized for the soil invertebrate toxicity testing. Soil samples were collected at the test start and end for determination of PHC content.

3.4.2. Invertebrate Species and Culture

Soil invertebrate tests organisms included: the surface soil earthworm *Eisenia fetida* (EC 2004), the anecic earthworm *Lumbricus terrestris* (EC 2004), the enchytraeid *Enchytraeus crypticus* (ISO 2003) the Collembola *Folsomia candida* (Willem, 1902) (EC 2014), the fungivory mite *Oppia nitens* (C.L. Koch) (Princz et al 2010) and the predatory mite *Hypoaspis aculeifer* (Canestrini 1883) (OECD 2008). *Folsomia candida* and *O. nitens* cultures originated from Environment Canada while the origin of the *E. crypticus* culture was from the Edgewood Chemical Biological Center. *Hyposaspis aculeifer* culture was provided by the ECT Oekotoxikologie laboratory. *Lumbricus terrestris* was field collected near Windsor, Ontario and *E. fetida* was sourced from a worm supplier in Saskatoon, Saskatchewan.

Soil invertebrate species were cultured in the dark at ambient temperature (ranging from 18-23˚C, typically 20˚C). Culture media for the earthworms was composed of sterilized, organic triple mix soil and fed organic material (combination of sterilized leaf litter, cooked oatmeal, bread crumbs and fruit/vegetables waste). *Folsomia candida* and *O. nitens* were cultured on substrate composed of moistened 8:1 plaster of Paris to activated charcoal and fed activated yeast biweekly. *Hyposaspis aculeifer* cultures were maintained on plaster of Paris and activated charcoal substrate with approximately 1 g of artificial soils and fed both cheese mites and Collembola (both *Onychiurus folsomi* and *F. candida*). *E. crypticus* culture media consisted of a mix of artificial soil and organic triple mix soil and fed bread crumbs and dry oats weekly.

3.4.3. Toxicity Tests

Range finding tests were initially conducted for every test organism. All soil invertebrate toxicity tests were performed in control chambers under the following conditions: daily average temperature of 20˚C (+/- 2˚C), 16h light, 8-hour dark cycle, with approximately 600 lux at surface and approximately 60% humidity. Each test consisted of a minimum of 9 to 11 concentrations plus a solvent and a negative control, with 5 replicates each, except *E. fetida*

which consisted of 10 replicates. For all species, except earthworms, 25 g (dry weight) of artificial soils was contained in 50 mL glass Schell vials with tight-fitting lids and a single hole for air exchange. Earthworms tests used 200 g (dry weight) of artificial soil wetted to 70% water holding capacity in unused polypropylene containers with tightened lids and a few holes.

3.4.4. Soil Invertebrates

Acute mortality and reproduction toxicity tests for *F. candida, E. crypticus* and *O. nitens* consisted of 28 days (EC 2014; ISO 2003; Princz et al 2010). For *F. candida*, 10 age synchronized 10 to 12 day old organisms were used while 10 fully clitellated unsynchronized adult individuals were added to soils for *E. crypticus*. For *O. nitens,* 15 age synchronized (within 1 to 2 days), young adult (approximately one week after light red brown in colour) organisms were added. Food (approximately 5 to 10 grains of dry yeast/test unit) was provided weekly for both for *F. candida* and *O. nitens.* For all tests, moisture losses were assessed by weight. At test completion, *F. candida* were counted utilizing floatation method with black ink and counted via photographs with ImageJ software (Rueden et al 2016). *O. nitens* were heat extracted from the soil over 2 days (22˚C for 24 hours, 25˚C for 24 hours) using a modified Tullgren apparatus and manually counted. At test completion *E. crypticus* were preserved in 70% ethanol solution and Bengal red dye for two days (ISO, 2003). Enchytraeids were separated and counted from soils via wet sieving.

Acute mortality and reproduction toxicity tests for *H. aculeifer* consisted of a 14-day duration (OECD 2008). Ten age synchronized (28 to 35 days old) females were added to test soils and fed approximately 10 Collembola (*O. folsomi*) every 3 days. At test completion, organisms were heat extracted from the soil using a modified Tullgren apparatus over 3 days at gradually increasing temperatures (22˚C for 24 hours, 25˚C for 24 hours, 27 ˚C for 24 hours) and manually counted.

Mortality toxicity tests were conducted for *E. fetida* over 28 days (EC 2004). Tests utilized sexually mature, fully clitellated adult earthworms from the culture on day 0. Worms were weighed prior to test start, sorted based on weight and distributed amongst containers randomly

at two worms per container. In compliance with EC (2004), only worms in the range of 250 to 600 mg were used. Earthworms were fed 1 teaspoon of cooked oatmeal on days 0 and 14. On day 28, the test ended, and adult mortality was assessed by hard sorting and counting the number of adult worms' present.

Mortality of *L. terrestris* was determined with a 14-day mortality tests (EC, 2004). Tests utilized sexually mature adult earthworms, weighing between 3 and 10 g. Three worms were assigned to each container randomly. Earthworms were not fed during this test. At test completion on day 14, soils were hand sorted and living adults counted.

3.4.5. Lubricating Oil and Soil Petroleum Hydrocarbon Analysis

This study reflects a fixed ratio design, where the ratio of individual PHC fractions are fixed but the total mixture concentration increases (van Gestel et al 2011). A lubricating oil composed of mainly F2 (C10- C16) and F3 (>C16-C34) PHC was utilized. Lubricating oil was supplied by Marsollier Petroleum (Saskatoon, Saskatchewan) and submitted in triplicates to Maxxam Analytical (Mississauga, Ontario) for analysis using an extended gas chromatography/mass spectrometry (GC/MS) protocol. Briefly, oil samples were extracted in hexane, concentrated to 1 mL and analyzed by GC/MS, following CCME (2008) for PHC and US EPA 8270 for polycyclic aromatic hydrocarbons (US EPA 2014a).

Soil PHC analysis was conducted in accordance with Canadian regulations (CCME 2008). Approximately 2 g of soil was extracted using 40 mL of hexane and acetone at a 1:1 volumetric ratio with 2 g sodium sulphate to remove water. Soil solvent samples were mechanically shaken at approximately 120 cycles per min for a minimum of 1.5 h (Schwab et al 1999). Following shaking, extracts were reduced under nitrogen to approximately 1.5 mL and transferred to GC vials. Quality control and assurance procedures included duplicates, matrix and blank spikes, method blanks and reagent blank every 20 samples. Spiked samples within 70-130% recoveries were considered adequate. Based on CCME (2008) guidance, the F2 fraction was considered carbon >10-16 and the F3 fraction is >16-34. Quantification of the F2 and F3 fractions was performed using a Varian CP3800 gas chromatograph fitted with a flame ionization detector

(GC-FID) with chromatogram interpreted performed on CompasCDS software (Varian, Santa Clarita, CA).

3.4.6. Test Endpoints, Dose Response to Mixture Analysis and Critical Body Residues

All soil invertebrate toxicity tests met test validity criteria for the negative control, with a survival of >90% or the minimum number of juveniles, and standard deviation less than 30%. For *F. candida, O. nitens, H. aculeifer, E. fetida* and *L. terrestris* the test endpoint was mean adult survival used to determine mortality. Inhibition of reproduction using mean juvenile production was determined for *E. crypticus, F. candida, O. nitens* and *H. aculeifer*. Students t-tests were conducted between the solvent and negative controls. The response in all tests' solvent control treatments were not significantly different than the negative control and, therefore, pooled.

The dose response analysis of soil invertebrates to the mixture followed guidance recommended by Environment Canada (EC 2005a). Determination of 25 and 50% lethal concentration (LC) or effective concentrations (EC) with 95% confidence intervals were conducted in Graphpad Prism® (version 6.0) by a four-parameter (minimum, maximum, EC50, slope) Hill logistic equation (Appendix A Equation [A-1]) (variable slope sigmoidal nonlinear regression model) and visualized in SigmaPlot (Version 12.0). Normality and homoscedasticity of residuals were assessed by the Shapiro-Wilk normality test and Levene's test, respectively. Full dose response was observed in all tests, a minimum response of 0% and maximum of 100%.

The organisms' critical body residue (CBR) at the mixture LC/EC25 values were estimated using a fugacity-based equilibrium three phase partitioning approach (Appendix A Equation [A-2]) (Mackay and Paterson, 1981). Elimination was assumed to be one compartment, first order elimination (Appendix A Equation [A-3]). Additional equations, parameters and rationale for the determination of CBR are summarized in Table A-1 and A-2 in Appendix A Supplemental Material.

3.4.7. *Mixture Toxicity Modelling*

Using the Solver Add-In for Microsoft Excel 2010, the response inhibition (y) for multiple PHC concentrations was calculated assuming concentration addition (Appendix A, Equation A-4) and independent action (Equation A-5) behavior of the mixture constituents. Initial estimates for individual F2 and F3 EC50 (Table A-3) were estimated from either available literature conducted with single substance PHC distillates on similar species (Cermak et al 2010; Cermak et al 2013; Erlacher et al 2013) or the regulatory estimated species sensitivity distribution for ecological soil contact when no available literature for single substance toxicity endpoints existed (CCME 2008) (Table B-4 to B-5). For the mite species, the mortality value was assumed to be double the ESSD to account for higher values observed for mortality. For available literature where there was an endpoint for mortality but not reproduction, EC50 for reproduction was assumed to be half the LC50 for effects on survival. For the individual F2 and F3 slopes (Table 3-2) for their dose response curves, the mixture toxicity slope values were assumed as available studies did not report dose response slopes. It was assumed that the individual F2 and F3 displayed full dose response relationships with a minimum response of 0 and maximum of 100%. The model with the best fit reported the lowest sum of squares between fitted model expected values and the observed response to the mixture, and a chi-squared test determined if the models were significantly different (p<0.05).

Once CA or IA was determined to be the best model fit, potential interactions were tested. The reference model was extended to include a deviation parameter to detect the following interactions: synergism, antagonism and dose level dependence as published by Jonker et al (2005) for binary mixtures. Synergism occurs when the mixture toxicity is greater than the toxicity expected from the single components and antagonism the opposite, when the mixture toxicity is less than the expected toxicity from the single components (van Gestel et al 2011). Dose level deviations indicate toxicity varies at low and high concentrations (Jonker et al 2005). Dose ratio dependent deviations from reference models occur when the single components illicit different mixture toxicity at varying ratios of the single components. Since the lubricating oil had a fixed ratio of F2:F3, dose ratio deviations were not assessed. The best model fit for the extended model was determined as the lowest sum of squares and a chi-squared test (p<0.05).

The individual PHC fraction toxicity endpoints were obtained from the optimized model fitted to the reference model. The single component LC/EC25 was determined from the optimized LC/EC50 by utilizing the slope and maximum response from the optimized model (Appendix A Equation [A-6]).

The predicted response from unfitted reference models was used to validate and assess which model predicted the observed response best. Individual fraction EC values and slopes used in determining toxic unit and for predicted responses from unfitted reference models were the same values utilized as initial estimates for the model described earlier (Appendix A; Table A-3 to A-5). Results were visualized by plotting observed response to the mixture with the predicted response from unfitted concentration addition and independent action reference models.

3.5 Results

3.5.1. Oil and Soil Analysis

Analysis of the lubricating oil indicates that it is dominated by F2 and F3 PHC fractions, consistent with literature (Table 3-1) (Wang et al 2006). The average (standard deviation) for each PHC fraction were as follows: 0.02% (8) as F1, 52% (0) as F2, 48% (0) F3 and 0.29% (0) as F4. Of the polycyclic aromatic hydrocarbons regulated by CCME (2008), eight were detected (naphthalene, acenaphthene, acenaphthylene, acridine, anthracene, fluorene, phenanthrene and pyrene) compromising an average (standard deviation) of 0.013% (0.8) of F2 PHC and 0.0058 % (0.5) of F3 PHC. The polycyclic aromatic hydrocarbons concentrations in the highest test concentration (*E. crypticus* reproduction) are 2.0 mg/kg naphthalene, 0.084 mg/kg acenaphthene, 0.027 mg/kg acenaphthylene, 0.17 mg/kg acridine, 0.019 mg/kg anthracene, 0.52 mg/kg fluorene, 0.036 mg/kg phenanthrene and 0.027 mg/kg pyrene. The total petroleum hydrocarbon (TPH) content was approximately 93% of nominal, differences likely due to loss of volatiles with acetone. Over the 14 and 28 day tests, degradation of F2 and F3 PHC within the artificial soil between the start and end of the test were minimal (5-10%).

Table 3-1. Aliphatic and aromatic percent composition of the lubricating oil used in toxicity tests and the Canada Wide Standards for Petroleum Hydrocarbons (CWS PHC) (CCME 2008) recommended composition for F2 and F3 petroleum hydrocarbons distillates for toxicity testing.

Fraction	Sub fraction	Branch type	% Composition (standard deviation)	
			Lubricating oil	CWS PHC
F2	>C10-12	aliphatic	8.6 (0.2)	36
		aromatic	2.0 (0.3)	9
		subtotal	11	45
	>C12-C16	aliphatic	78 (1.8)	44
		aromatic	12 (1.7)	11
		subtotal	90	55
		F2 total	100	100
F3	>C16-C21	aliphatic	53 (1.4)	56
		aromatic	20 (1.4)	14
		subtotal	73	70
	>C21-C34	aliphatic	22 (0.59)	24
		aromatic	5 (0.50)	6
		subtotal	27	30
		F3 total	100	100

CWS PHC=Canada Wide Standards for Petroleum Hydrocarbons (CCME 2008)

The PHC product used in our study varied from the composition used in the toxicity testing for derivation of regulatory guidelines (CCME, 2008) (Table 3-1). The aliphatic:aromatic ratio for F3 fraction was similar between products, however, the ratio for F2 varied from the composition used for in toxicity testing for regulations. The aliphatic: aromatic ratio of the PHC products used for regulatory toxicity testing was 4:1 across F2 and F3 and subcategories. The average aliphatic: aromatic ratio for F2 was 5.5 and F3 3.5 in the lubricating oil tested.

3.5.2. Dose Response to Mixture and Mixture Toxicity

Mixture LC25 values ranged from 1,558 to >186,000 mg/kg TPH and the range for inhibition of reproduction EC25 values was from 142 to 7,346 mg/kg TPH (Table 3-2). The CBRs ranged from 0.05 mmol/kg TPH for *L. terrestris* mortality to 21.8 mmol/kg TPH for *E. crypticus* reproduction inhibition. Mixture mortality, but not reproduction, showed a trend with the

fugacity predicted CBR and lipid content (Figure B-1). Species with a greater LC25 value also had higher CBRs and lipid contents, except for *E. crypticus* (Table 3-2). The coefficient of determination between LC25s and lipid contents was 0.74 (Figure B-2).

Table 3-2. The 50th percentile lethal or effective concentration values with 95% confidence intervals, slope, coefficient of determination, 25th percentile lethal or effective toxicity values, lipid content and fugacity predicted critical body residue for soil invertebrates (*Eisenia fetida, Lumbricus terrestris, Enchytraeus crypticus, Folsomia candida, Oppia nitens, Hypoaspis aculeifer*) exposed to soil spiked with a fixed ratio petroleum hydrocarbon product mixture.

Organism	Test type	Mixture LC/EC (mg/kg TPH)						Lipid content (% ww)	CBR (mmol/kg TPH)
		LC/EC50	Lower CI	Upper CI	Slope	r^2	LC/EC25		
Lumbricus terrestris	mortality	2,158	2,022	5,562	4.3	0.97	1,558	1.2[a]	0.050
Eisenia fetida	mortality	2,860	2,369	3,451	17	0.97	2,493	1.8[b]	0.11
Folsomia candida	mortality	6,172	5,247	7,261	1.3	0.93	2,514	8.5[c]	0.53
Hypoaspis aculeifer	mortality	24,969	18,393	33,897	0.7	0.80	4,328	13[d]	1.4
Oppia nitens	mortality	11,293	9,943	12,826	2.0	0.96	5,982	13[d]	1.9
Enchytraeus crypticus	mortality	>186,000	nd	nd	nd	nd	nd	na	nd
Oppia nitens	reproduction	1,210	853	1,715	0.66	0.88	142	13[d]	1.9
Hypoaspis aculeifer	reproduction	1,850	1,345	2,545	0.51	0.87	206	13[d]	1.4
Folsomia candida	reproduction	3,160	2,783	3,587	1.9	0.95	1,658	8.5[c]	0.53
Enchytraeus crypticus	reproduction	31,736	22,974	43,840	0.79	0.81	7,346	4.7[e]	21.8

mg/kg TPH=mg total petroleum hydrocarbons per kg of dry soil; LC=lethal concentration; EC=effective concentration; r^2=coefficient of determination; CBR= critical body residue for LC/EC25 (additional information in Appendix A); nd=not determined; na=not applicable; ww=wet weight; [a] Kraus et al 2000; [b] Wagman et al 2001; [c] Holsmstrup et al 2002; [d] Convey 1992, [e] Rodriguez and Verdonschot 2001

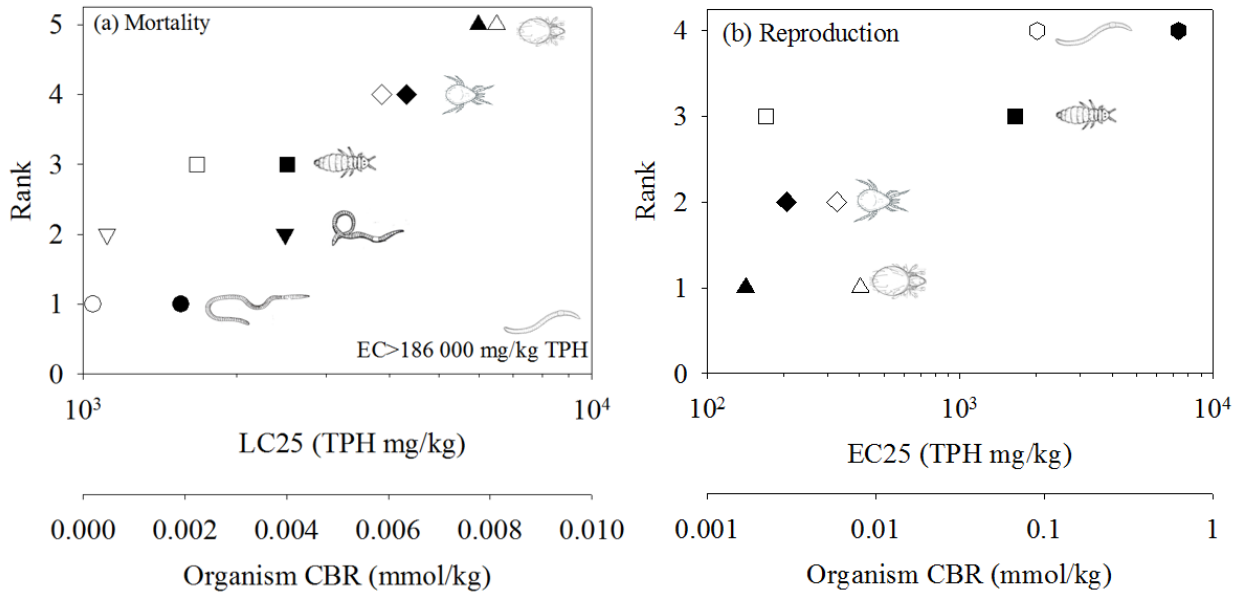

Figure 3-1. Rank of lethal concentration (LC) 25 for mortality from exposure to petroleum hydrocarbon mixture contaminated soil as a function mortality LC25 and organism critical body residue (CBR) (a), and rank of effective concentration (EC) 25 for reproduction (b) from exposure to the petroleum hydrocarbon mixture as a function of reproduction EC25. The lowest rank is most sensitive species. Circles represent *Lumbricus terrestris*, diamonds represent *Hypoaspis aculeifer*, squares represent *Folsomia candida*, triangles represent *Oppia nitens*, inverted triangles represent *Eisenia fetida* and hexagons represent *Enchytraeus crypticus*. Solid represents EC/LC25 and open represents critical body residue.

Across species and tests, both CA and IA reference models fit the observed mixture response to the PHC-contaminated soils similarly, with no significant differences between models based on the chi test (Figure 3-2, Table 3-3, Figure A-2 and A-3). Of the nine toxicity tests assessed, CA reported lowest sum of squares for five tests, IA for three and one test both models produced the same sum of squares. For instance, the sum of squares from CA fit to *E. fetida* mortality was 2,354 and for IA was 2,465 (Table 3-3). For all tests, no further interactions such as dose dependence beyond the reference models fit the observed response with a lower sum of squares. When validating CA and IA models using individual PHC fractions from literature, across test organisms and types, both CA and IA models overestimated toxic responses to the PHC mixture as observed with the consistently negative average residual (average of difference between observed response and predicted response; Figure 3-1, Table 3-3). The range in average residuals

for CA ranged from -0.5 to -33 and for IA the values ranged from -0.2 to -37 (Table 3-3). The organisms and test type with the lowest average residuals was CA for *O. nitens* reproduction with a value of -0.5.

Table 3-3. The sum of squares from fitted reference models, individual PHC fraction toxicity values from fitted reference models and average residuals between observed response and expected response from unfitted reference models.

Species	Test type	Fitted Model Sum of Squares		Single LC/EC25 (mg/kg)				Unfitted Model Average Residuals	
				CA		IA			
		CA	IA	F2	F3	F2	F3	CA	IA
Eisenia fetida	mortality	2,354	2,475	2,466	2,533	1,317	1,370	-17	-16
Folsomia candida	mortality	2,272	2,127	1,464	3,448	1,106	17,078	-11	-11
Hypoaspis aculeifer	mortality	4,507	5,790	39,269	1,927	1,673	33,313	-29	-37
Lumbricus terrestris	mortality	3,885	3,885	1,593	1,545	11,434	759	-12	-0.20
Oppia nitens	mortality	3,169	3,273	4,482	10,907	2,895	7,000	-25	-27
Enchytraeid crypticus	reproduction	2,541	2,265	2,714	67,184	2,422	44,875	-33	-36
Folsomia candida	reproduction	3,666	3,635	1,687	1,631	851	7,113	-14	-13
Hypoaspis aculeifer	reproduction	5,370	5,618	237	234	460	457	-10	-18
Oppia nitens	reproduction	2,292	2,351	32	1,799	2,504	29	-0.50	-5

CA=concentration addition; IA=independent action; na=not applicable; mg/kg=mg F2 or F3 per kg of dry soil

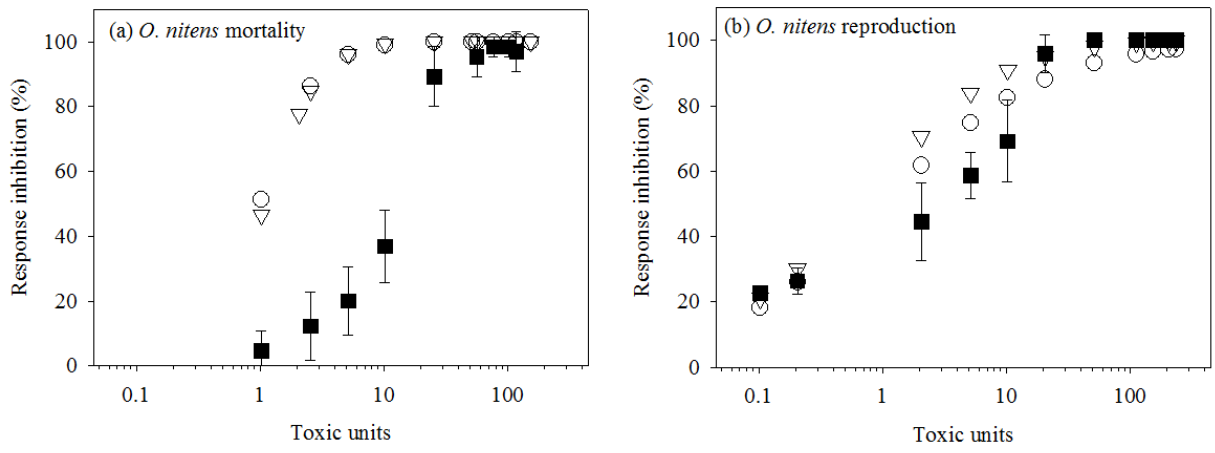

Figure 3-2. *Oppia nitens* dose response relationship for mortality (a) and reproduction (b) as a function of toxic units, exposed to petroleum hydrocarbon mixture contaminated soils for 28 days. Solid squares represent average observed responses from the mixture with standard deviation intervals, open circles represent concentration addition predicted mixture toxicity and open triangles represent independent action predicted mixture toxicity.

The single component toxicity endpoints predicted by the optimized, fitted CA model are more consistent with the trends across species observed in response to the mixture toxicity, and literature, than IA (Figures 3-3 and A-4). For instance, with CA predicted F2 LC25 values for *F. candida* and both earthworms are lower than both mites, a similar trend observed with responses to the mixture (Figure 3-3). The opposite was true for the trend across species for IA predicted single component values, most notably, the single F2 LC25 values predicted by the IA model indicated *L. terresris* as the most tolerant species when this species displayed the most sensitive survival response to the mixture (Figure A-4). Concentration addition predicted individual fraction EC values appeared consistent with literature, except for F2 mortality. Concentration addition overpredicted F2 mortality and the IA overpredicted both F2 mortality and reproduction. The range in values predicted by CA and IA for F2 mortality for earthworms were 1,464 to 1,593 mg/kg and 1,317 to 11,434 mg/kg F2, respectively, while the ranges found in literature and regulations were 237 to 776 mg/kg F2 (CCME 2008; Cermak et al 2010; Cermak et al 2013; Erlacher et al 2013; ESG 2003) (Table 3-3). The range in values predicted by CA and IA for F2 reproduction for all species were 32 to 2,714 mg/kg F2 and 460 to 2,504 mg/kg F2, respectively, while the ranges in literature and regulations for two species (*E. andrei* and

O. folsomi) were 132 to 211 mg/kg F2 (CCME 2008; Cermak et al 2010; Cermak et al 2013; Erlacher et al 2013; ESG 2003).

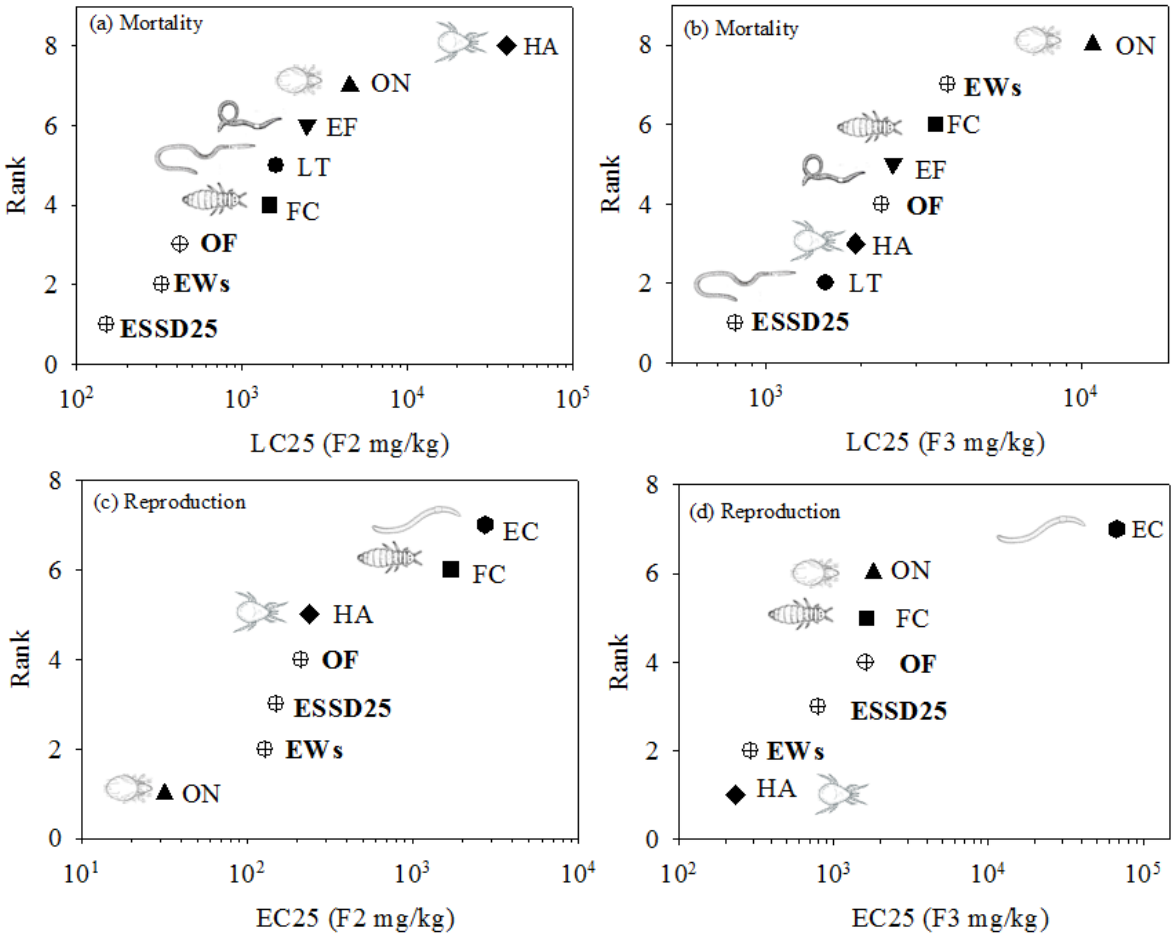

Figure 3-3. Rank of individual F2 (a, c) and F3 (b, d) PHC lethal concentration (LC) for mortality and effective concentration (EC) 25 for reproduction, predicted from an optimized concentration addition model fit as function of LC or EC25 predicted from an optimized concentration addition model fit. The lowest rank is most sensitive species. Empty circles with crosses and bold font indicates value from average of available literature or regulatory soil invertebrate ecological soil contact value for single F2 distillates. Solid circles represent *Lumbricus terrestris* (LT), solid diamonds represent *Hypoaspis aculeifer* (HA), solid squares represent *Folsomia candida* (FC), solid triangles represent *Oppia nitens* (ON), inverted solid triangles represent *Eisenia fetida* (EF) and solid hexagons represent *Enchytraeus crypticus* (EC). ESSD represents the estimated species sensitivity distribution from CCME (2008) regulations, OF represents *O. folsomi* and EW represents average values from literature for earthworm species.

3.6 Discussion

3.6.1. Mixture Toxicity

In the current study, toxicity of a PHC mixture of F2 (C10-C16) and F3 (>C16-C34) PHCs to soil invertebrates fit models of similar or dissimilar mechanism of actions equally well, despite the consensus that PHC have similar modes of action. Other studies on toxicants with similar and dissimilar mechanisms of action found comparable results, that IA and CA fit observed mixture toxicity data similarly (Qiu et al 2016; Schnug et al 2014; Backhaus et al 2004; Cedergreen et al 2008; Mo et al 2014; Faust et al 1994). Of the limited studies on binary PHC mixture toxicity to soil invertebrates, Cermak et al (2013) found that mixtures of F2 and F3 PHC followed CA based on earthworm organism tissue concentration. However, on a soil concentration basis, antagonism was observed, likely due to decreased bioavailability from preferential partitioning to non-aqueous phases in soil (Cermak et al 2013). Concentration addition was also a better model fit when testing mixtures of oil fractions on sea urchin embryos tests (Rial et al 2013). The equivalence of CA and IA models in our system highlights the limitations of these models as outlined by others (Backhaus et al 2012; van Gestel et al 2011). Limitations exist when applying mathematical approaches to biological processes. Although the reference models fit comparably with experimental data and tended to overestimate toxicity, this study found the models a tool for effectively predicting individual PHC fraction toxicity endpoints using responses from toxicity testing with the mixture.

The single substance toxicity values predicted from the optimized CA model were more consistent with the observed mixture toxicity trends across species and literature than the IA predicted values. The species EC25's from exposure to the mixture were ranked to validate the accuracy of models predicting individual F2 toxicity values, since it is well established that F2 PHC are more toxic to soil invertebrates than F3 (Cermak et al 2010; Cermak et al 2013; Erlacher et al 2013; ESG 2003). Concentration addition predicted individual F2 EC values for mortality and reproduction which followed similar interspecies trends as observed with mortality responses to the mixture. For example, *F. candida* and both earthworms were the most sensitive species to the PHC mixture and the F2 LC25 predicted by CA. Both models underestimated toxicity of F2 mortality, as the average literature values and regulatory values were below the

lowest model predicted F2 EC value (Figure 3-3, Figure B-4). However, the toxicity endpoint values from literature (Cermak et al 2010; Cermak et al 2013; Erlacher et al 2013; Agnell et al 2012) and regulations (CCME 2008) are lower relative to the species tested in this study as these data points reflect averages of two species (*E. andrei* and *O. folsomi*), with the earthworms being a species sensitive to PHC-contaminated soils.

3.6.2. Interspecies Sensitivities

In our study, three categories of organism functional traits conceptually explain interspecies toxic response to PHC- contaminated soils: (1) traits related to external exposure, (2) intrinsic sensitivity and (3) life history population demographics (van Straalen 1994; Rubach et al 2011) (Figure 3-4, Table B-6). Key traits that influence an organism's external exposure to PHC in soil include body size and diet (de Bello 2010; Hedde et al 2012; Andriuzzi et al 2016). The author hypothesized body size as a trait influencing which soil phase an organism will receive exposure, as the smaller body size of *E. crypticus* allows this organism to remain in soil pore water where less PHC components exist, accounting for its reproductive tolerance and lack of mortality observed in our study and others with low water solubility PHCs (Sverdrup et al 2007; Bleeker et al 2003). Although in aquatic environments organisms with small body size and small surface area to volume ratio often exhibit greater sensitivity (Rubach et al 2011), our study found the opposite was true in highly variable soil environments. Due to their relatively large body size, earthworms contact PHC constituents from both soil solids and soil water, receiving greater exposure than species who reside mainly in pore space or soil water (Rombke et al 2005). Of the species studied, only earthworms ingest soil in addition to the uncontaminated food source provided during laboratory tests, an additional exposure route contributing to their sensitivity (Hedde et al 2012).

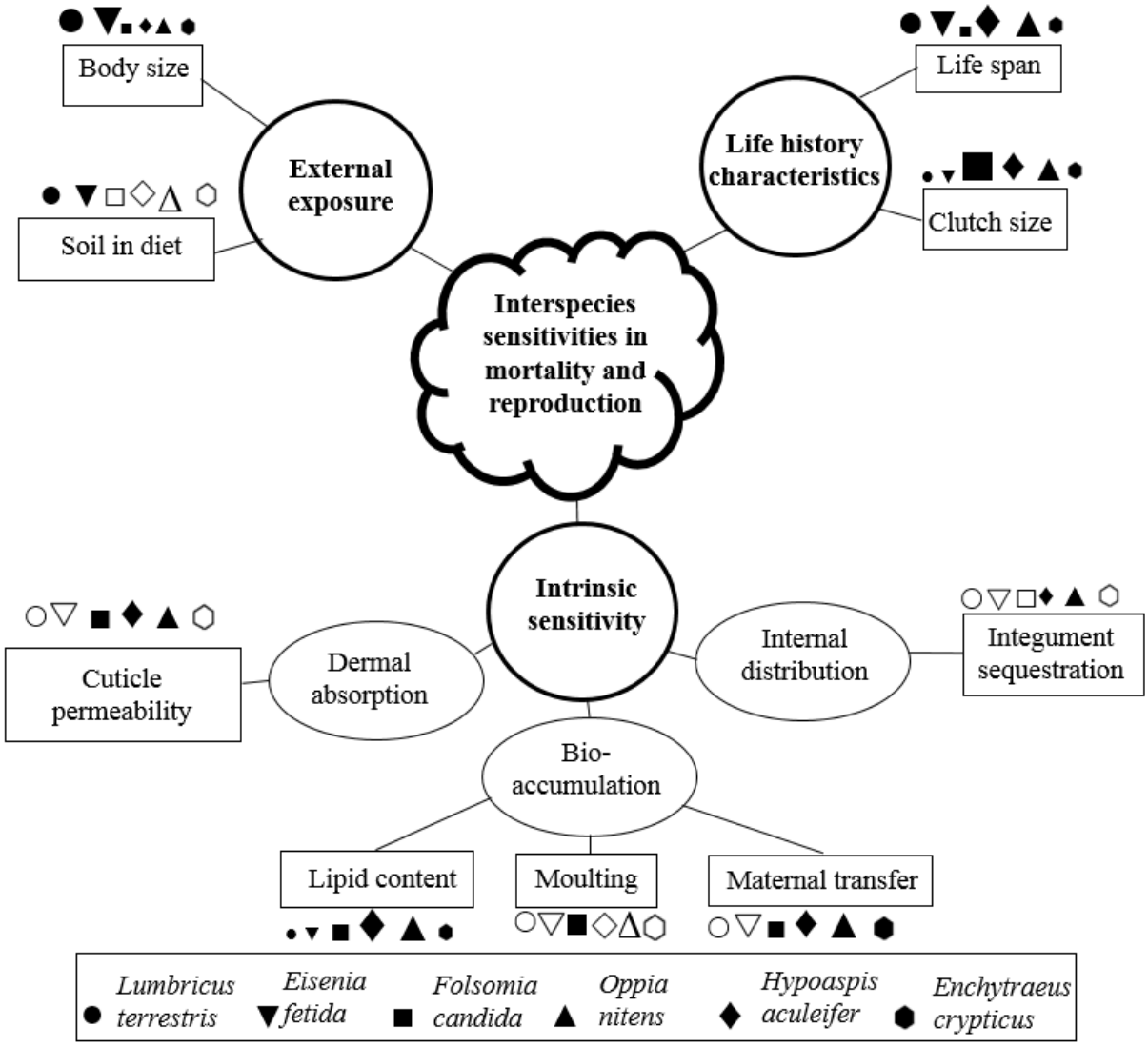

Figure 3-4. Conceptual diagram of key traits with literature explaining the interspecies sensitivity in mortality and reproduction observed in standardized laboratory organisms exposed to petroleum hydrocarbon contaminated soils (adapted from van Straalen (1994) and Rubach et al (2011)). The three main categories of traits are external exposure, intrinsic sensitivity and life history characteristics. The intrinsic sensitivity category contains the subcategories dermal absorption, bioaccumulation and internal distribution. Solid symbols indicate yes or presence of a trait, open symbols indicate no or absence. Small symbol size reflects a trait that is small, short or low and larger sizes indicate large, long or high traits.

Traits influencing intrinsic sensitivity varied across the species tested in our study. Given dermal absorption is the primary exposure pathway for soil invertebrates in laboratory conditions (Vijver et al 2002; Jager et al 2003), traits affecting this exposure pathway were identified. For soil invertebrates, the cuticle surrounding the exoskeleton represents a biological barrier between external soil contamination and internal components (Hedde et al 2012). The permeability of the cuticle determines the degree of absorption. When referring to cuticle permeability, it is the diffusion directly across the cuticle or via gaps in the cuticle (Walton 1980; Huguier et al 2015; Lilywhite and Maderson 1988). The cuticle of earthworms and nematodes is composed of collagen and is permeable to allow for osmoregulation while arthropods, like mites and Collembola, contain water impermeable chitin cuticles (Johnstone 1994; Norton and Behan-Pelletier, 2009). Species with permeable cuticles, like the earthworms and enchytraeids, are vulnerable to desiccation from water losses, and hence need to be in close proximity or direct contact with soil pore water. Of the test species with impermeable, chitinous cuticles, this study and others observed that *F. candida* survival responded sensitively to PHC-contaminated soil (Cermak et al 2010; Eom et al 2007; Sverdrup et al 2002b; Princz et al 2012). However, this is not true for all Collembola, as other multiple species studies found some Collembola species (*O. folsomi, F. nivalis* and *Proisotoma minuta*) more sensitive to PHC-contaminated field soils than earthworms and *F. candida* (Princz et al 2012). *F. candida* possess two unique traits contributing to their sensitivity, a ventral tube and a waxy cuticle (Hopkin 1997). The ventral tube is an opening on the underside of *F. candida* with a permeable membrane where water absorption occurs (König and Varma 2006; Eisenbeis 1982; Hopkin 1997). Although this is a key trait influencing *F. candida*'s sensitivity to water soluble toxicants like pesticides (Jegede et al 2017; Lock et al 2002), in our study, the organism absorbs only the water soluble PHC components. In addition, *F. candida* possess a waxy cuticle that provides anti wetting capabilities, preventing drowning and aiding dispersal. The concentration of lipids on the epicuticle of their integument facilitates diffusion of hydrophobic contaminants across the integument (Walton 1980). Mites experience less cuticle absorption of PHCs in soils due to their hardened, sclerotised, chitinous exoskeleton (Norton and Behan-Pelletier, 2009). Hugueir et al (2015) hypothesized that dermal absorption in mites occurs through permeable regions in the joints of their legs, as observed in a soil dwelling sclerotized spider (Everts et al 1991). Another well-researched trait reflective of intrinsic sensitivity this study identified was lipid content, as this is the cellular target for PHCs.

Interspecies sensitivity trends in mortality, but not reproduction, agree with the "survival of the fattest concept" theory (Lassiter 1990), that species with higher lipid contents withstand higher internal concentrations of contaminants that act via non polar narcosis in acute tests (Geyer et al 1994). However, lipid content did not completely account for the observed mortality, as critical body burdens were not near the internal threshold of approximately 3 mmol/kg known to cause mortality (CCME 2008; Baas et al 2015). This discrepancy is potentially due to relatively small differences in lipid contents across tests species compared to aquatic or mammalian species. As observed with aromatic PHCs in *F. candida* (Broerse et al 2012), the intrinsic differences in species ability to biotransform PHCs influences toxic responses. However, literature is currently lacking on the biotransformation traits such as enzyme activities in the species studied.

Traits indicative of excretion influence the extent of intrinsic bioaccumulation of PHCs in soil invertebrates (van Straalen 1994; Rubach et al 2011). *Folsomia candida* was the only species in our study who likely excreted internal PHC through moulting, as they moult every 3 to 8 days (EC 2014). Although not studied in the soil invertebrates tested in this study, excretion via moulting occurs for heavy metals (Schmidt and Ibrahim 1994) and organics (Gaylor et al 2012) in terrestrial invertebrates like grasshoppers and crickets. An additional excretion route observed in all species tested except the earthworms was maternal transfer of toxicants, a well-documented form of excretion for heavy metals in aquatic invertebrates (Schmidt and Ibrahim 1994; Conley et al 2009; Keteles and Fleeger 2001; Cid et al 2010). The last intrinsic sensitivity trait proposed reflects an organism's ability to withstand toxicity by storing toxicants in inert body parts. Soil invertebrates, like mites, possess the ability to sequester PHCs into their sclerotised integument (Gaylor et al 2012; Schmidt and Iberhim 1994). Heavy metals in the integument of other sclerotised invertebrates (e.g. crayfish) ranked as the second tissue, with highest heavy metal content, following the hepatopancreas (Alcorlo et al 2006; Vogt 2002; Alikhan et al 1990; Keenan and Alikhan 1991).

Lastly, this study identified two life history traits, life span and clutch size, as key traits to explain the trends in reproduction test responses across species. With a maximum life span of 140 days and large average clutch size per adult, *F. candida* represents an opportunistic species who invests energy into reproduction rather than survival, apparent by their relatively tolerant

reproductive response to PHC-contaminated soils (EC 2014; Rubach et al 2011; van Straalen 1994). Based on our findings and other studies on earthworm reproduction (Agnell et al 2012; Cermak et al 2010; Princz et al 2012), the mites and earthworms' reproduction was sensitive to PHC-contaminated soils. Their life history traits reflect they allocate more energy into survival rather than reproduction (EC 2004; Norton and Behan-Pelletier 2009; Princz et al 2012; Murphy and Sardar 1991). Other multiple species studies with *O. nitens, H. aculeifer* and earthworm reproduction confirm that these species reproduction response is more sensitive than *F. candida* to PHC soil contamination (Owojori et al 2014; Droge et al 2006; Crouau et al 1999; Princz et al 2012).

When assessing how functional traits influence interspecies interactions with the environment, phylogenetic signals are considered. In an ecotoxicology context, phylogenetic signal describes the tendency for evolutionarily close species to respond similarly to contaminants (Blomberg et al 2003). For instance, this study observed similar tolerance to PHC-contaminated soil in both earthworm species and mite species. However, phylogeny does not fully explain sensitivity as closely related species with important trait differences can vary substantially in their sensitivity. For example, *E. crypticus* is evolutionarily similar to earthworms yet can tolerate orders of magnitude greater levels of PHC contamination in soil.

3.6.3. Implications for Soil Ecolgoical Risk Assessment

Our study on mixture toxicity illustrates an approach to conducting site specific risk assessments in PHC mixture contaminated soils in Canada. Regulations in Canada require PHC-contaminated sites be regulated based on fractions and fail to account for mixture toxicity to soil invertebrates. This study highlights how sites with soils contaminated with mixtures of F2 and F3 PHCs can derive individual fraction toxicity endpoints considerate of mixture toxicity by using a battery of toxicity tests and a simple mixture toxicity model, concentration addition. These predicted individual fraction toxicity endpoints for the soil invertebrates are promising for application in risk assessment.

A question we often face when using laboratory toxicity tests to protect field organisms from contamination is: are we protecting the most sensitive species? This research highlights the importance of conducting a battery of toxicity tests on soils with varying traits when assessing the risks to organisms from soil contaminants. For instance, inclusion of an earthworm and mite species in multiple species toxicity tests is recommended, not only because of their ecological relevance to soil ecosystems, but that their populations are sensitive to PHC-contaminated soils. An additional benefit of our findings on identifying sensitive traits is the potential application to field settings. The presence of species with sensitive traits like a permeable cuticle at a PHC-contaminated field location indicates minimal risks to soil invertebrates is present.

4. Manuscript 2: Soil Invertebrate Avoidance Behavior Identifies Petroleum Hydrocarbon-Contaminated Soils Toxic to Sensitive Plant Species

4.1 Preface

The influence of a petroleum hydrocarbon product towards plants and soil invertebrates was assessed. Plant toxicity was assessed in numerous species from agronomic and forested settings. Soil invertebrate behavior towards contaminated soils was assessed using their avoidance response. The mixture toxicity effects on plant toxic responses as well as the avoidance response was determined.

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4.2 Abstract

In recent years, laboratory soil toxicity testing has advanced with the introduction of ecologically relevant boreal forest soil invertebrate and plant species, as well as increased adoption of avoidance toxicity tests. In this study, the author investigated the toxicity of a binary petroleum hydrocarbon (PHC) mixture to six agronomic and boreal forest plant species (*Elymus lanceolatus*, *Lactuca sativa*, *Medicago sativa*, *Raphanus sativus*, *Pinus banksiana* and *Picea glauca*) and the avoidance response of five soil invertebrate species *(Eisenia fetida, Enchytraeus crypticus, Folsomia candida, Oppia nitens and Hypoaspis aculeifer)*. The study assessed concentration addition and independent action mixture toxicity reference models, with potential interactions, using observed responses from the mixture and individual component toxicity endpoints from literature. Our key finding was soil invertebrate avoidance of PHC-contaminated soil was in the similar range of growth measurements for plant species sensitive to PHC-contaminated soils. This study further supports the inclusion of avoidance tests in toxicity test batteries for assessing PHC toxicity as invertebrate avoidance response appears to be linked to plant growth and informative of plant habitat quality.

4.3 Introduction

In recent years, standardized soil toxicity tests incorporated ecologically-relevant soil invertebrate and plant test species. For instance, Environment Canada introduced phytotoxicity protocols for seven herbaceous and woody boreal forest species (EC 2013). Boreal forest plant species are lacking in literature with long-lived tree species, like *P. glauca*, being especially underrepresented (Princz et al 2010). Compared to agronomic plant species, boreal forest species were more sensitive to PHC-contaminated soils but displayed similar sensitivities to salt-contaminated soils (Princz et al 2010). The oribatid mite, *Oppia nitens*, is currently being standardized for laboratory toxicity testing in Canada (Princz et al 2012). This species is highly relevant to Canadian soils as it inhabits surface soils of boreal and northern regions (Princz et al 2010; Princz et al 2012). Inclusion of boreal species improves ecological relevance of risk assessments, especially in Canada where a significant amount of industrial activities occurs in the boreal forest (Princz et al 2010). However, toxicity testing of boreal plants is time consuming as they require longer test durations, up to 42 days for tree species like *P. glauca*. Shorter toxicity tests indicative of soil habitat quality, like soil invertebrate avoidance behavior tests, exist that may be an option to reduce the number of plant toxicity tests required when conducting soil ecological risk assessments (Da Luz et al 2004; Owojori et al 2011; Owojori et al 2014).

Soil invertebrate avoidance toxicity tests provide rapid and valuable ecotoxicological information about contaminated soils. Avoidance tests rely on an organisms' ability to actively avoid contaminants in their habitat by detecting a chemical using chemoreceptor organs (Moment and Johnson 1979) and altering movements (Amorim et al 2005; Araújo et al 2016). The test provides a toxicity endpoint reflective of habitat suitability, the ability of soil to provide habitat for soil invertebrates, microorganisms and plants (ISO 2008). Contaminated soils of limited habitat function experience greater than 80% avoidance (Hund-Rinke et al 2003; Hentati et al 2013). The avoidance test can be viewed as a mechanism to prevent further exposure and a sublethal indicator of potential species survival in these environments (Hellou et al 2005). Contrasting avoidance endpoint values with sublethal and lethal endpoints provides insight into population-level impacts (Lopes et al 2014). Complete avoidance or failure to avoid contaminated areas may result in localized population declines or extinctions (Araújo et al 2016;

Lopes et al 2014; Araújo et al 2014a). The absence of key functional organisms from soils reduces soil quality by reducing organic matter quality, negatively impacting soil ecosystem services (Hopkin 1994; Gillet and Ponge 2002; Gillet and Ponge 2004).

While soil invertebrate avoidance tests provide indications of soil organism habitat quality (Hund-Rinke et al 2003; Hentati et al 2013), limited studies consider whether soil invertebrate avoidance tests are informative of plant habitat quality. Where both soil invertebrate avoidance and phytotoxicity were examined in the same study (Hentati et al 2013), few species of both soil invertebrates and plants were assessed, providing limited inference on whether avoidance is informative of plant habitat. If linkages exist between soil invertebrate avoidance and plant toxicity to contaminants, it further supports the inclusion of avoidance toxicity tests in risk assessment. Consideration of avoidance data in SSRAs makes this management option more desirable to site managers due to its ecological relevance, rapid time frame and minimal soil and labour resources. The study evaluated mixture toxicity and whether soil invertebrates' avoidance response to PHC-contaminated soils was indicative of plant toxicity.

Minimal aliphatic petroleum hydrocarbon (PHC) avoidance studies exist, and those available reflect earthworm avoidance (Hentati et al 2013; Schaefer 2001), despite validation of numerous species for soil invertebrate avoidance tests (Owojori et al 2011; Owojori et al 2014; Amorim et al 2005; Heupel 2002). Assessment of soil invertebrate behavior to PHC-contaminated soils is important as many species, like predatory mites, are attracted to volatile compounds as a mechanism to find food (Aratchige et al 2004; Pfeffer and Filser 2010; Araújo et al 2014a). Existing PHC avoidance literature include single polyaromatic hydrocarbons (Owojori et al 2011; Owojori et al 2014; Hellou et al 2005; Araújo et al 2014a; Araújo et al 2014b), or PHCs in mixtures with other contaminants like metals (Aldaya et al 2006) or nutrients (Bori et al 2016). Even though, PHC contamination typically occurs as mixtures, few existing studies examine whether avoidance responses follow intrinsic mechanism of action-based mixture toxicity theories like concentration addition or independent action (Loureiro et al 2009). Although mixture toxicity relies on chemical compositions, soil invertebrate avoidance maybe more influenced by physical properties of the contaminant in soil such as vapor pressure, alteration of soil wetting properties, or limiting oxygen diffusion.

Our objectives in this study were to (1) test the toxicity of PHC-contaminated soil to a suite of agronomic and ecologically relevant plant species, (2) assess avoidance response of soil invertebrates with varying traits and trophic levels to PHC-contaminated soils and (3) determine which mixture toxicity models fit plant toxicity and soil invertebrate avoidance to PHC-contaminated soils.

4.4 Materials and Methods

4.4.1. Soil Preparation

The test soil utilized was Organization for the Economic Cooperation and Development (OECD) artificial soils, compromised of approximately 70% silica sand, 20% kaolin clay and 10% 2 mm sieved, air-dried *Sphagnum* sp. peat moss, calcium carbonate and distilled water. The moisture content for plant toxicity tests was approximately 90% water-holding capacity while avoidance tests were 70%. Soil pH was determined by shaking soil solutions with 0.01 M $CaCl_2$ in a 1:5 soil: solution ratio (EC 2005a). Soil pH was assessed at test start and completion to monitor consistency across concentrations and within acceptable range of 6.5 to 7.5 (EC 2005a).

This study reflects a fixed ratio design, where the ratio of individual PHC fractions is fixed, with the total mixture concentration increasing (van Gestel 2011). The PHC product utilized was a lubricating oil dominated by F2 and F3 PHC. When spiking soils, acetone was utilized as a carrier solvent to ensure even distribution of oil. Initially, mineral components of soil were spiked with oil and acetone and left in a fume hood overnight for dissipation of acetone. The following day peat and calcium carbonate was added. This process allows quicker removal of acetone and improved homogenization of oil amongst mineral and organic components.

4.4.2. Toxicity Tests

Plant species approved by Environment Canada for phytotoxicity tests were utilized and included the following economic and ecologically-relevant species: northern wheat grass (*Elymus lanceolatus)*, lettuce *(Lactuca sativa)*, alfalfa (*Medicago sativa)*, radish (*Raphanus sativus)*, jack pine (*Pinus banksiana)* and white spruce (*Picea glauca)* (EC 2005a; E 2012). Standardized or

common methodologies were applied for the avoidance test on the following soil invertebrate: *E. fetida* (ISO 2008), *F. candida* (ISO 2011) [31], *O. nitens* (Owojori et al 2011), *H. aculeifer* (Owojori et al 2014), and *E. crypticus* (Amorim et al 2008). The organisms used in avoidance tests were unsynchronized adults. For *E. fetida* and *E. crypticus*, adult organisms were distinguished by a clitellum. Individual *E. fetida* used for tests were individual wet weights between 250 and 600 mg (EC 2004). For additional information on culturing of soil invertebrates see Gainer et al (2018).

Range finding tests were initially conducted for every plant species to determine an appropriate range of concentrations. Plant toxicity test chamber conditions were 24˚C (+/- 2˚C), 16h light, 8h dark cycle and full spectrum fluorescent lighting (18,750 +/- 6,250 lux). Avoidance test chamber conditions were 20˚C (+/- 2˚C), 16h light, 8h dark cycle and with approximately 600 lux at surface. Humidity was maintained at approximately 60%. Each toxicity test consisted of a minimum of 9 concentrations plus a solvent (acetone) control and a negative control. Plant and avoidance toxicity tests consisted of five replicates. Replicates and treatments were randomly placed in their locations on the same level in the growth chamber.

Test soil samples were collected at the start and end of tests for PHC analysis. Soil PHC analysis was conducted in accordance with Canadian regulations (CCME 2008) in which hydrocarbons are extracted from 2 g of soil via shaking with 40 mL of 1:1 hexane:acetone followed by reduction under nitrogen gas. Quality control and assurance procedures included duplicates, matrix and blank spikes, method blanks and reagent blank every 20 samples. Spiked samples within 70-130% recoveries were considered adequate. Based on Canadian Council of Ministers of the Environment guidance (CCME 2008), the F2 fraction was considered carbon greater than 10 to 16 and the F3 fraction is greater than 16 to 34. Quantification of the F2 and F3 fractions was performed using a Varian CP3800 gas chromatograph fitted with a flame ionization detector (GC-FID) with chromatogram interpreted performed on CompasCDS software (Varian, Santa Clarita, CA). Total petroleum hydrocarbon (TPH) content was determined as the sum of F2 and F3 PHCs.

Seeds were either commercially purchased or donated from BrettYoung Seeds (Winnipeg, Manitoba). Vegetable seeds used organic (Earlys, Saskatoon, Saskatchewan) and boreal forest species were purchased from a tree nursery (Smoky Lake, Alberta). All seed lots utilized had a minimum of 95 % purity, with at least a 95 % germination rate. Seeds were stored in the dark at approximately 4˚C and tree species (*P. banksiana* and *P. glauca*) were cold stratified in wet paper towel a minimum of three weeks prior to test commencement (EC 2012). The amount of seeds per container varied by species: 5 seeds/vessel for *L. sativa*, *R. sativus* and *E. lanceolatus*, and 10 seeds/vessel for *M. sativa*, *P. banksiana* and *P. glauca* (EC 2005a; EC 2012). Seeds were randomly distributed across treatments and replicates. Consistent with protocols, seeds were planted at a depth approximately twice the average diameter of the seed itself using forceps. Test duration varied by species: 14 days for *R. sativus* and *L sativa*, 21 days for *E. lanceolatus* and *M. sativa*, 35 days for *P. banksiana*, and 42 days for *P. glauca*. Emergence measurements were made one week after test start for non-boreal species and at test completion for boreal species. At test completion, plants were gently removed from soil by saturating soil and gently shaking to loosen soil, followed by measurement of endpoints. Following measurement of shoot and root length, samples were dried in an oven at 90°C for 48 hours. Plant toxicity endpoints included: emergence, shoot dry mass, root dry mass, shoot length and root length.

All soil invertebrate responses were determined using two-chambered containers. The dimensions of the chamber, amount of soils used and duration varied by organism (Appendix B Table B-1). The containers were divided into two sections using a plastic divider and drawing a line on the outside. The outside of the container was labeled as either control or treatment. With the plastic divider in place, the container was filled with control soil on one side and treated soil on the opposing side. The plastic divider was removed and test specimens added in the center. Animals were not fed during test duration. During test duration and completion, care was taken to minimize movements as this can cause disturb behavior of soil invertebrates, such as *F. candida*. At test completion, the plastic divider was inserted to separate control and treated soils. Soil was gently removed from each side. Method of extraction of individuals from the soil varied by organism (Appendix B Table B-1). During heat extraction, care was taken to keep the plaster of Paris within the receiving extraction container moist.

4.4.3. Statistical Analysis

The measurements obtained from the plant toxicity tests included inhibition of emergence, root length, shoot length, root dry mass and shoot dry mass. All plant species endpoints met test validity in negative and solvent controls (EC 2005a; EC 2012). For both plant toxicity and avoidance tests, students t-tests were conducted between the solvent and negative controls. For all tests, responses were not significantly different between solvent control and negative control treatments, and therefore, data were pooled. Statistical analysis could not be performed on select species endpoints (*L. sativa* emergence, *M. sativa* emergence and *P. banksiana* shoot length) since an inhibition response greater than 50% was not observed at the maximum testing concentrations.

Avoidance was determined as the number of organisms avoiding the contaminated soils as a fraction of the total number of organisms introduced, the net response. Net response is determined as: NR=((C-T)/(N))*100, where NR=net response, C=number of organisms in the control soil, T=number of organisms in the treated soil and N=total number of test organisms (Owojori et al 2011; Owojori et al 2014; ISO 2008; Hund-Rinke et al 2003; ISO 2011). Positive net response values indicate avoidance while negative indicate an attraction to contaminated soil. ISO (ISO 2008; ISO 2011) considers limited habitat function of contaminated soil if NR greater than 70%. An avoidance test was considered valid if recovery of individuals was at least 80%, mortality was less than 20% and proportion of organisms was 50 +/- 10% in dual control containers (Owojori et al 2011; ISO 2008; ISO 2011). Normality and homogeneity of variance was assessed with box plots and, Shapiro-Wilks test and Levene's test, respectively. To determine effect concentrations (EC), negative net response were considered 0% avoidance (ISO 2008; ISO 2011). A non-avoidance response was observed in *E. crypticus* and test endpoint not determined.

The whole PHC mixture's dose response analysis followed guidance recommended by Environment Canada (EC 2012; EC 2005b). Determination of 25 and 50% effective concentrations (EC) with confidence intervals were conducted in Graphpad Prism® (version 6.0) by a four-parameter (minimum, maximum, EC50, slope) logistic Hill equation (variable slope

sigmoidal nonlinear regression model) (Appendix B Equation [B-1]). Graphics were visualized in SigmaPlot (Version 12.0).

4.4.4. Mixture Toxicity Modelling

Methods for mixture toxicity followed the same procedure outlined in Gainer et al [34] with equations summarized in Appendix A. Briefly, the Solver Add-In for Microsoft Excel 2010 was utilized to assess and fit the response inhibition for multiple PHC concentrations with concentration addition (Appendix B Equation [B-2]) and independent action (Appendix B Equation [B-3]). The model with the best fit reported the lowest sum of squares between the optimized models expected values and observed mixture responses. A Chi-squared test determined if the sum of squares between models were significantly different ($p<0.05$). Following determination of the which reference model was best fit, potential interactions were tested by extending to include a deviation parameter to detect the following interactions: synergism, antagonism and dose level dependence as published by Jonker et al (2005) for binary mixtures. Synergism occurs when the mixture toxicity is greater than the toxicity expected from the single components and antagonism is if the mixture toxicity is less than the expected toxicity from the single components (van Gestel 2011). Dose-level deviations indicate toxicity varies at low and high concentrations (Jonker et al 2005). Since the PHC product used in this study has a fixed ratio of F2:F3, dose ratio deviations were not assessed. Like the reference model, the best model fit for the extended model was determined as the lowest sum of squares and a chi-squared test ($p<0.05$).

The initial model estimates for the individual F2 and F3 EC50s were obtained from literature or regulatory documents (Appendix B Tables B-2 to B-4). Individual fraction toxicity endpoints were only available for three plant species: *E. lanceolatus*, *M. sativa* and *Horduem vulgare* (CCME 2008; Cermak et al 2010; Angell et al 2012). If available, values from the same species were used. For species lacking individual fraction toxicity endpoints, the average of available literature was used (Appendix B Table B-2). For emergence from F3 contaminated soils, all plant species used *H. vulgare* value as this was the only plant species with that endpoint. No individual fraction endpoints were available from literature for avoidance tests. The individual fraction endpoints were assumed to be half the mixture value (Table 4-3). Available studies on individual

F2 and F3 toxicity tests did not report slopes so the slope of the mixture was assumed (Appendix B Table B1-B3). Available studies also did not report if a full dose response curve was reached for each test, so it was assumed the full dose response was obtained with a minimum of 0 % and maximum of 100% inhibition or avoidance.

The individual F2 and F3 PHC toxicity endpoints were obtained as the values from the optimized reference models. The EC25 for the individual fraction from the optimized model was determined using the EC50 for the individual fraction with the slope, maximum response and minimum response from the mixture (Appendix B Equation [B-4]). The maximum response for all tests were 100%. Full dose response (between 0 and 10% as a minimum and 90 to 100% as maximum) was observed in all tests with the mixture except a few. *R. sativus* emergence reached a maximum response of 80 % and *R. sativus* shoot length reached a maximum response of approximately 70%. To validate individual F2 and F3 PHC toxicity endpoints obtained from the optimized models, the CA or IA predicted values were compared with the same individual fraction endpoints from literature previously mentioned (Appendix B Table B-2 to B-4). The difference between the model predicted values and literature were determined. Results were visualized by plotting observed response of the mixture with the predicted response from unoptimized concentration addition and independent action reference models.

4.5 Results

4.5.1. Oil and Soil Analysis

The TPH content at test start was approximately 93% of nominal, for detailed oil composition see our previous study (Gainer et al 2018). The composition of the lubricating oil used in toxicity testing was mainly F2 (52%) and F3 (48%) PHCs. Over the 14 and 42 days tests, degradation of F2 and F3 PHC within the artificial soil between the start and end of the test was minimal (5-20%).

4.5.2. Toxic Responses to Mixture

Root dry mass was the most sensitive endpoint across plant species, with the lowest EC25 values and smallest range values (Figure 4-1; Table 4-1, 4-2). Root dry mass EC25 values ranged from 180 mg/kg TPH for *L. sativa* to 6,330 mg/kg TPH for *P. banksiana*. Emergence was the most tolerant endpoint for all plant species exposed to the PHC mixture, with values ranging from 21, 620 mg/kg TPH observed in *E. lanceolatus*, to over 186,000 mg/kg TPH soil for *M. sativa* and *L. sativa*. The EC25 values for shoot length ranged from 230 mg/kg TPH in *M. sativa* to greater than 69, 750 mg/kg TPH for *P. banksiana*. Root length EC25 values ranged from 140 mg/kg TPH for *M. sativa* to 22,770 mg/kg TPH for *P. glauca*.

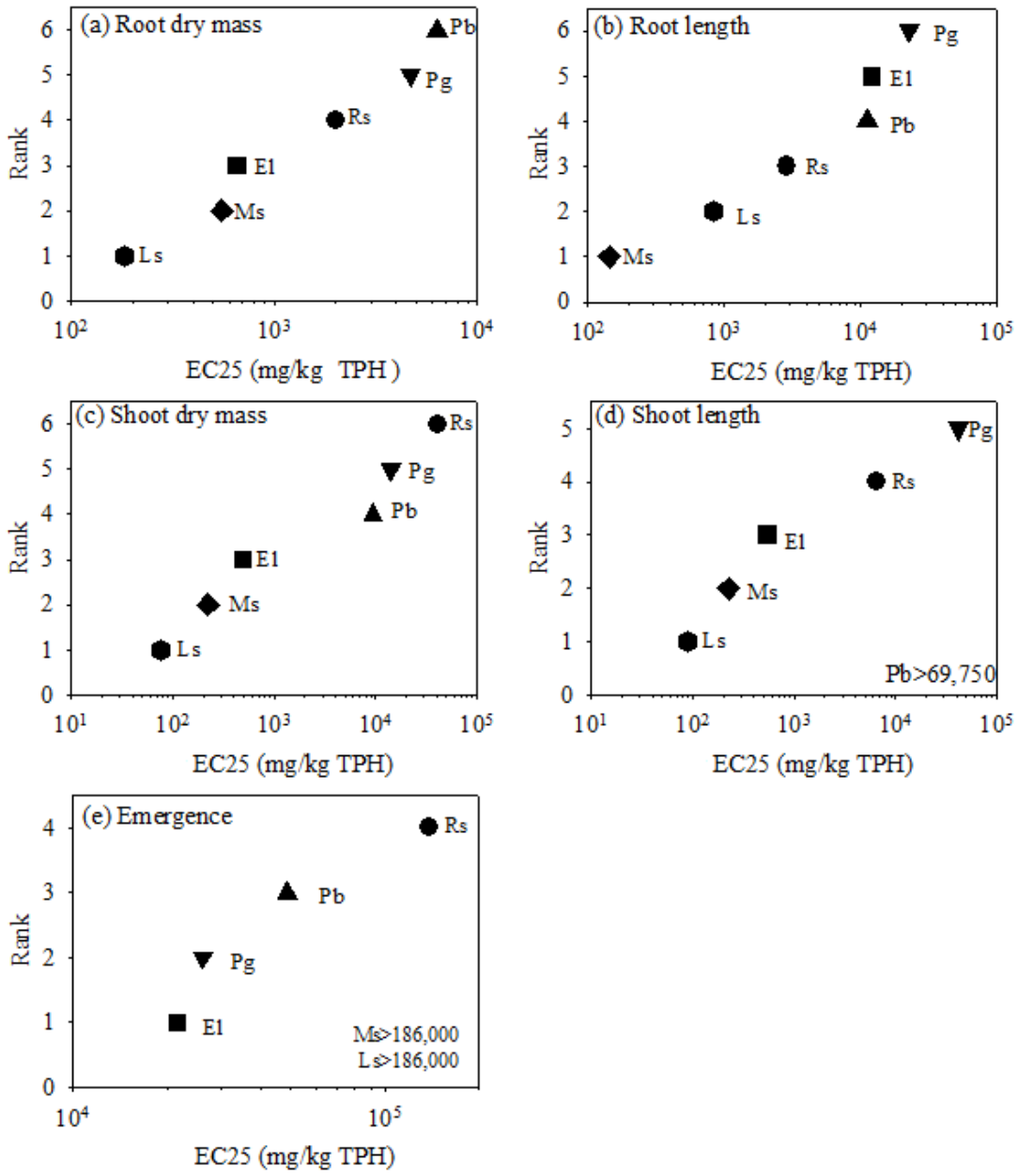

Figure 4-1. Rank of mixture effective concentration (EC) 25 for plant (a) root dry mass, (b) root length, (c) shoot dry mass, (d) shoot length and (e) emergence from exposure to the petroleum hydrocarbon mixture contaminated soil as a function of effective concentration (EC) 25. The lowest rank is most sensitive species. Circles represent *Raphanus sativus* (Rs), diamonds represent *Medicago sativa* (Ms), squares represent *Elymus laceolatus* (El), triangles represent *Pinus banksiana* (Pb), inverted triangles represent *Picea glauca* (Pg) and hexagons represent *Lactuca sativa* (Ls). TPH represents total petroleum hydrocarbons.

Table 4-1. The 25th percentile effective concentration values, 50th percentile effective concentration values with 95% confidence intervals, coefficient of determination and dose response curve slope emergence for plants (*Raphanus sativus, Elymus laceolatus, Medicago sativa, Pinus banksiana, Lactuca sativa* and *Picea glauca*) exposed to soil spiked with a fixed ratio petroleum hydrocarbon product mixture.

Species	Mixture EC values (mg/kg TPH)					
	EC25	EC50	Lower 95% CI	Upper 95% CI	r^2	slope
Raphanus sativus	138,210	172,180	164,430	180,290	0.89	5.0
Elymus lanceolatus	21,620	32,900	28,010	38,640	0.88	2.7
Medicago sativa	na	>186,000	na	na	na	na
Pinus banksiana	48,790	60,690	54,380	58,470	0.92	7.5
Lactuca sativa	na	>186,000	na	na	na	na
Picea glauca	26,080	35,650	33,360	38,090	0.97	4.6

mg/kg TPH= mg total petroleum hydrocarbons per kg soil; CI=confidence interval; EC=effective concentration; r^2=coefficient of determination; na=not applicable; greater than value indicates full response not obtained

Table 4-2. The 25th percentile effective concentration values, 50th percentile effective concentration values with 95% confidence intervals, coefficient of determination and dose response curve slope growth (length and dry mass) for plants (*Raphanus sativus, Elymus laceolatus, Medicago sativa, Pinus banksiana, Lactuca sativa* and *Picea glauca*) exposed to soil spiked with a fixed ratio petroleum hydrocarbon product mixture.

Species	Endpoint	Mixture EC values (mg/kg TPH)					
		EC25	EC50	Lower 95% CI	Upper 95% CI	r^2	slope
Raphanus sativus	Root length	2,910	18,710	13,370	26,170	0.92	0.59
	Shoot length	6,640	100,800	72,000	141,130	0.85	0.40
	Root dry mass	2,020	9,320	6,370	13,630	0.92	0.72
	Shoot dry mass	41,140	85,580	73,580	99,530	0.89	1.5
Elymus lanceolatus	Root length	11,990	24,600	20,550	29,460	0.91	1.7
	Shoot length	550	3,370	2,530	5,210	0.89	0.61
	Root dry mass	660	3,170	2,300	5,060	0.87	0.70
	Shoot dry mass	490	2,430	1,890	3,600	0.92	0.69
Medicago sativa	Root length	140	1,260	890	1,790	0.92	0.50
	Shoot length	230	1,840	1,340	2,510	0.92	0.53
	Root dry mass	550	2,410	1,820	3,200	0.92	0.75
	Shoot dry mass	220	1,540	1,020	2,340	0.86	0.86
Pinus banksiana	Root length	11,153	45,290	35,440	57,870	0.77	0.83
	Shoot length	na	>69,750	na	na	na	na
	Root dry mass	6,330	22,190	17,750	27, 750	0.84	0.93
	Shoot dry mass	9,470	36,000	27,930	46,400	0.75	0.87
Lactuca sativa	Root length	840	14,670	9,290	23,180	0.75	0.39
	Shoot length	90	1,120	850	1,700	0.86	0.44
	Root dry mass	180	610	470	800	0.89	0.97
	Shoot dry mass	80	730	510	1030	0.86	0.50
Picea glauca	Root length	22,770	35,310	31,970	38,990	0.93	3
	Shoot length	44,540	47,930	45,210	50,810	0.92	15.3
	Root dry mass	5,080	11,260	9,030	14,030	0.90	1.4
	Shoot dry mass	14,150	23,140	20,150	28,560	0.93	2.6

mg/kg TPH= mg total petroleum hydrocarbons per kg soil; CI=confidence interval; EC=effective concentration; r^2=coefficient of determination; na=not applicable; greater than value indicates full response not obtained

All soil invertebrates except, *E. crypticus*, avoided the PHC-contaminated soil (Figure 4-2). The soil invertebrate with the most sensitive avoidance response to the PHC mixture contaminated soil was *O. nitens* while *H. aculeifer* was most tolerant (Figure 4-2, Table 4-3). The range in EC25 values for soil invertebrate avoidance to PHC-contaminated soils was 1,140 to 11,800 mg/kg TPH (Table 4-3). A non-avoidance response was observed in *E. crypticus* up to a concentration of 46, 500 mg/kg TPH. For all organisms, at low test concentrations there was slight attraction to the PHC-contaminated soil.

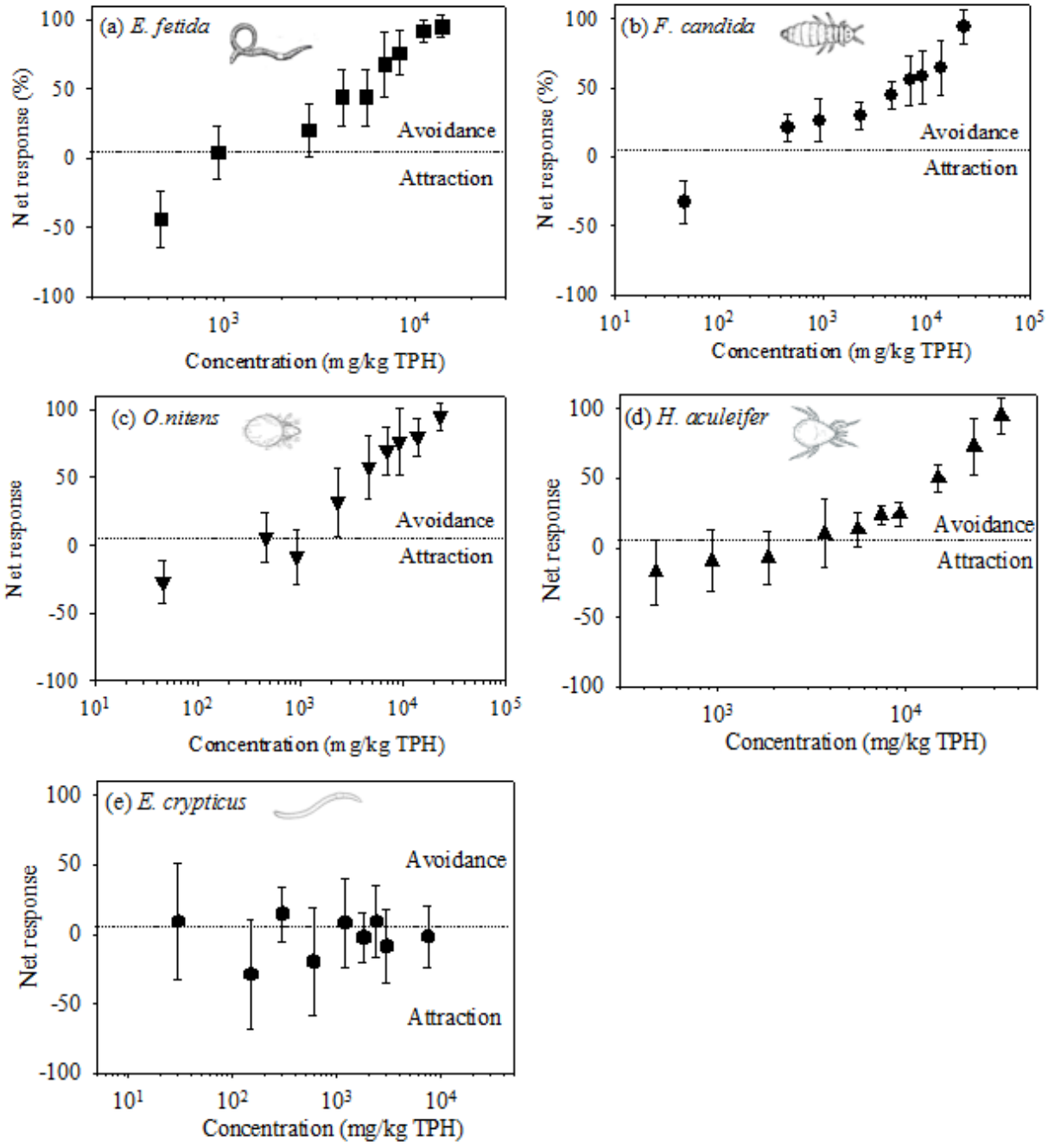

Figure 4-2. Avoidance net response of (a) *Eisenia fetida*, (b) *Folsomia candida*, (c) *Oppia nitens*, (d) *Hypoaspis aculeifer* and (e) *Enchytraeus crypticus* to petroleum hydrocarbon-contaminated soil. TPH represents total petroleum hydrocarbons.

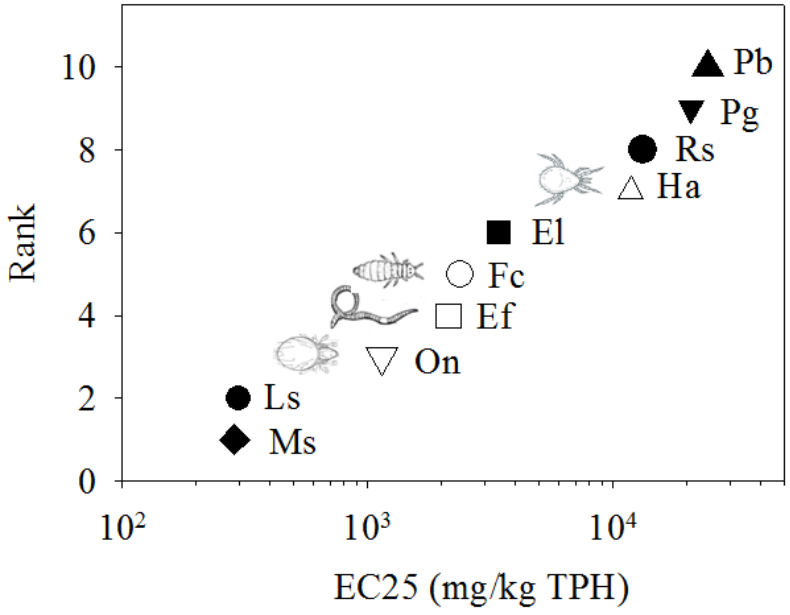

Figure 4-3. Rank of effective concentration (EC) 25 total petroleum hydrocarbon (TPH) values from exposure of soil invertebrate's avoidance and average of plant endpoints to petroleum hydrocarbon contaminated soil as a function of EC25, from most sensitive to least. Empty circles depict soil invertebrate avoidance response and solid circles represent the average of plant length and biomass endpoints for: solid circle represents *Raphanus sativus* (Rs), solid diamond represent *Medicago sativa* (Ms), solid square represent *Elymus laceolatus* (El), solid triangle represent *Pinus banksiana* (Pb), solid inverted triangle represent *Picea glauca* (Pg), solid hexagon represents *Lactuca sativa* (Ls), empty square represents *Eisenia fetida* (Ef), empty circle represents *Folsomia candida* (Fc), empty inverted triangle represents *Oppia nitens* (On) and empty triangle represents *Hypoaspis aculeifer* (Ha).

Table 4-3. The 25th percentile effective concentration values, 50th percentile effective concentration values with 95% confidence intervals, coefficient of determination and dose response curve slope for soil invertebrate (*Eisenia fetida, Enchytraeus crypticus, Folsomia candida, Oppia nitens* and *Hypoaspis aculeifer*) avoidance of soil spiked with a fixed ratio petroleum hydrocarbon product mixture.

Species	Mixture EC values (mg/kg TPH)				r^2	Slope
	EC25	EC50	Lower 95% CI	Upper 95% CI		
Eisenia fetida	2,130	3,950	3,000	5,210	0.67	1.78
Folsomia candida	2,370	5,750	4,560	7,260	0.63	1.25
Oppia nitens	1,140	2,390	1,790	3,180	0.89	1.48
Hypoaspis aculeifer	7,110	11,800	10,040	13,860	0.84	1.90
Enchytraeus crypticus	na	>46,500	na	na	na	na

mg/kg TPH= mg total petroleum hydrocarbons per kg soil; CI=confidence interval; EC=effective concentration; r^2=coefficient of determination; na=not applicable; greater than value indicates full response not obtained

The soil invertebrate avoidance EC25 values are in the same range of the average of plant growth endpoints (mass and length only) (Figure 4-3). The average EC25 for growth endpoints for the four most sensitive plant species (*M. sativa, L. sativa, E. lanceolatus* and *R. sativus*) ranged from 290 to 13, 180 mg/kg TPH. The EC25 values for soil invertebrate that showed an avoidance response ranged from 1,140 to 11, 900 mg/kg TPH.

4.5.3. Mixture Toxicity

When validating CA and IA models using individual PHC fractions from literature and regulations, both models overestimated toxic responses to the PHC mixture in all test species and types, except *O. nitens* avoidance (Appendix B Figure B-1 to B-6, Table B-5 and B-6). Both CA and IA models fit the observed mixture responses of plants and soil invertebrate avoidance, with no significant difference between models (Appendix B Table B-5 and B-6). For all tests, no further interactions (antagonism, synergism or dose level) beyond the reference models fit the models. The plant species and endpoint where the model produced the lowest differences between the observed response and the model predicted response was CA for *M. sativa* shoot length with an average residual value of 0 (Appendix B Figure B-3, Table 4-3). In contrast, the highest average residual was observed in *R. sativus* emergence, with a value of -42 for CA and -38 for IA (Appendix B Figure B-1, Table B-5). The lowest average residuals observed for soil

invertebrate avoidance test responses was *O. nitens*, with both reference models reporting a value of 2.3.

The ability of the CA and IA reference models to predict individual F2 and F3 toxicity endpoints was assessed for plant species only as no comparable literature for soil invertebrate avoidance was available. For predicting individual F2 toxicity endpoints across all plant toxicity and soil invertebrate avoidance tests, the differences between models was small for the majority of tests with a few exceptions (Figure 4-4). Greater differences were observed between CA and IA reference model predicted F3 plant toxicity and avoidance response endpoints. The CA and IA predicted individual F2 and F3 toxicity endpoints were compared with select species (*E. lanceolatus* and *M. sativa*) who had available studies from literature or regulations to determine if predicts were valid (Figure 4-5). For both F2 and F3 predicted toxicity endpoints, the majority of predicted values were close to other studies with a few exceptions. Deviations from near zero were observed more in CA predicted values than IA, with CA tending to overestimate toxicity.

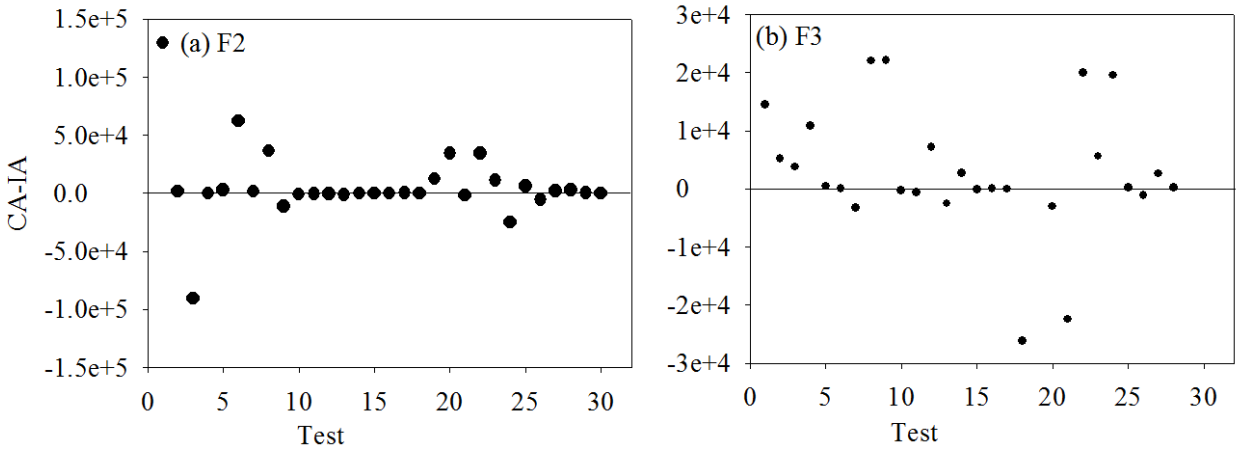

Figure 4-4. The difference between the concentration addition and independent action predicted (a) F2 and (b) F3 toxicity endpoints for all plant species toxicity endpoints and soil invertebrate avoidance with a few outliers not presented.

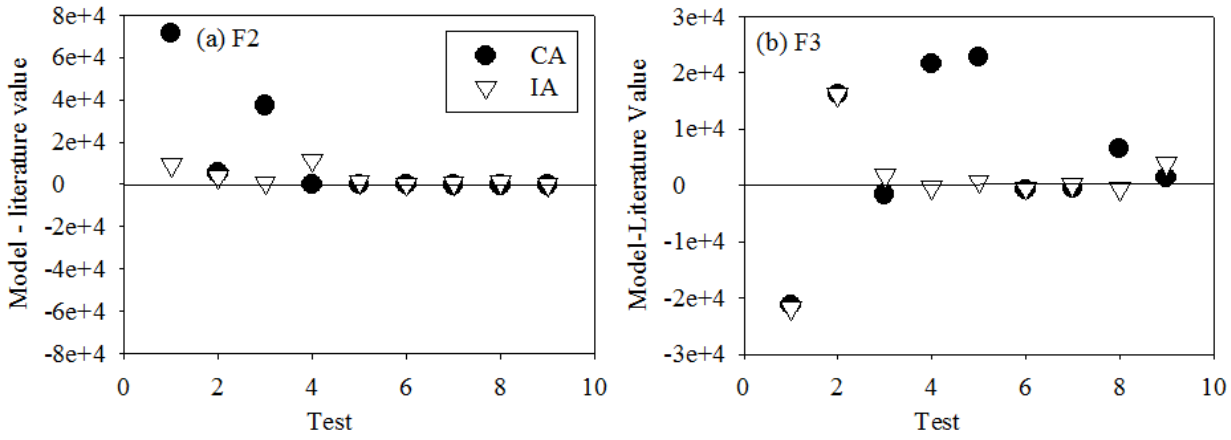

Figure 4-5. The concentration addition and independent action predicted (a) F2 and (b) F3 toxicity endpoints subtracted from the average literature toxicity values for *E. lanceolatus* and *M. sativa.*

4.6 Discussion

4.6.1. Plant Toxicity and Soil Invertebrate Avoidance

In our study, the avoidance response of four soil invertebrate species reflected plant toxic responses of plant species sensitive to PHC-contaminated soil. The study observed that the total petroleum hydrocarbon EC25 values of soil invertebrate species avoiding PHC-contaminated soil were within the range of growth measurements for the four most sensitive plant species. The present study is the first to look at such trends in PHC-contaminated soils, with minimal related studies existing. Literature across contaminants is mixed suggesting this relationship between sensitive plant species and soil invertebrate avoidance behavior may be specific to PHC-contaminated soils. A study with a sensitive plant species, *Brassica napus*, as well as *Avena sativa*, reported shoot length EC50 values an order of magnitude lower than the avoidance response of *F. candida* to boric acid-contaminated soils (Becker et al 2011). However, the differences between this study and ours may be due contaminant specific mechanism of action or the fact our study used an average of length and biomass endpoints while Becker et al (2011) used only shoot length. As observed in our study, shoot length can be either a sensitive endpoint, like with *M. sativa*, or tolerant, as observed with *P. banksiana*. In another study (Velicogna et al 2016), soil invertebrate avoidance responses were more sensitive than plant toxicity responses.

Eisenia andrei avoidance of silver nitrate and silver nanoparticles was approximately an order of magnitude less than the average of the length and biomass test endpoints from both *E. lanceolatus* and *Trifolium pretense* (Velicogna et al 2016). More studies are necessary to determine if the relationship between soil invertebrate avoidance response and plant species sensitive to contamination extends beyond PHC-contaminated soils.

The toxic responses of the six plant species tested ranged orders of magnitudes. The study explored potential traits that may explain these findings, as trait-based approaches in plant ecology successfully explain community compositions following disturbances (Hevia et al 2017; Chapin et al 1993). Consistent with literature, emergence is the least sensitive test endpoint (Princz e tal 2012; Angell et al 2012). *Lactuca sativa* (lettuce) and *M. sativa* (alfalfa) emergence displayed the greatest tolerance to PHC-contaminated soils, with minimal comparable studies, as PHC phytotoxicity literature is currently dominated by germination tests. The emergence tolerance of *L. sativa* and *M. sativa* to PHC-contaminated soils is likely due to their smaller seed size as smaller seeds contain more limited resources, requiring rapid initial growth to access above and below ground resources, rather than staying dormant in adverse conditions (Robson et al 2010; Fenner and Kitahima 1999; Swanborough and Westoby 1996). For growth response to PHC-contaminated soils, the study hypothesized life-cycle duration as a key trait influencing the interspecies variation, reflecting findings from trait studies in plant ecology (Hevia et al 2017). In our study, as well as others (CCME 2008; Saterbak et al 1999; Dorn et al 1998; Eom et al 2007; Fatokun and Zharare 2015), annuals like *L. sativa* and *M. sativa*, were the most sensitive species across root and shoot endpoints. Long-lived species, *P. banksiana* and *P. glauca,* displayed tolerant growth responses to PHC contamination, a finding also observed by the first phytotoxicity study on these species (Princz et al 2010). Field (Chapin et al 1993; Robson et al 2004) and laboratory (Princz et al 2017) studies with other contaminants agree that longer-lived species exhibit resistance to unfavorable environmental conditions like contamination or drought. The mechanism for tolerance of longer life-cycles maybe a slower growth rates and less carbon demands, allowing for more allocation of energy to stress resistance such as chemical defense, nutrient uptake (Lambers and Poorter 1992) or detoxification (Chapin et al 1993). Alternatively, the tolerance to PHC-contaminated soils observed in *P. banksiana* and *P. glauca* maybe conifer specific rather than a life-cycle trait. Other studies also observed conifers were

tolerant to PHC-contaminated soils (Princz et al 2010; Mackey and DePuit 1985; Olson and Fletcher 2000) which is possibly due to the higher lipid content of coniferous xylem (Odabasi et al 2015). An additional trait potentially influencing how plant species respond to PHC-contaminated soil is root lipid content, as the PHC site of toxic action is cellular membrane lipids. Other plant toxicity studies on organics report a positive correlation between root lipid content and concentration of organic contaminants in roots (Gao and Zhu 2004; Trapp et al 1990; Zhang and Zhu 2009). Lastly, varying detoxification processes also likely accounts for plant interspecies differences in toxic responses to PHC-contaminations as activities of enzymes, like glutamate dehydrogenase and superoxide dismutase, vary across agronomic and woody plant species in uncontaminated and PHC-contaminated soils (Sadunishvili et al 2009; Song et al 2011; Zhang et al 2007). This study recognizes how trait-based discussions may also be conflated with phylogenetic influences on modes of toxic action and toxicokinetics as phylogenetic distance is often linked with trait similarity (Swanborough and Westoby 1996).

Our plant toxicity findings did not support the common observation that a flowering plants embryo containing a single or double cotyledon as a trait influencing the sensitivity of species to PHC-contaminated soils. Other studies (Princz et al 2017) and regulations (EC 2005a; ASTM 1999) suggest dicots as the most sensitive plant species to boric acid followed by monocots and finally gymnosperms. In our study, the only monocot (*E. lanceolatus*) displayed moderate sensitivity to PHC-contaminated soils across all endpoints. The most sensitive species were both dicots, however; our results agree with findings of Princz et al (2017) that gymnosperms are tolerant.

Soil invertebrate avoidance is typically thought to be a sensitive endpoint relative to acute and chronic soil toxicity tests. This is not universally true as this study found that avoidance response of three species was greater than their corresponding reproduction toxicity responses. The soil avoidance response for *H. aculeifer* (11,799 mg/kg TPH) was over an order of magnitude greater than the reproductive inhibition observed in a previous study (206 mg/kg TPH) (Gainer et al 2018). A similar trend, but to a lesser extent, was evident between *O. nitens* and *E. fetida* avoidance response and reproduction inhibition (Gainer et al 2018; Angell et al

2012). This study found no avoidance response with *E. crypticus* whereas reproduction inhibition occurred in soils contaminated with the same PHC product (Gainer et al 2018). This contrasts some available literature with aromatic and aliphatic PHC avoidance that found similar values between avoidance and reproduction. In a field study with weathered hydrocarbons and metals, the avoidance response of *E. fetida* was more similar to the reproduction toxic response than mortality (Bori et al 2016). Studies with the single aromatic PHC, phenanthrene, on both *H. aculeifer* (Owojori et al 2014) and *O. nitens* (Owojori et al 2011), reported similar avoidance EC50s with overlapping confidence intervals. The discrepancies with these studies and the findings in this chapter are likely due to the increased volatility from the PHC product this study tested.

Traits explain differences in soil invertebrate avoidance response across species. In this study, *E. crypticus* displayed no avoidance of PHC-contaminated soil, likely due to their lack of exposure. Sediment toxicity tests with crude oil also found non-avoidance in sediment dwelling juvenile fish (Moles et al 1994). As suggested in Gainer et al (2018), the small body size and close association with soil pore water allows the organism to remain in soil pore water where minimal exposure to PHCs occurs due to the limited water solubility (approximately <1 mg/L (CCME 2008)) of the PHC product this study tested, a lubricating oil (solubility ~ 0.01 mg/L). Previously, Gainer et al (2018) observed similar mortality and reproductive inhibition responses between *H. aculeifer* and *O. nitens,* potentially due to the phylogenetic relatedness of traits governing exposure between species, despite these species being from different trophic levels. However, phylogenetic relatedness does not appear to influence the avoidance response of these mites to PHC-contaminated soils, as *H. aculeifer* avoidance response EC25 was an order of magnitude greater than *O. nitens*. Other studies with phylogenetic similar species, like Collembola, found a range in avoidance responses to soil contaminated with naphthalene (Boitaud et al 2006) or a herbicide (Heupel 2002). The tolerance observed in the *H. aculeifer* is likely due to its attraction to hydrocarbon volatiles, a mechanism aiding these predators in locating prey (Aratchige et al 2004; Pfeffer and Filser 2010; Hall and Hedlund 1999). Predatory mites detect volatiles from olfactory sensilla located on their front legs (van Wijk et al 2006). The remaining species, *O. nitens, E. fetida* and *F. canada,* all displayed avoidance responses at similar PHC concentrations, even though these organisms display varying functional traits and

phylogeny. Since these species mainly consume parts of decompising soil organic matter, the similarity in avoidance is likely a reflection of their food searching behavior. Like the predatory mite, but to a lesser extent, species like Collembola are also attracted to volatile odours released from organic matter and fungus (Wenke et al 2010; Bengtsson et al 1988) and this is also evident in their observed attraction to the PHC-contaminated soils at low test concentrations. Other studies showed *F. candida* and *E. andrei* avoid PHC-contaminated soils (Hentati et al 2013).

4.6.2. Mixture Toxicity

Our study shows that different mathematical models produce similar outcomes across a range of species and test types. This is consistent with previous studies on soil invertebrate toxicity where authors found both models fit the mortality and reproduction inhibition response to PHC-contaminated soil (Gainer et al 2018). Due to the similar mechanism of action for F2 and F3 PHC, non-polar narcosis, the concentration addition model was expected to fit best. As discussed in our previous research (Gainer et al 2018), other studies with similar and dissimilar mechanisms of toxic action reported comparable findings (Qiu et al 2016; Backhaus et al 2004; Cedergreen et al; 2008) and this is potentially due to the mathematical nature of these models (van Gestel et al 2011, Backhaus and Faust 2012). In addition, perhaps the models were not able to differentiate between CA and IA due to the many assumptions incorporated about the individual fraction toxic responses to organism in the mixture toxicity. Like a previous study (Gainer et al 2018), this study found these models advantageous as a tool for predicting individual component toxicity using responses to the mixture. While Gainer et al (2018) previously suggested CA as the best model for predicting individual component toxicity concentrations for soil invertebrate mortality and reproduction responses to PHC-contaminated soils, both CA and IA were appropriate for predicting plant toxicity and soil invertebrate avoidance responses.

5. Manuscript 3: Site Specific Risk Assessment, Persistence and Toxicity of a Lubricating Oil in Canadian Soils

5.1 Preface

Canadian regulations on setting guidelines protective of soil dwelling organisms vary relative to other jurisdictions like the European Union. Currently, minimal studies assess the impact of guidelines values on the soil receptors they are suppose to protect. In this study, the impact of guidelines values on two soil invertebrates is investigated following an extensive literature review to construct a species sensitivity distribution curve. While constructing the curve, we also assessed the influence of species assemblage on guideline values for different Canadian land use; another area lacking literature in soil ecotoxicology. Lastly we investigate whether organic carbon normalization, a common approach used in sediment ecotoxicology literature for organic contaminants, can be applied to soil PHC contamination toxicity data and incorporated into guideline derivation.

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5.2 Abstract

Sediment toxicity studies and ecological risk assessments on organic contaminants routinely use organic carbon normalization; however, no studies examine its potential for use in soils with petroleum hydrocarbon (PHC) contamination. Limited studies in soil ecotoxicology assess the influence of species assemblages used in species sensitivity distribution construction on the resulting guideline designated to of soil dwelling organisms. An extensive literature review was conducted to compile recent toxicity data for soil dwelling organisms (soil invertebrates and plants) for three groups of petroleum hydrocarbon (medium mixture, F2 fraction and F3 fraction). Species sensivity distribution curves were constructed for each petroleum hydrocarbon group using the conventional total contaminant concentrations approach and an organic carbon normalization approach. To test the level of protection current Canadian regulations provide to soil dwelling organisms, two standardized soil invertebrate test species (*Folsomia candida* and *Oppia nitens*) were exposed to a medium PHC mixture (lubricating oil) in twelve field soils at concentrations equivalent the two hazardous concentration (HC) percentiles used in Canada. Persistence of the medium PHC mixture in field soils was also investigated to determine the duration of toxic effects. We found organic matter influenced PHC toxicity to soil invertebrates, but persistence was influenced more by soil cation exchange capacity. Incorporating organic carbon normalization into species sensitivity distribution curves provided a higher level of protection to soil dwelling receptors in low organic matter soils as well as reduced the variability of PHC soil toxicity data. Soil remediation guidelines derived for protection of soil dwelling organism using a diverse species assemblage provided similar levels of protection as guidelines developed with test species that would never occur on certain Canadian land uses. We conclude that: (i) Canadian HC values should be revisited as they may not be protective and (ii) that soil guidelines for PHC protective of soil dwelling organisms should be expressed as carbon normalized values.

5.3 Introduction

The level of protection offered by soil toxicity benchmarks in ecological risk assessments in Canada varies relative to other regulatory agencies like the European Union. The preferred method for conducting soil ecological risk assessments in most countries is the weight of evidence approach through construction of a species sensitivity distribution (SSD) curve (CCME 2006), or ranked percentiles curve, defined as a statistical distribution of toxicity data represented as a cumulative distribution function (Posthuma et al 2002; Maltby et al 2005; Checkai et al 2014). In Europe the data points used in SSD construction include test measurement endpoints of the 10^{th} percentile of effects, while Canada uses the 25^{th} percentile (CCME 2006; Posthuma et al 2002). Another discrepancy between Canada and other countries ecological risk assessment regulations exists in the choice of hazard concentration (HC) percentile utilized to reflect guideline values. The HC percentile selected statistically translates as the proportion of species affected by the contaminant; for example, an HC5 assumes 95% of the organisms are protected from adverse effects (Posthuma et al 2002; Frampton et al 2006). In Canada, the HC percentile applied to the SSD for protection of soil dwelling organisms (soil invertebrates and plants) varies depending on land use, with the 25^{th} percentile applied to more sensitive land uses (agricultural, residential/parkland and natural) and the 50^{th} percentile HC utilized for establishing guidelines for industrial and commercial lands. In contrast, aquatic toxicologists within Canada, and soil ecotoxicologists outside Canada, use the 5^{th} percentile HC, also referred to as the predicted no effect concentration (Vega et al 1999; Posthuma et al 2002; Wheeler et al 2002; Frampton et al 2006; Checkai et al 2014). Currently, there are limited studies (CCME 2008; Princz et al 2012; Agnell et al 2012) assessing the influence of Canadian soil quality guidelines for PHCs on populations of soil invertebrates in a variety of field soils. Given this literature gap and combined with the large difference in level of protection between Canada and Europe, how well are guidelines protecting soil-dwelling receptors in Canada?

Incorporating organic carbon normalization into soil ecological toxicity data may improve how Canada conducts site-specific soil ecological risk assessments of PHC contamination. Soil organic carbon content modify toxicity by influencing the bioavailability of petroleum hydrocarbons to soil biological receptors as it affects the partitioning between the solid, liquid and gas phases (Di Toro 1991; Alexander 2000; Harwood et al 2013). Sediment ecotoxicologists

routinely normalize hydrophobic contaminants (log k_{ow} 4/5) by sediment organic carbon content, which reduces the variability in toxicity test data (Landrum and Faust 1994; Swartz 1999; Weston et al 2004; Du et al 2014; Harwood et al 2013; Seudel et al 1993). For example, Landrum and Faust (1994) reduced variation in polycyclic aromatic hydrocarbon bioavailability in sediment and a soil from 10 to 2 using carbon normalization. Some regulations in Canadian (ARBCA 2012) incorporate porewater equilibrium and partitioning into sediment quality guidelines for PHCs, however, this approach is less effective on moderately insoluble, medium PHC with high organic carbon partition coefficients (log k_{oc}>10) and low water solubility. Another benefit to organic carbon normalization of PHC contaminated soils, is an ability to compare toxicity data across differing soil or sediment types, which commonly occurs in soil ecotoxicology (Swartz et al 1999; Paige et al 2002). Canadian regulations for PHC contaminated soils permit incorporation of soil organic carbon content when determining site specific guidelines for human health exposure pathway calculations like vapour inhalation (CCME 2006; CCME 2008; HC 2010) but not for derivation of guidelines protective of soil dwelling organisms. Yet both the EU and Australia allow for organic carbon normalization to soil properties for ecological soil guideline development (NEPC 2011; Checkai et al 2014). Due to its benefits and global use, rationale exists to investigate the use of carbon normalization in soil ecotoxicology, especially for hydrophobic contaminants like PHCs whose toxicity to soil organisms is highly influenced by organic matter content (Princz et al 2010).

In Canada, site specific risk assessment (SSRA) is an ideal approach to assessing complicated contaminated sites. For many PHC contaminated sites in Canada, SSRA is the only approach to modify the exposure pathway protective of soil flora and fauna. Modifying the guidelines protective of soil dwelling organisms involves compiling data from literature and/or collected from privately contracted, non-published toxicity testing to construct an SSD curve (CCME 2006; Posthuma et al 2002). Canadian guidance documents for developing soil remediation guidelines (CCME 2006), indicate the requirements for constructing an SSD for protection of soil organisms is a minimum of ten data points from at least three studies. Since many of the regulatory guidelines in Canada reflect historical studies, a benefit to conducting toxicity testing as part of an SSRA is the freedom to incorporate current trends in literature and derive guidelines that are site specific. In the last decade, numerous advances in soil ecotoxicology literature have

occurred such as standardization of toxicity tests for both soil invertebrate and plant species ecologically relevant to Canada (EC 2012; EC 2014; EC 2018) in addition to the standardization and adoption of behavioral tests (Amorim et al 2005; ISO 2011; Owojori et al 2011; Owojori et al 2014; Gainer et al 2019).

Through a SSRA, not only can toxic effects to receptors be investigated in a site-specific manner, but also the persistence of the effect. Persistence of contaminants in soil is important to assess as it is informative of the duration of stress an organism experiences from exposure (Posthuma et al 2002). Posthuma (et al 2002) suggested that the choice of HC percentile used on SSDs consider the persistence of the contaminant. In soils, a persistent toxicant has a degradation half-life greater than 120 days (Mermond et al 2012; EUC 2011). Depending on the type of PHC contamination (light versus heavy PHCs), soil properties and climate, degradation of PHCs in soil may be classified as persistent or non-persistent. Within a SSRA, the ability to assess management options to accelerate PHC degradation also exists. For instance, soil contaminated with medium PHC products, like diesel or lubricating oils, typically degrade through volatization and biodegradation (Wang et al 1999). A simple remediation management option to accelerate PHC losses to biodegradation is through nutrient additions to surface soils (Chang et al 2010; Karppinen et al 2017; Kim et al 2018). Thus, a SSRA provides a well-rounded investigation of PHC contaminated soils – inclusive of toxicity, persistence and remedial options.

The ecological relevance of plant and soil invertebrate species included on an SSD across Canadian land uses has not been studied. Often debated in ecotoxicology, is whether species assemblages on SSDs should reflect species that actually occur in those ecosystems or whether to adopt a nondiscriminatory approach, mixing species from different geographic regions (Posthuma et al 2002; Maltby et al 2005; Hose and van den Brink 2004; Smith and Cairns 1993). Differences between marine and freshwater are apparent (del Signore et al 2016), so these species are treated separately, but there are additional considerations when combining tropical standardized tests species with temperate species on freshwater SSDs, intended for use in the tropics (Forbes and Calow 2002). Although well studied in aquatic ecotoxicology, the relevance of species assemblages on SSDs has only been briefly addressed in soil ecotoxicology (Princz et al 2012), largely due to limited available data and fewer standardized test species. Earthworms

and cultivated plants are the most well studied organisms in soil ecotoxicology, yet, absent from the boreal forest areas of Canada; which represent the majority of undisturbed, natural lands in the Canada and circumpolar countries in Scandinavia and Russia (EC 2004). Due to glaciation, earthworm distribution in Canada is currently limited to southern regions (EC 2004; Orgiazzi et al 2016). Whether soil ecotoxicologists should consider species assemblage representative of a certain land uses in Canada also extends to plant species. Most plant species represented on soil SSDs in Canada reflect agronomic and garden test species, yet many contaminated sites occur on natural land uses where the only receptors are ecological (CCME 2008; CCME 2017). Since soil invertebrate and plant species reflective of natural land uses can be both more sensitive or tolerant than other standard test species (Gainer et al 2018; Gainer et al 2019; Princz et al 2010), it is unclear if species assemblages will result in different guidelines protective of these organisms.

The study is exploratory and interpretative in nature, with four varying objectives. The first objective was to assess the current level of protection from Canadian regulations by testing the impact of soil contaminated with a medium PHC mixture at concentrations in field soil equivalent to guidelines, on two standardized soil invertebrate test species (Collembola *Folsomia candida* and oribatid mite *Oppia nitens*). We also aimed to determine if organic carbon normalization reduces variability in the toxicity data points collected and its feasibility for site specific ecological risk assessments of PHC contaminated soils. The third objective involved investigating the persistence of PHCs in surficial field soils and whether degradation improved following fertilizer additions. Lastly, we examined whether ecologically relevant species assemblages on SSDs influence the guidelines derived for natural land uses located in the Canadian boreal forest.

5.4 Materials and Methods

5.4.1. Soil Collection, Characterization and Preparation

Soil collected from six locations across Canada were used in a laboratory experiment to assess persistence and toxicity of a medium PHC product to soil invertebrates (Table 1). At each location, soils were either collected as subsoil in disturbed areas where topsoil was stripped, or as

topsoil from undisturbed locations. Soil samples were oven dried at 80˚C for 48 hours to remove any soil fauna and sieved to 2 mm and homogenized in bulk prior to experimental trials. In clay-abundant soils, the clumps were broken down through grinding. No fauna was observed during the processing of field soils. The water-holding capacity of each test soil was determined according to Environment Canada protocols (EC 2004). These soils were subsequently used for either the persistence study or the soil invertebrate toxicity study.

Soil samples were submitted for analysis of various properties. The following soil properties were determined from a commercial laboratory (ALS Laboratories, Saskatoon, SK): pH (1:2 Soil:$CaCl_2$), organic matter content (%) (loss on ignition), cation exchange capacity ($_c$mol/kg)(NH_4OAC), % sand (0.05-2 mm), silt (2um-0.05 mm), clay (<2um) and texture (Table 1). For results below detection limit, values were considered an order of magnitude less than detection limit, to allow for statistical analysis of this data. For instance, a detection limit of <1 was considered 0.1. To convert soil organic matter content to organic carbon content, a conversion factor of 2 was used (Pribyl 2010; Tipping et al 2010).

Table 5-1. Physical and chemical properties of the field soils used to assess persistence and toxicity of lubricating oil to soil invertebrates.

Geographic Location	Soil Type	Organic Matter (%)	Organic Carbon (%)[a]	CEC (cmol/kg)	pH	Sand (%)	Silt (%)	Clay (%)	Texture
Nordegg AB	ts	1.8	0.9	16.8	7.7	52.4	28.0	19.5	Loam / Sandy loam
Nordegg AB	ss	1.8	0.9	18.6	7.9	45.7	31.8	22.5	Loam
Martin, ON	ts	<1.0	0.05	2.02	4.9	86.4	12.8	0.7	Sand
Martin, ON	ss	<1.0	0.05	1.8	5.9	86.9	12.4	0.7	Sand
Ignace, ON	ts	1.2	0.6	3.3	4.7	88.1	11.1	0.8	Sand
Ignace, ON	ss	<1.0	0.05	0.9	6.5	95.2	2.9	1.9	Sand
Stittsville, ON	ss	<1.0	0.05	1.4	7.7	90.5	6.3	3.2	Sand
Stittsville, ON	ts	1.8	0.9	6.5	7.6	70.9	23.6	5.5	Sandy loam
Burstall, SK	ts	4.8	2.4	16	7.4	67.1	22.2	10.7	Sandy loam
Burstall, SK	ss	1.8	0.9	11.9	7.5	68.2	24.5	7.4	Sandy loam
Caron, SK	ss	1.2	0.6	24	8.2	64.2	23.2	12.6	Sandy loam
Caron, SK	ts	1.2	0.6	28	7.5	35.0	29	37	Clay loam

ts=topsoil; ss=subsoil; [a]conversion factor of 2 used to determine organic carbon from organic matter

5.4.2. Persistence Study Experimental Setup

For the persistence study, field soils were spiked with a lubricating oil to a PHC content of approximately 1400 mg/kg F2 (>C10-C16) and F3 (>C16-C34). Both these initial concentrations are elevated relative to guidelines for sensitive Canadian land uses (agricultural, residential, parkland, natural) however the concentration of F3 PHC is not considered elevated on less sensitive land uses (industrial or commercial). Lubricating oil was supplied by Marsollier Petroleum (Saskatoon, Saskatchewan). A detailed composition of the lubricating oil is presented in Supplemental Material Table S-1 and Gainer et al (2018). The lubricating oil was added to dry soil using acetone as a carrier solvent to ensure even distribution. The acetone was allowed to volatize in a fume hood overnight prior to water or fertilizer additions.

The fertilized treatment for contaminated soils was amended with dissolved fertilizer in stock solution. Fertilizer additions were calculated on a CNP ratio of 100:9:1. This ratio was selected based on other research in Canadian soils and climates (Chang et al 2010; Karppinen et al 2017; Kaur et al 2017; Leys et al 2005). The fertilizer used was a combination of monoammonium phosphate (11-52-0) and urea (NH_4N_2O; 46-0-0), the most common fertilizer forms used for agricultural soils in Canada. Following fertilizer addition to the contaminated soils, water was added to maintain water content at 50% water holding capacity.

Treated soils were placed in polypropylene microcosms and incubated in a growth chamber at 20˚C for 10 weeks to reflect an average southern Canadian summer when peak degradation occurs in PHCs surface soils (Kim et al 2018). Other conditions within the growth chamber include 16h light, 8-hour dark cycle, with approximately 600 lux at surface and approximately 60% humidity. Soils were watered every three to four days to maintain consistent soil moisture. Samples were collected and analyzed for F2 and F3 PHC content every two weeks. To ensure a representative sample was collected, the soils were mixed prior to sample collection. Following sample collection, soils were lightly packed back into microcosms.

5.4.3. Species Sensitivity Distribution Curves

Compilation of available toxicity test data from literature primarily followed guidance outlined by CCME (2006). The guidance indicates that for the weight of evidence approach a minimum of ten data points from at least three studies is required and the data must represent a minimum of two soil invertebrates and two plant species (CCME 2006). Microorganism tests were exempt since they are considered under the nutrient cycling to ecological exposure pathway in Canadian regulations (CCME 2006). The preferred effects data form is expressed as lethal or effective concentration (LC/EC) at the 25th percentile. Canadian regulatory guidance for soils , not aquatics, indicates acute and chronic effects endpoints can be complied on the same SSD and to obtain the geometric mean of values when numerous endpoints are available for a single species (CCME 2006).

A literature search was conducted in Web of Science to collect data on laboratory toxicity tests for soil invertebrates and plants exposed to soils contaminated with whole mixtures of medium PHCs, F2 (>C10-C16) fraction PHCs and F3 (>C16-C34) fraction PHCs in the last 25 years. Medium PHC products were considered products of mainly F2 and F3 PHCs like diesel, motor oil or lubricating oil, and both fresh and weathered contamination was considered. Soil invertebrates and plants were considered as test organisms for this study. Due to the limited comparisons of available data in literature, effects percentiles between 20 and 30 and two 50th percentiles (Cermak e al 2013; Ramadass et al 2015) were also included in this study. Only studies that reported data as actual contaminant concentrations as well as organic matter or organic carbon in their test soils were considered. The organic carbon content of OECD artificial soils was assumed to be 2.65% based on the average of two studies (ESG 2003 and Agnell et al 2012). Soil invertebrate test endpoints included mortality, reproduction inhibition, inhibition of progeny body weight and avoidance test. Avoidance tests were included as they are standardized and have been incorporated into recent soil ecological SSD construction studies (Princz et al 2017). Avoidance tests were considered as their own distinct data point reflective of behavioural tests. Plant toxicity test types included were inhibition of root length, shoot length, root dry mass, shoot dry mass, stem dry weight and fruit dry weight. Seed germination and emergence were not included in data collection as these endpoints are relatively insensitive to PHC contamination and are not routinely used in Canadian PHC regulations (CCME 2008; Checkai et al 2014).

The determination of organic carbon normalization followed the US EPA sediment quality criteria (Supplemental Material Equation 1) and involved dividing toxicity endpoints by the total organic carbon in dry weight sample (decimal form) (US EPA 2014). To assess if organic carbon normalization influenced variability of toxicity test data, the coefficient of variation was determined on the log transformed data sets for each species assemblage used to construct an SSD.

One of our objectives was to compare HC values from SSDs constructed using only species ecologically relevant for a natural land use in the boreal forest to the results produced when all test species available in literature are included, irrespective of ecological relevance. A natural land use is defined by AEP (2016) as lands without human inhabitation or activities where the primary receptors are ecological. The plant species considered ecologically relevant to natural land uses were species native to Canada and included: *Elymus lanceolatus, Medicago sativa, Picea glauca* and *Pinus banksiana* (AAF 2016). Soil invertebrate species were considered ecologically relevant to northern remote forested areas if they were representative of native species to boreal forests of Canada such as oribatid mites or Collembola. Earthworms were not considered ecologically relevant to a boreal forest natural land uses (see Introduction) (EC 2004; Orgiazzi et al 2016). The criterion to determine significant differences between SSD curves was nonoverlapping 95% confidence intervals. In addition, a retrospective power t-test analysis with a significance level of 0.05 was conducted to determine the power associated with the log-transformed data sets for each species assemblage used to construct an SSD (Thomas 1997). Low effect size was considered 0.2 and a high effect size was 0.8. The power test was conducted using the minimum and maximum number of data points plotted on the SSDs.

Species sensitivity distribution curves were generated using the CCME SSD Master version 3.0 (CCME 2013). An SSD was created for total and organic carbon normalized TPH, F2 and F3 concentrations. Within the CCME SSD Master, the following models were evaluated: normal, logistic, extreme value (Gompertz) and Gumbel distribution curves (CCME 2013). Best model fit was determined based on the lowest Anderson-Darling Goodness-of-Fit test statistic (A^2) and spread of residuals was also assessed visually. The 5^{th}, 25^{th} and 50^{th} percentile from the model

with best fit was obtained to reflect Canadian and global regulations. For comparing species assemblage differences, the 25th percentile was reported as this is the percentile used for natural land uses in Canada.

5.4.4. Toxicity Testing Experimental Setup

The toxicity of the lubricating oil was assessed in field soils at two concentrations plus a control. Lubricating oil was spiked into moistened field soils using acetone as a carrier solvent at concentrations similar to the HC25 (1200 mg/kg TPH) and HC50 (3000 mg/kg TPH) from the SSD curve generated for the mixture with all species. Each treatment contained five replicates.

Two soil invertebrate tests organisms were assessed, the Collembola *Folsomia candida* (Willem, 1902) (EC 2014) and the fungivorous mite *Oppia nitens* (C.L. Koch) (ECCC 2018). *Folsomia candida* and *O. nitens* cultures originated from Environment Canada and both were reared on 8:1 plaster of Paris and activated charcoal substrate and fed active yeast weekly in mostly dark conditions at an average temperature of 20˚C.

Mortality and reproductive toxicity tests for *F. candida* and *O. nitens* consisted of 28 days (EC 2014; ECCC 2018). For *F. candida*, ten age synchronized 10 to 12 day-old organisms were added to test soils. The *O. nitens* test utilized 15 age synchronized (within 1 to 2 days), young adult (approximately one week after individual pigmentation became light red brown in colour). All tests containers were 50 mL glass Schell vials with tight-fitting lids and a single hole for air exchange. All soil invertebrate toxicity tests were performed in controlled environmental chambers under the following conditions: daily average temperature of 20˚C (+/- 2˚C), 16h light, 8-hour dark cycle, with approximately 600 lux at surface and approximately 60% humidity. Food was provided weekly for both for *F. candida* and *O. nitens.* For all tests, moisture losses were assessed by weight. At test completion, the number of live adult and juvenile *F. candida* were determined using a floatation method with black ink and enumerated with ImageJ software (Rueden et al 2016). *O. nitens* were heat extracted from the soil using a modified Tullgren apparatus with increasing heat (25 to 30˚C) over three days and manually counted.

5.4.5. Petroleum Hydrocarbon Analysis

The lubricating oil utilized for the study was composed of mainly F2 (C10- C16) and F3 (>C16-C34). Soil PHC analysis was conducted in accordance with Canadian regulations (CCME 2008). Briefly, soil samples were shaken with a 1:1 ratio of hexane and acetone, reduced under nitrogen gas and transferred to GC vials. Quality control and assurance procedures included duplicates, matrix and blank spikes, method blanks and reagent blank every 20 samples. Spiked samples within 70-130% recoveries were considered adequate. Quantification of the F2 and F3 fractions was performed using a Varian CP3800 gas chromatograph fitted with a flame ionization detector (GC-FID) with chromatogram interpreted performed on CompasCDS software (Varian, Santa Clarita, CA). For further information on the soil analysis see Gainer et al (2018).

5.4.6. Persistence Data Analysis

Since degradation followed first order kinetics, a first order rate constant (k) for each soil was determined as the slope of natural logarithm of the PHC fraction concentration (mg/kg) as a function of time (days). Half life ($t_{1/2}$) was determined as 0.693 divided by the first order rate constant.

General linearized modeling (Zuur et al 2007) was the statistical model utilized to assess the effects of fertilizer additions and soil properties on F2 and F3 PHC degradation rate constants, with consideration of interactions. Criteria to determine optimal model fit was assessment of residuals followed by the lowest determined based on lowest Akaiki information criterion (AIC) values (Zuur et al 2007; Zuur et al 2010). Data exploration identified all soil properties (sand, clay, organic matter content, CEC) assessed were correlated, so each property was assessed independently, along with fertilizer treatment, on degradation rate constant. Pseudo r^2, or explained deviance, for each model was determined as difference between the null deviance and residual deviance divided by null deviance expressed as a percent. Data was processed in R studio using the nlme (Pinheiro et al 2018) and MASS (Venables and Ripley 2002) packages, and visualized in SigmaPlot (Version 12.0).

5.4.7. Toxicity Testing Data Analysis

General linearized modeling (Zuur et al 2007) was conducted to determine the effects of PHC contamination and soil properties on adult survival and juvenile production for both test species. Similar methods as persistence data analysis were followed, where data was initially explored for residuals and lowest AIC identified best model fit (Zuur et al 2007; Zuur et al 2010). Many soil properties were correlated (sand, clay, organic matter content, CEC), so models were assessed for a single soil property in addition to contamination levels effects on number of adults or juveniles (Zuur et al 2007). The model with best fit was determined based on lowest AIC. For all models, organic matter content, with no interactions, provided best fit. A quasi-poisson cumulative distribution model provided best fit for adult *F. candida* and *O. nitens*, while negative binomial provided best fit for juvenile production for both test species. Pseudo r^2 and R packages used were the same as previously described for the persistence data analysis.

5.5 Results

5.5.1. Persistence

Fertilizer additions increased removal of both F2 and F3 PHCs over a ten week period (Table S-2, both $p<0.05$). Considering all soils, the average k for F2 PHC when fertilized (0.019 d^{-1}(0.0093)) was greater than the unfertilized (0.015 d^{-1} (0.0067) control, corresponding to a half life difference of approximately 13 d between treated soils (Figure 1, Supplemental Information S-3). A similar trend was observed in F3 PHC degradation, with an average k of 0.018 d^{-1} (0.0072) in fertilized soils and 0.013 d^{-1} (0.0049) in unfertilized soils. The half life for F3 PHC in fertilized soils was 47 (19) d and in unfertilized soils was 60 (22) d.

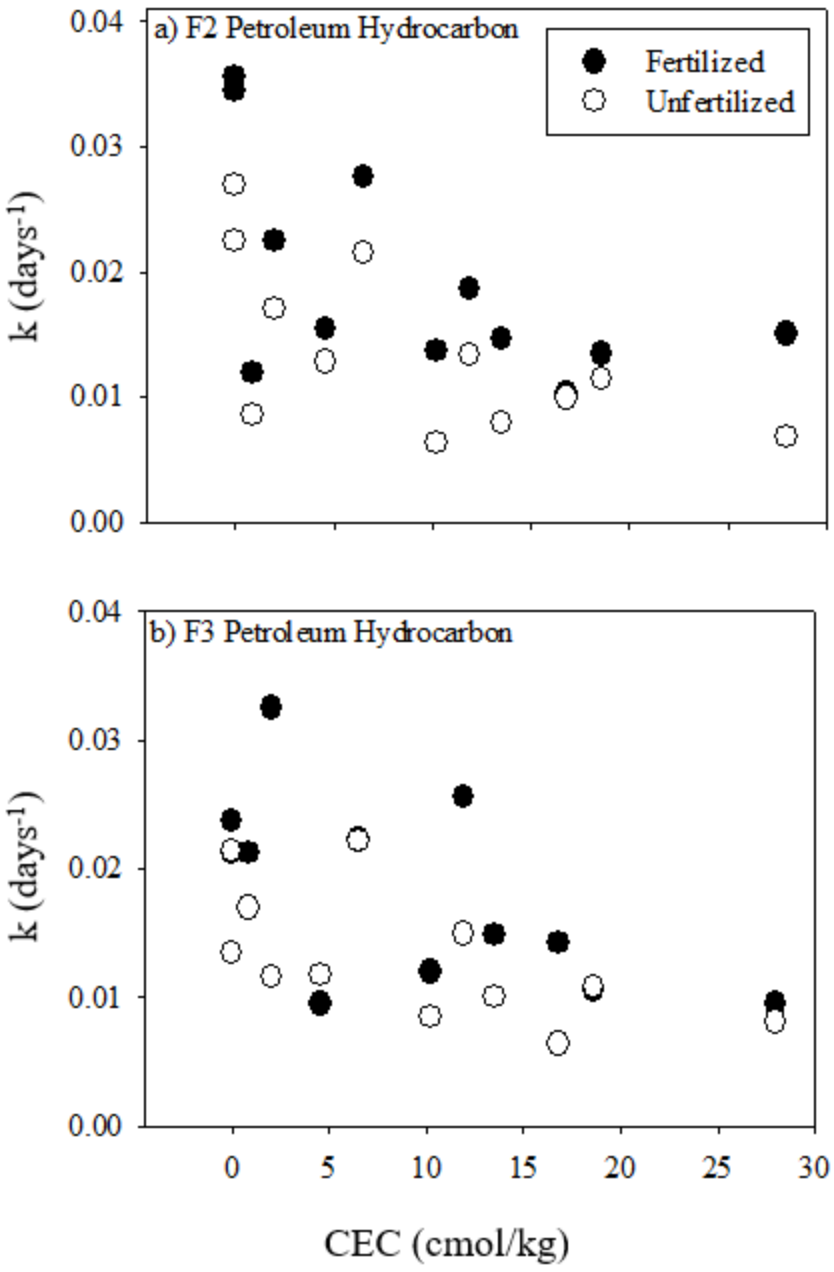

Figure 5-1. First order degradation rate constants (k) (days^{-1}) for (a) F2 and (b) F3 Petroleum Hydrocarbon when fertilized (solid circles) at a CNP ratio of 100:9:1 and unfertilized (open circles) in field soils with a range of soil cation exchange capacities (CEC). Initial petroleum hydrocarbon concentrations for each soil was approximately 1400 mg/kg F2 and 1300 mg/kg F3.

The degradation of both F2 and F3 PHCs was also influenced by soil CEC (Table S-2, $p<0.05$). Soils with a low CEC displayed higher degradation rate constants than low CEC soils, across both contaminants. The degradation rate constant for F2 PHCs in soils with a CEC ranging from

detection limit to 15 cmol/kg (0.021 (0.0098)) was almost twice the degradation rate constant for soils with a CEC range from 16-28 cmol/kg (0.012 (0.0025)) (Table S-3). This translates to a half life difference of approximately 20 days between tests soils with a CEC less than 15 cmol/kg and a CEC greater than 15 cmol/kg. The differences between degradation rate constants and soil CEC increased in F3 PHC contaminated soils. The degradation rate constant for F3 PHCs in soils with a CEC range from detection limit to 15 cmol/kg (0.018 (0.0065)) was almost an order of magnitude greater than the degradation rate constant for soils with a CEC range from 16-28 cmol/kg (0.0099 (0.0027)). For F3 PHCs, the half life difference between soils with a low CEC (<15 cmol/kg) and high CEC (>16-28 cmol/kg) was approximately 30 days.

5.5.2. Species Sensitivity Distribution

From the literature search for medium PHC mixtures, a total of 43 endpoints from four studies were selected (Supplemental Material Table S-4) (Ramadass et al 2015, Chaineau et al 1997, Gainer et al 2018, Gainer et al 2019). The geometric average of species with multiple endpoints was determined for the medium PHCs mixture, resulting in 19 data points for all test species and 11 data points for species native to natural land uses in boreal forest (Table S-5). Results from the literature search for F2 PHC distillates yielded 90 individual endpoints from a total of eight studies which resulted in a total of 18 data points after determining the geometric average species with all test species from the literature review, with only 11 data points for species reflective of boreal forest species (Table S-5 and S-6)(CCME 2008; ESG 2003; Agnell et al 2012; Cermak et al 2010; Cermak et al 2013; Erlacher et al 2013; Gainer et al 2018; Gainer et al 2019). Results from the literature search for F3 PHC distillates yielded 62 individual endpoints from seven studies (Table S-7) (CCME 2008; Agnell et al 2012; Cermak et al 2005; Cermak et al 2010; Cermak et al 2013; Erlacher et al 2013; Gainer et al 2018; Gainer et al 2019). Following the average of multiple endpoints for the same species, 19 data points were available for all test species and 13 data points available for the natural land use species of the boreal forest (Table S-8). The literature search for all three PHC groups met the minimal data requirements outlined by Canadian regulations (CCME 2006); a minimum of 10 data points from at least three studies, with approximately equal proportions of plant and soil invertebrate species.

For all PHC groups (mixture, F2 and F3 fractions), carbon normalization reduced the variability in toxicity test data points plotted on the SSDs. Following organic carbon normalization, the coefficient of variation for toxicity data available for whole mixtures of medium PHCs, F2 and F3 PHCs was reduced from 0.15 to 0.1, from 0.2 to 0.15 and from 0.16 to 0.12, respectively. The lowest Anderson-Darling statistic for best model fit ranged from 0.17 to 0.38 across all models for PHC groups, species assemblages and expression of concentrations (Figure 2, 3; Figure S-1 to S-6, Table 2; Table S-10). For both actual and carbon normalized concentrations, the best model fit when including all species with available data for the medium PHC mixture SSD was extreme value while Gumbel produced best fit the F2 and F3 PHC SSDs. The best model fit when considering only species relevant to a natural land use were consistent with the all species SSD with the exception that normal and logistic regression fit the TPH SSD data better for total and carbon normalized concentrations, respectively.

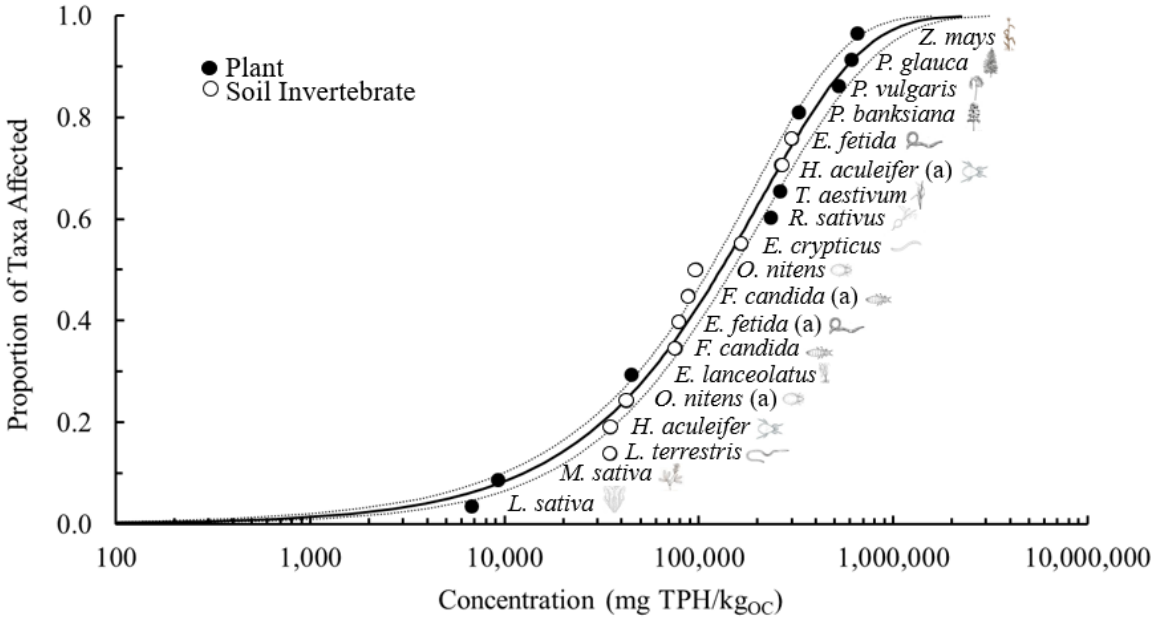

Figure 5-2. Species sensitivity distribution for soil invertebrates and plants exposed to whole mixtures (total petroleum hydrocarbon (TPH)) normalized to soil organic carbon content in soil from all species in available literature. Soil invertebrate data points (open circle) represent the geometric mean of available endpoints (mortality, inhibition of juvenile production and/or inhibition of juvenile growth) for each species. Plant species data points (solid circle) represent the geometric mean of available growth endpoints (inhibition of root length, shoot length, root dry mass, shoot dry mass and fruit production) for each species. Soil invertebrate avoidance tests indicated as distinct data points and represented by (a). Solid line indicates model fit and dotted lines represent 95% confidence intervals.

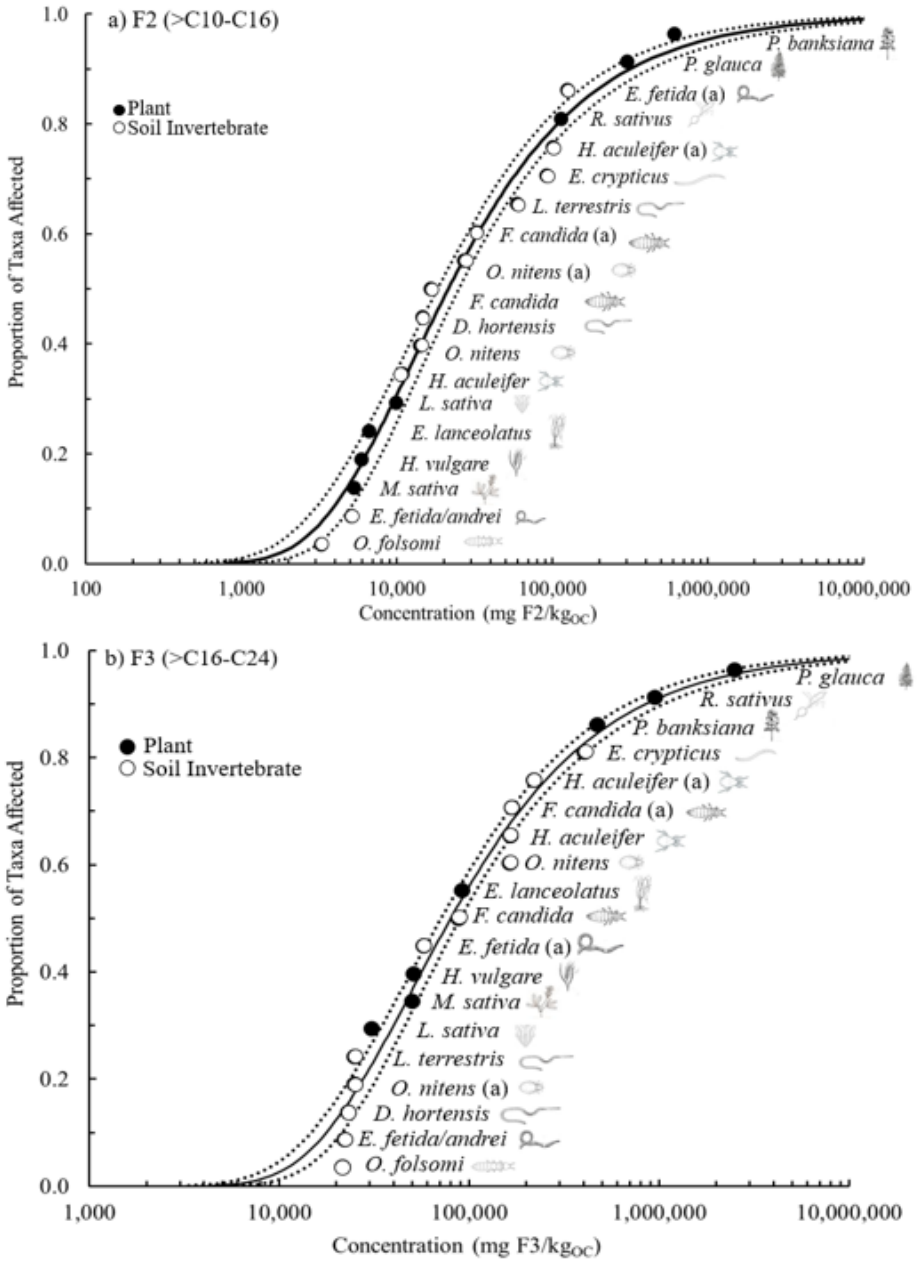

Figure 5-3. Species sensitivity distribution constructed for soil invertebrate and plant toxic responses to individual petroleum hydrocarbon (a) F2 fraction and (b) F3 fraction normalized to soil organic carbon content from all species in available literature. Soil invertebrate data points (open circle) represent the geometric mean of available endpoints (mortality, inhibition of juvenile production and/or inhibition of juvenile growth) for each species. Plant species data points (solid circle) represent the geometric mean of available growth endpoints (inhibition of root length, shoot length, root dry mass, shoot dry mass and fruit production) for each species. Soil invertebrate avoidance tests indicated as distinct data points and represented by (a). Solid line indicates model fit and dotted lines represent 95% confidence intervals.

For both actual and carbon normalized concentrations, the inclusion of ecologically relevant species for a natural land use in the boreal forest did not alter HC25 were observed across all PHC groups (Table 5-2, Figure 5-4). The 95% confidence interval for the HC25 generated with total concentrations from the SSD including all species was 1252 to 1857 mg/kg TPH, overlapping the confidence intervals for the HC25 from the SSD with only species representative of natural land use, 1175 to 2305 mg/kg TPH. Although differences in HC25s were not observed between species assemblages used on the SSDs, the calculated power was low (<0.8) for both a low or high effect size. However, we found differences in HC5 values between species assemblages for one set of data, specifically between organic carbon normalized concentrations for the PHC mixture. The OC normalized HC5 values for the PHC mixture when all species were considered was 5120 mg/kg_{oc} TPH and this value almost doubled to 10, 500 mg/kg_{oc} when only ecologically relevant species were included in SSD construction.

Table 5-2. Summary of best model fit and different Hazard Concentration (HC) percentile values (95% confidence intervals) from species sensitivity distribution curves derived using total or carbon normalization constructed for total petroleum hydrocarbon, F2 petroleum hydrocarbons and F3 petroleum hydrocarbons.

Petroleum Hydrocarbon Composition	Hazard Concentration Percentile	Total concentration (mg/kg)		Actual concentration normalized to soil OC (mg/kg$_{OC}$)	
		All species	Boreal forest species	All species	Boreal forest species
TPH	HC5	200 (150-260)	290 (200-430)	5,120 (3,640-7,220) *	10,500 (7,940-13,900) *
	HC25	1,250 (1,090-1,430)	1,060 (720-1,560)	43,480 (36920-51,190)	40,800 (34,800-47,830)
	HC50	3,190 (2,840-3,590)	na	129400 (113070-148090)	na
	Model	Extreme Value	Normal	Extreme Value	Logistic
F2	HC5	130 (100-160)	120 (80-170)	2,640 (1,970-3,530)	2,840 (2,120-3,740)
	HC25	320 (280-370)	310 (250-400)	7,930 (6,480-9,710)	8,730 (7,110-10,720)
	HC50	730 (650-820)	na	21,360 (18,240-25,000)	na
	Model	Gumbel	Gumbel	Gumbel	Gumbel
F3	HC5	424 (350-510)	500 (380-650)	12,730 (10,320-15,700)	15,440 (11, 200-19,880)
	HC25	1,070 (940-1,230)	1,270 (1,050-1,540)	33,470 (2,8860-38,800)	40,560 (33,910-48,520)
	HC50	2,480 (2,230-2,750)	na	79,850 (71,090-89,700)	na
	Model	Gumbel	Gumbel	Gumbel	Gumbel

HC=hazard concentration; F2=C>10-C16; F3=C>16-C34; na=not applicable since HC level not applicable to a natural land use; model indicates model with best fit using CCME SSD Master v3; *indicates values are different based on non-overlapping confidence intervals

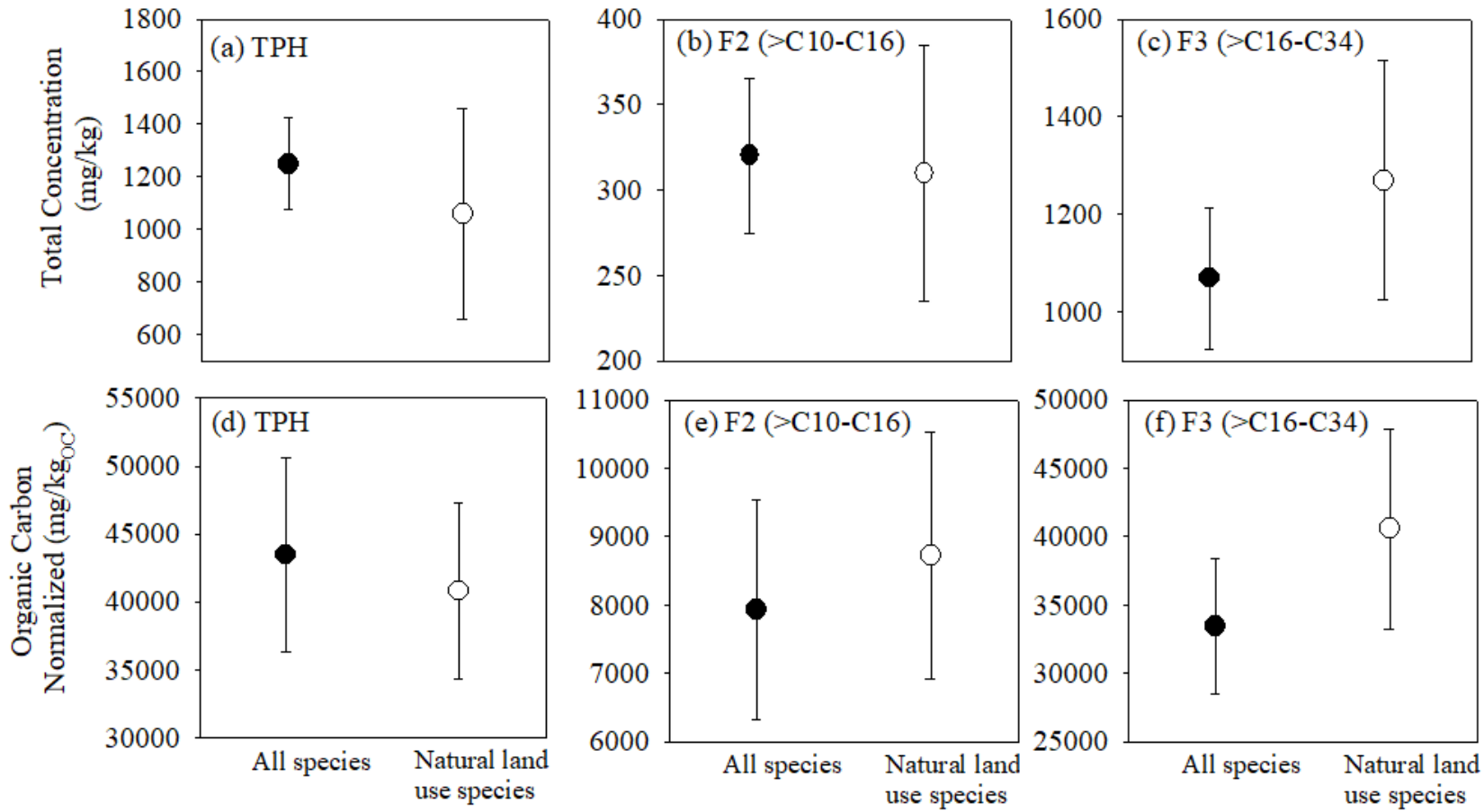

Figure 5-4. Distribution of median hazardous concentration for 25% of species (HC25 mg/kg or mg/kg$_{oc}$) estimated from species sensitivity distributions constructed using soil invertebrates and plants species from different habitats: all species (solid circles) and species just representative of the boreal forest region (open circles). Values were determined organisms exposed to whole mixtures of total petroleum hydrocarbon (TPH) (a, d), F2 fraction distillates (b, e) and F3 fraction distillates (c, f) assessed as total concentrations (a, b, c) or organic carbon normalized concentrations (d, e, f).

Soils with higher organic carbon content resulted in higher site-specific guidelines, consistent across each PHC group (Table 3). When the fraction of organic carbon is greater, like 7%, the resulting guideline from the HC50 for a medium PHC mixture was 9058 mg/kg TPH, almost an order of magnitude greater than the corresponding value (1294 mg/kg TPH) for low organic carbon content (1%) soils. A soil with an organic carbon content near 3% would result in a site-specific guideline similar to those obtained using the conventional total concentration approach to toxicity data. However, at lower and higher organic carbon content levels, the guidelines from using organic carbon normalization diverge from the values from the conventional approach, across all PHC groups. At 7% organic carbon content, the site specific HC50 value for F2 fraction PHC is 1495 mg/kg F2, double the conventional approach guideline value of 730 mg/kg PHC. Conversely, using a low (1%) site-specific organic carbon content yields a HC50 value of 214 mg/kg F2, less than half the aforementioned conventional value.

Table 5-3. Site specific remediation guidelines expressed as total petroleum hydrocarbons, F2 fraction and F3 fraction PHCs using conventional approaches with no carbon normalization and when using carbon normalization with incorporation of site-specific fraction organic carbon.

Petroleum Hydrocarbon Composition	Hazard Concentration Percentile	fOC (%)			Conventional approach (mg/kg)
		1	3	7	
		(mg/kg)			
TPH	HC25	435	1,304	3,044	1,250
	HC50	1,294	3,882	9,058	3,190
F2	HC25	79	238	555	320
	HC50	214	641	1,495	730
F3	HC25	335	1,004	2,343	1,070
	HC50	799	2,396	5,590	2,480

fOC= fraction soil organic carbon; TPH=total petroleum hydrocarbons; F2=C>10-C16; F3=C>16-C34

5.5.3. Soil Invertebrate Toxicity

Both test species of soil invertebrates met the validation criteria in all field soils tested. For both *F. candida* and *O. nitens* tests, the mean adult survival was greater than 70%, the mean number of juveniles was 100 and coefficient of variation was less than 30% (ECCC 2018, EC 2014). Adult survival and juvenile production of *F. candida* and *O. nitens* in soils contaminated with lubricating oil was significantly influenced by contaminant concentration and soil organic matter

content (Figure 5; Table S-11; all $p<0.001$). The general linearized models applied to *F. candida* and *O. nitens* adult survival and juvenile production explained at least 70% of the variance of the responses for all four tests. Survival of adult *F. candida* and *O. nitens* in control field soils was consistent across organic matter levels. A low number of adult *F. candida* and *O. nitens* survived in field soils with organic matter content below detection limit in both lower (HC25) and higher (HC50) PHC contaminated soils. This trend was also apparent at 1.25 % organic matter level for *F. candida* adult survival in lower (HC25) PHC-contaminated soils; however, *O. nitens* adult survival was greater at the same contamination and organic matter level. Across both PHC soil contamination levels, adult survival of *F. candida* and *O. nitens* was highest in field soils with high organic matter.

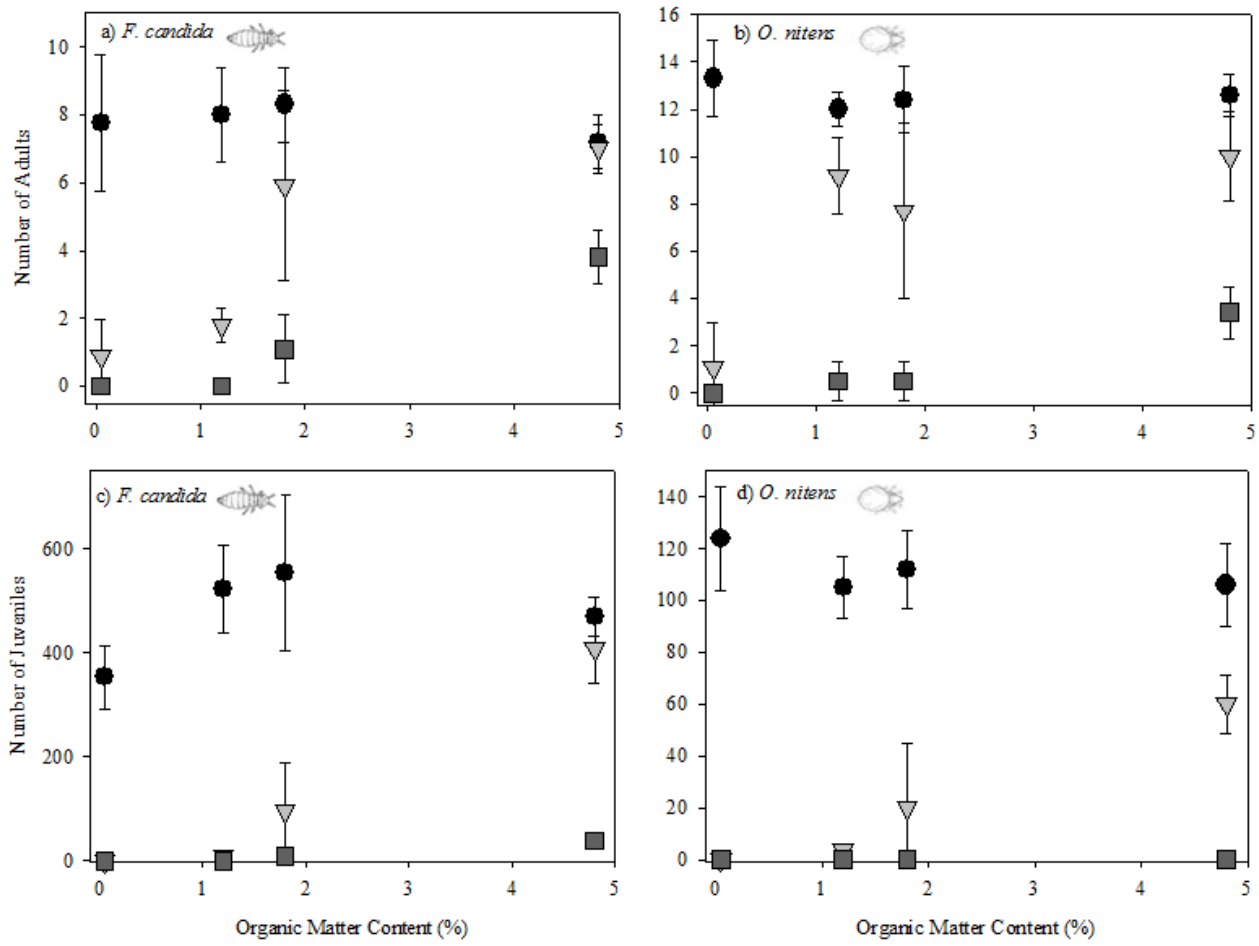

Figure 5-5. The average number (standard deviation intervals) of adult (a) *F. candida* and (b) *O. nitens*, and the average number (standard deviation intervals) of juvenile (c) *F. candida* and (d) *O. nitens* exposed to uncontaminated field soils (circle) or contaminated fields soils with varying organic matter content. The total petroleum hydrocarbon content of contaminated soils at approximately the same concentration as the 25th percentile (inverted triangle) or 50th percentile hazard concentration (square).

The adverse effects of PHC contaminated soils across soils with a range of organic matter content was more pronounced in juvenile production for *F. candida* and *O. nitens* (Figure 5; Table S-11; all $p<0.001$). The number of juvenile *F. candida* in uncontaminated field soils ranged from 309 to 554 individuals across organic matter contents, with the lowest in soils with organic matter content less than detection limit. Production of juvenile *O. nitens* in uncontaminated field soils ranged from 104 to 124 across organic matter contents. In field soils contaminated with either low or high levels of PHCs, juvenile production of both *F. candida* and

O. nitens was minimal, with one exception. In soils with low PHC contamination (HC25) and high organic matter, *F. candida* and *O. nitens* juvenile production was greater, with juvenile production of *F. candida* similar to control values.

5.6 Discussion

This study reinforces the influence of soil organic matter on PHC toxicity to soil invertebrates, as well as its role in habitat quality. We found the toxicity of PHC-contaminated soils to adult and juvenile *O. nitens* and *F. candida* in twelve field soils was mediated by soil organic matter content. Of all the soil properties influencing toxicity to soil invertebrates assessed in this study, including sand, clay, CEC and organic matter content, the organic matter content explained the most variation for both populations of adult and juvenile *O. nitens* and *F. candida*. Organic matter influences the degree of exposure soil- or sediment-dwelling organisms receive in PHC contaminated soils, as it has a high sorption affinity for organic contaminants (Di Toro 1991; Harwood et al 2013; Princz et al 2010; Princz et al 2018). Not only does organic matter influence an organisms exposure to PHCs but also their habitat, as humus form has been linked to oribatid mite community structure (Princz et al 2010; Maraun and Scheu 2000).We observed larger adult and juvenile populations in contaminated soils with higher OM levels, a trend consistent with organic contaminant sediment and soil toxicity literature (Princz et al 2018; Landrum and Faust 1994; Di Toro 1991; Zhao et al 2011; Harwood et al 2013). In a similar study with perfluorooctane sulfonate-contaminated soils, juvenile populations of *F. candida* and *O. nitens* doubled when organic matter content increased from 2.6 to 15% (Princz et al 2018). Beyond the influence of soil properties on PHC toxicity in soil invertebrates, these findings illustrate how the current Canadian framework provides minimal protection for two soil dwelling organisms in low organic matter soils. Other studies with inorganic mercury in soil also identified how current regulatory guidelines in Europe and Australia provided low protection levels for soil organisms (Mahbub et al 2018; Tipping et al 2010). A potential approach to improving protection of soil-dwelling organisms in low organic matter soils is by incorporating organic carbon normalization into guideline development for organic contaminants.

Organic carbon normalization of toxicity data reduces variability in PHC soil toxicity data and provides higher levels of protection to soil dwelling receptors in low organic matter soils. The reduction in variability following organic carbon normalization we observed is consistent with observations from other studies who adopted this approach for organic contaminants (Landrum and Faust 1994; Landrum 1995; Swartz 1999; Du et al 2014; Harwood et al 2013; Weston et al 2004). Landrum and Faust (1994) observed the uptake clearance from PAH-contaminated sediments was reduced by a factor of two following carbon normalization. In low organic carbon soils, site-specific guidelines using organic carbon normalization produced lower guidelines than the corresponding values from the conventional total concentration approach, providing greater protection to soil dwelling receptors. No studies in literature use carbon normalization in soil guideline development for PHC contaminated soils, although it has been applied to mercury (II) in soil (Tipping et al 2010). Incorporating organic carbon normalization into PHC risk assessments is an approach already used in many Canadian regulations as well as literature for human health, aquatic and sediment toxicity, highlighting the current acceptance of this approach in toxicology (CCME 2006; HC 2010; US EPA 2014). However, follow-up studies are needed to assess the effects of site-specific guidelines considering organic carbon content on soil dwelling organisms in field soils, to determine the level of protection offered.

Soil remediation guidelines derived for protection of soil dwelling organisms using a diverse species assemblage appear to provide similar levels of protection as guidelines developed with test species that would never likely occur on certain land uses in Canada. We found no differences between guidelines derived using a diverse range of species available from literature and species relevant for the boreal forest land use, despite differences in taxonomic composition, an influential factor reported in aquatic ecotoxicology literature (Maltby et al 2005; Kwok et al 2007). Although the 95% confidence intervals for the HC25 values substantially overlapped, and the data met the minimum number of points (10) for each SSD recommended by Canadian regulations (CCME 2006) and literature (Wheeler et al 2002; Posthuma et al 2002; del Signore et al 2016), a retrospective power analysis for high and low effect size, reported the power was low, suggesting we potentially failed to identify a difference between species assemblages on SSDs. In addition, a similar experiment (Princz et al 2012) observed guidelines derived using species compositions representative of the boreal forest were more sensitive to PHC contamination than

those lacking boreal forest species. However, the study by Princz et al (2012) included mostly boreal forest species with only two non-boreal forest-inhabiting test species. Perhaps the HC25 are similar due to the robustness of model fitting in that region of the curve (Green et al 2018; Xing et al 2014). Results of similar studies from aquatic toxicology are mixed. Maltby et al (2005) found no statistical differences across SSDs constructed with species assemblages representative of geographical regions for 16 pesticides. Similarly, Hose and van den Brink (2004) found that endosulfan SSDs generated with Australian fish and arthropod species were not significantly different than those with non-Australian taxa. Conversely, Kwok et al (2007) found that for most metals temperate species are more sensitive than tropical, with the opposite being true for phenol, ammonia and chlorpyrifos.

For one pair of species assemblage data, the organic carbon normalized PHC mixture data, species assemblages influenced the HC5. This difference is likely due to variations in how the models utilized fit the lower tail region of the SSD, an area of the distribution curve sensitive to model choice (Green et al 2018; Xing et al 2014). Due to the sensitivity of HC5 to model selection, some researchers suggest a minimum of 15 or 20 studies for HC5 calculations using parametric or nonparametric bootstrapping approaches (Xing et al 2014; Newman et al 2000; Wang et al 2008). For all PHC groups we studied, available data for plotting on the SSD was between 11 and 19, thus, some data sets in the study did not meet the minimum requirements recommended by some researchers for assessing HC5. Xing et al (2014) observed that the Gumbel method was consistently the best parametric model for estimating HC5 values, consistent with most of this studies findings, except the PHC mixture data. Using the Gumbel model, the HC5 values for the organic carbon normalized PHC mixture data between species assemblages produced similar values with overlapping confidence intervals (Table S-12). We emphasize further research is needed to determine if this trend, that species assemblages does not influence remedial guidelines protective of soil dwelling organisms, is consistent within PHCs and across contaminants.

Although organic matter content was the dominant soil property affecting soil invertebrate toxicity, cation exchange capacity was more influential on PHC degradation. Higher degradation was observed in lower cation exchange capacity soils, likely due to greater availability of PHCs

(Alexander 2000; Kim et al 2018). In the data exploration, we found low cation exchange capacity correlated with minimal amounts of clay and organic matter, both of which sorb PHCs, limiting their availability (Alexander 2000). During data exploration we also found cation exchange was negative associated with sand content. Sandy soils possess greater fractions of macropore space, promoting gas exchange and facilitating hydrocarbon degradation losses to abiotic and biotic processes (Wang et al 1999; Kim et al 2018). Abiotic degradation for F2 and F3 PHC contamination in low cation exchange capacity soil is primarily attributed to volatization (Wang et al 1999). We hypothesize that microbial degradation also likely contributed to degradation of PHCs, given the increased losses in fertilized soils we observed in most test soils and findings from comparable studies (Walworth and Reynolds 1995; Chang et al 2010; Karppinen et al 2017; Kim et al 2018). Across all test soils, the average degradation half life for both F2 and F3 PHCs was less than 120 days, indicating they are not considered persistent soil contaminants (Mermond et al 2012; EUC 2011). However, findings from this study reflect controlled environmental conditions within a laboratory growth chamber and are not fully representative of field conditions. In consideration of the non-persistent nature of the medium PHC product we tested in surface soils, perhaps the impacts to soil dwelling organisms will be short term, largely affecting adult reproduction rather than survival. At moderate levels of PHC contamination (HC25) in soils with organic matter content of at least 3%, adult *F. candida* and *O. nitens* survived, suggesting that following degradation, population recovery may occur.

6. Manuscript 4: Avoidance Response of Juvenile and Adult Soil Invertebrates to Soil Contaminants

6.1 Preface

Since juveniles are considered more sensitive to contaminants than adults, they are an important demographic group to study for behavioural responses. Currently, the avoidance response of juvenile soil invertebrates is unknown as no literature exists. This study assessed the juvenile avoidance response of three soil invertebrates to common soil contaminants. The study includes a discussion on the use of sodium chloride as a reference toxicant for avoidance tests and potential extrapolation of findings from avoidance tests to field situations.

Gainer, A., R. Akre, O.J. Owojori and S. Siciliano. 2019b. Protecting vulnerable individuals in a population: Is juvenile soil invertebrate avoidance to contaminated soil more sensitive than adults? Chemosphere. 220: 658-667.

Amy Gainer: Conceptualization, methodology, validation, formal analysis, data curation, writing (original draft), visualization
Robyn Akre: Investigation, writing (review and editing)
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6.2 Abstract

Juveniles are generally considered more sensitive to contaminants than adults. However, it is unknown if the behavioural responses of juvenile soil invertebrates is different than the adults. The absence of juvenile or adult soil invertebrates in contaminated soils due to avoidance adversely impacts the soil quality. Here, this study assessed the avoidance response in two life stages (juvenile and adult) of three standardized soil toxicity test invertebrates (*Folsomia candida*, *Enchytraeus crypticus* and *Eisenia fetida*) exposed to phenanthrene, copper and sodium chloride-contaminated soil. Interestingly, this found the juvenile's avoidance response could be more sensitive, less sensitive and the same as the adult's avoidance response, depending on the contaminant and test species. The juvenile avoidance response of *E. fetida* to sodium chloride, and *E. crypticus* and *E. fetida* to copper was more sensitive than the adult's response. In contrast, the avoidance response of juvenile *F. candida* to sodium chloride was less sensitive than the adult's response. No life stage differences were observed in the avoidance response of *E. crypticus* individuals exposed to sodium chloride, *F. candida* individuals exposed to copper and *E. fetida* individuals exposed to phenanthrene. Although life stage differences in avoidance responses were evident for some species and contaminants, it was not consistent. In terms of avoidance, the assumptions that juveniles are the most sensitive individuals in a population is not always true. Sodium chloride was identified as an ideal reference toxicant for avoidance tests.

6.3 Introduction

Avoidance response tests with contaminated soils provide an ecologically relevant test for inclusion in soil ecological risk assessments. They are informative of the ability of a range of soil invertebrates to sense contaminants in their environment and indicative of both soil invertebrate and plant habitat quality (Hund-Rinke et al 2003; Amorim et al 2005a; Amorim et al 2005b; Aruajo et al 2016a; Gainer et al 2019a; Owojori et al 2011; Owojori et al 2014). The turnaround time is quick due to a short test duration, of typically two days, with some tests species only requiring one day (ISO 2011; Amorim et al 2005a, Owojori et al 2011; Owojori et al 2014). Unlike the laboratory mortality and reproduction tests where organism exposure is forced, avoidance tests are more field realistic and ecologically relevant scenarios since individuals have the ability to reduce exposure through avoidance if the contaminant can be sensed (Aldaya et al 2006; Aruajo et al 2016b). In addition, avoidance test design more accurately reflects the heterogeneous nature of soil contamination (Meli et al 2013). Results from avoidance tests on populations of different organisms with similar functional roles provides insight into how contaminants impact community structure and function, with implications on short and long-term soil quality (Aruajo et al 2016b; Lopes et al 2004). Due to juvenile's demographic role in populations (Heckmann et al 2005), the avoidance response of juvenile individuals complements existing soil toxicity endpoints like mortality and reproduction. However, currently, minimal literature exists on the juvenile invertebrate avoidance response to contaminated soils. Available studies on juvenile soil invertebrate avoidance response to contaminants provide limited scope since they only studied one species, earthworms, and one contaminant group, organophosphate pesticides (Jordaan et al 2012; Hodge et al 2000).

Juveniles compromise a large demographic of populations, yet, are not highly represented in soil ecotoxicology laboratory literature or standardized soil toxicity or behavior testing. Although the *Folsomia candida* avoidance ISO (2011) protocol suggests use of 10 to 12 day old individuals in avoidance tests, this age is still considered an adult as it corresponds to fifth instar sub adults (Riepert 1996; Fountain and Hopkin 2001). Juvenile earthworms in agricultural soils compose between 50-80% of the population (Peigne et al 2009; Schmidt et al 2003; Pelosi 2015). For some species, like oribatid mites, juveniles account for one third of the total population (Norton 1994). Thus, juvenile's avoidance response influences future population growth. The avoidance

response of both life stages of soil invertebrates is informative of potential changes in soil quality of contaminated and surrounding uncontaminated soils. Avoidance of contaminated areas, or failure to avoid contaminated soils leading to mortality or reproduction inhibition, both potentially result in a localized population collapse. The absence of soil invertebrates in contaminated soils results in deleterious soil conditions, whether it be loss of soil structure or reduced litter decomposition (Aldaya et al 2006; Amorim et al 2006; Amorim et al 2008).

Most existing toxicity literature suggests juveniles are more sensitive than adults to numerous contaminants across a range of environments and species. From a meta-analysis of fish toxicity literature across several contaminants, Hutchinson et al (1994) determined that for 92% of contaminants, juvenile fish were more sensitive than adults. Similar life stage relationships exist in terrestrial invertebrates. Fountain et al (2007) reported juvenile Collembola and spiders were either absent or present in low amounts on soils exposed to chlorpyrifos, proposing that this maybe due to the greater ability of adults to detect and avoid insecticides (Fabian and Petersen 1994). Exposure of the predatory mite, *Hypoaspis aculeifer*, to dimethoate elicited greater toxicity to two immature forms than the adults (Heckmann et al 2005). In the earthworm, *Eisenia fetida*, sexual development and cocoon production are inhibited at lower concentrations of metals than adult mortality (Spurgeon et al 1996; van Gestel et al 1991). The hatching of embryos and juvenile growth in *Enchytraeus crypticus* in response to cadmium-contaminated soils were more sensitive endpoints than the adults mortality (Bicho et al 2015a). The organophosphorus pesticide, dimethoate, and the pyrethroid pesticide, cypermethrin, were more toxic to juvenile forms of a leaf beetle, *Gastrophysa polygoni,* than adults (Kjaer et al 1998). Two studies on the juvenile earthworm avoidance response to pesticide-contaminated soils found non-avoidance (Jordaan et al 2012; Hodg et al 2000), however, adults were not included simultaneously in the study, limiting comparisons between life stages. Despite evidence that juveniles toxic responses are more sensitive individuals than adults, it is unknown if this pattern extends to avoidance responses to contaminated soils which may lead to population shifts in contaminated soils.

The mechanism of difference between life stages is not fully understood in soil invertebrates but likely due to different physiological and behavioral abilities to detect contaminants in their environment. Therefore, behavior may vary with age and species due to different abilities to

detect contaminants (Heckmann et al 2005). For instance, due to their higher surface area to volume ratio and slower movements, greater absorption is likely occurring in juveniles. No information was available in literature regarding life stages differences in chemosensing for *F. candida* and *E. crypticus*. However, juvenile earthworms possess less and smaller sized sensory nerves than the adults, potentially reducing their ability to detect contaminants in soil (West 1978; Moment and Johnson 1970). Although it is unclear if life stage differences in soil invertebrate avoidance behavior exist, limited available literature on earthworm physiology suggests that juveniles they may not have same capacity as adults to detect contaminations.

The present study is the first of its kind to test the avoidance response of juveniles from a range of species to soil contaminants. Our primary objective was to determine if avoidance response to three varying contaminants (copper, phenanthrene and sodium chloride) diverge between the juvenile and adult life stages in three standardized soil invertebrate toxicity test species (*Folsomia candida, Enchytraeus crypticus* and *E. fetida*).

6.4 Materials and Methods

6.4.1. Test Organisms

The three soil toxicity test organisms used included: *E. fetida, F. candida* and *E. crypticus*, with culture origins described in Gainer et al (2018). Cultures for *F. candida* and *E. crypticus* were maintained at an average temperature of 21°C with a photo period of approximately 12:12 h light:dark. *Eisenia fetida* was reared in the dark at an average temperature of 20°C. Culture media for the earthworms was composed of topsoil and newspaper and fed varying organic materials consisting of organic wastes, sterilized leaf litter and cooked oatmeal. The *F. candida* population was cultured on a substrate composed of moistened 8:1 plaster of Paris to activated charcoal and fed activated yeast biweekly. Enchytraeids were cultured in sterilized topsoil and fed with a combination of ground oats and breadcrumbs weekly.

Eisenia fetida and *E. crypticus* test juveniles and adults were not age synchronized. Juvenile *E. fetida* were identified by the absence of a clitellum and weight ranged between 100 and 250 mg. The upper mass range was selected based on Environment Canada (2004) protocol that indicates

250 mg is the minimum weight of sub-adults lacking clitellums that can be utilized in tests. Adult *E. fetida* used in tests possessed clitellum and were at least 250 mg in weight (EC 2004). Adult and juvenile *E. crypticus* were differentiated based on presence or absence of clitellum as well as body length. Adult *E. crypticus* were identified by the presence of a clitellum with body lengths between 8 and 10 mm, consistent with low organism density sizes reported by Goncalves et al (2017). Juvenile *E. crypticus* were identified by the absence of clitellum and a body length less than adults, between 5 and 6 mm. As indicated by Goncalves et al (2017), consideration of organism density is important when selecting juvenile *E. crypticus* individuals from non-age synchronized cultures since density influences organism size.

Juvenile *F. candida* were age synchronized to be 4 to 5 days old and adults 10 to 12 days. Juvenile *F. candida* are defined by Environment Canada (2014) as sexually immature; therefore, this study selected the age for juveniles as half the age of sexually mature sub-adults, fifth instars used for adult toxicity tests. Synchronization of *F. candida* juveniles and adults occurred by collecting eggs in a separate culture, allowing eggs to hatch over three days and removing unhatched eggs.

6.4.2. Test Substances and Soils

Three test chemicals were used in this experiment that reflect different types of global environmental contamination: copper, sodium chloride and phenanthrene. Copper was added as $CuSO_4 \cdot 5H_20$ (purity=99.99%; CADS 7758-98-7), phenanthrene (purity 98% Sigma-Aldrich; CAS 85-01-8) and sodium chloride (purity 99% Sigma-Aldrich; CAS 7647-14-5). Copper sulfate was selected to be consistent with current literature, improving comparability of results. Stock solutions in deionized water were made for copper and sodium chloride.

Organization for the Economic Cooperation and Development artificial soils were utilized for all test organisms according to Environment Canada (EC 2004; EC 2014). The composition was approximately: 70% silica sand, 20% kaolin clay and 10% sieved (2mm), air dried *Sphagnum* sp. peat moss, calcium carbonate and distilled water. Soils moisture content was maintained at 70% water holding capacity. At the test start, soil pH was determined and adjusted with calcium

carbonate to be within the acceptable range of 6 to 7.5 and all replicates within a 0.5 range of each other (EC 2004; EC 2014). Soil pH was determined by shaking soils with 0.01 M $CaCl_2$ in a 1:5 soil: solution ratio for one hour. Definitive test concentrations were selected based on preliminary results from range finding tests. The range in sodium chloride concentrations were 0.9 to 30.9 dS/m, in copper was 0 to 1800 mg/kg and phenanthrene was 0 to 8800 mg/kg (Appendix D Tables D-1 to D-3). Copper and sodium chloride were mixed into soil from stock solutions. Phenanthrene was mixed into soil using acetone as a carrier solvent. Phenanthrene was initially mixed into soil without peat and left in fume hood overnight for removal of acetone. The following day, peat was mixed into the soil. This process allowed for faster dissipation of acetone and greater homogenization between mineral and organic fractions. Solvent controls were included against the negative control during tests conducted with phenanthrene. Test samples were collected at start and end for determination of measured concentration of phenanthrene, copper and electrical conductivity.

Phenanthrene in soil was quantified by gas chromatography with mass spectrometry, following the United States Environmental Protection Agency (US EPA) 3540C/8270D method (US EPA 2014a). Soluble electrical conductivity was determined from saturated paste using method Standard Method 22 2510 in 0.01 molar potassium chloride (Standard Methods Committee 1997). Total copper content was determined using US EPA 200.8 R5.4 method (US EPA 1994). Soil was digested in acid and metal content determined with an inductively coupled plasma mass spectrometry. Quality control and assurance included matrix spike, spiked blank and method blanks. Spiked recoveries were within 50 to 130% for phenanthrene and 75 to 125 % for copper and electrical conductivity.

6.4.3. Experimental Setup

The avoidance tests consisted of dual chambers containing a combination of test chemical and control soils. Chamber size varied with organism. Chambers for *E. fetida* tests were rectangular with dimensions of 20 x 12 x 5 cm (Loureiro et al 2005; ISO 2008), *F. candida* tests chambers were circular with a diameter of 7 cm (ISO 2011) and *E. crypticus* test chambers were circular with diameter of 8 cm (Amorim et al 2008). The exterior of the chamber was marked into two

sections indicating control or test soils. Using a divider, half the chamber was filled with test soil and the other half control soil. The negative control consisted of uncontaminated soils on both sides. For *E. fetida* the soil mass on each side was 250 g dry weight while both *F. candida* and *E. crypticus* contained approximately 25 g dry weight. After the chamber was filled with both soils, the divider was removed, and 10 individuals were introduced to the surface at the dividing line. The vessels were closed with a single hole for air circulation. Each test consisted of a range of 9 to 12 concentrations and a negative control with each test concentration consisting of 5 replicates. The tests were carried out under the following conditions: daily average temperature of 20˚C (+/- 2˚C), 12:12 hours light:dark cycle, with approximately 600 lux at surface and approximately 60% humidity (EC 2014).

After 48 hours, dividers were inserted back into the containers to separate the control soil from the test soil. *Eisenia fetida* were manually counted by hand sorting through the soil. *Enchytraeus crypticus* in test soils were preserved in 70% ethanol solution and Bengal red dye. Enchytraeids were separated and counted from soils via wet sieving (ISO 2003). *Folsomia candida* were determined by flooding the soil with water and counting the floating individuals by sight.

6.4.4. Statistical Analysis

Normality and homoscedasticity (homogeneity) of residuals were assessed by the Shapiro-Wilk normality test and Levene's test, respectively. Avoidance response was expressed as a net response calculated from the number of individuals on the contaminated side subtracted from the number of individuals in the control side and divided by the total number of recovered organisms multiplied by 100 (ISO 2008; ISO 2011). A positive net response indicates avoidance of the contaminated soil while a negative net response indicates attraction to the contaminated soil. Tests were considered valid if the average net response in negative and solvent controls was 50% (+/- 10%). Tests were considered invalid if average recovery of organisms was less than 80% (ISO 2008; ISO 2011). Based on students t-tests, the net response with solvent controls and negative controls were the same as net response with dual negative controls.

Near full dose response (70 to 100%) was observed in most tests with some tests displaying non-avoidance, providing a suitable data set for non-linear regression. For determination of toxicity effective concentration (EC) values, negative net responses were considered zero (ISO 2008; ISO 2011). Determination of EC25 and 50 with 95% confidence intervals were conducted in Graphpad Prism® (version 6.0) by a four-parameter (minimum, maximum, EC50, slope) Hill logistic equation (variable slope sigmoidal nonlinear regression model) and visualized in SigmaPlot (Version 12.0). Differences between juvenile and adult endpoints (α=0.05) was determined using unpaired t-tests on the EC50 and standard error from the non-linear regression in Graphpad Prism®.

6.5 Results

Avoidance occurred in both juvenile and adult life stages for all test contaminants, with a few exceptions (Figure 6-1 to 6-3; Table 6-1). Adult and juvenile *F. candida* and *E. crypticus* avoided copper and sodium chloride (Figure 6-1, 6-2). Adult and juvenile *F. candida* and *E. crypticus* displayed non-avoidance to phenanthrene at any concentrations. Adult and juvenile *E. fetida* displayed avoidance behavior to all contaminants tested (Figure 6-3).

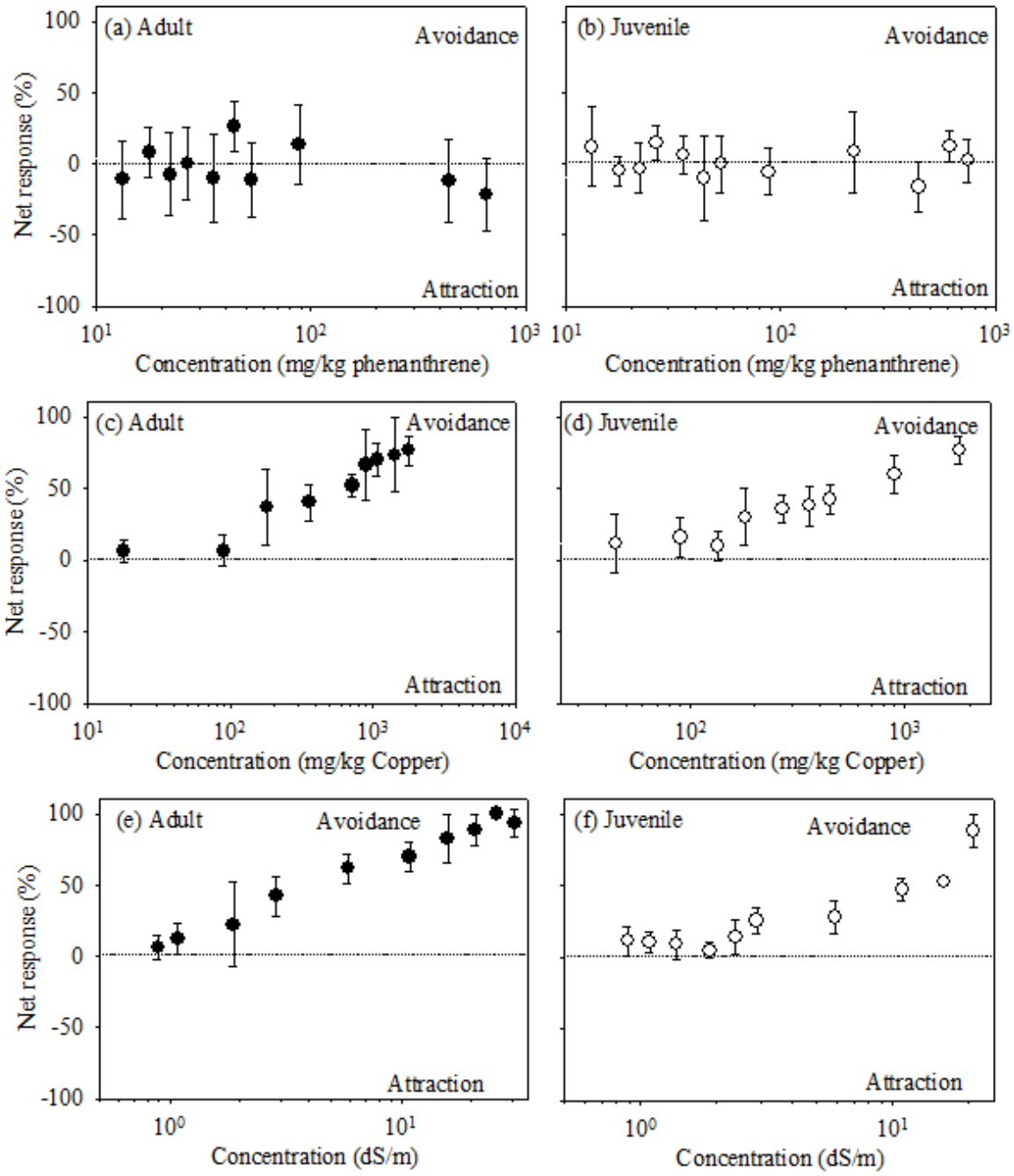

Figure 6-1. Avoidance net response of *Folsomia candida* adults to phenanthrene (a), juveniles to phenanthrene (b), adults to copper (b), juveniles to copper, adults to sodium chloride (e) and juveniles to sodium chloride (f). Sodium chloride concentration expressed as electrical conductivity (EC).

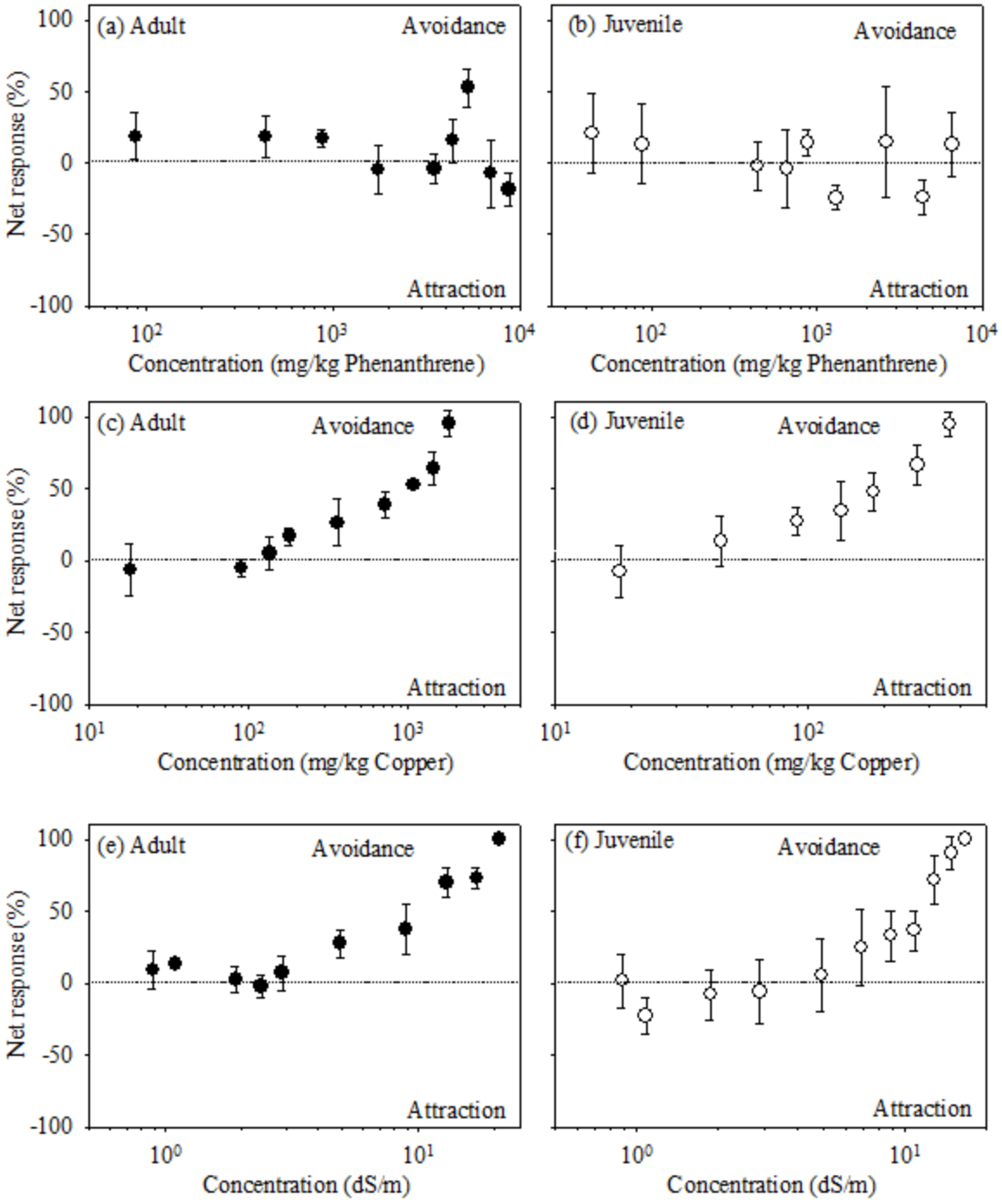

Figure 6-2. Avoidance net response of *Enchytreaus crytpicus* adults to phenanthrene (a), juveniles to phenanthrene (b), adults to copper (b), juveniles to copper, adults to sodium chloride (e) and juveniles to sodium chloride (f). Sodium chloride concentration expressed as electrical conductivity (EC).

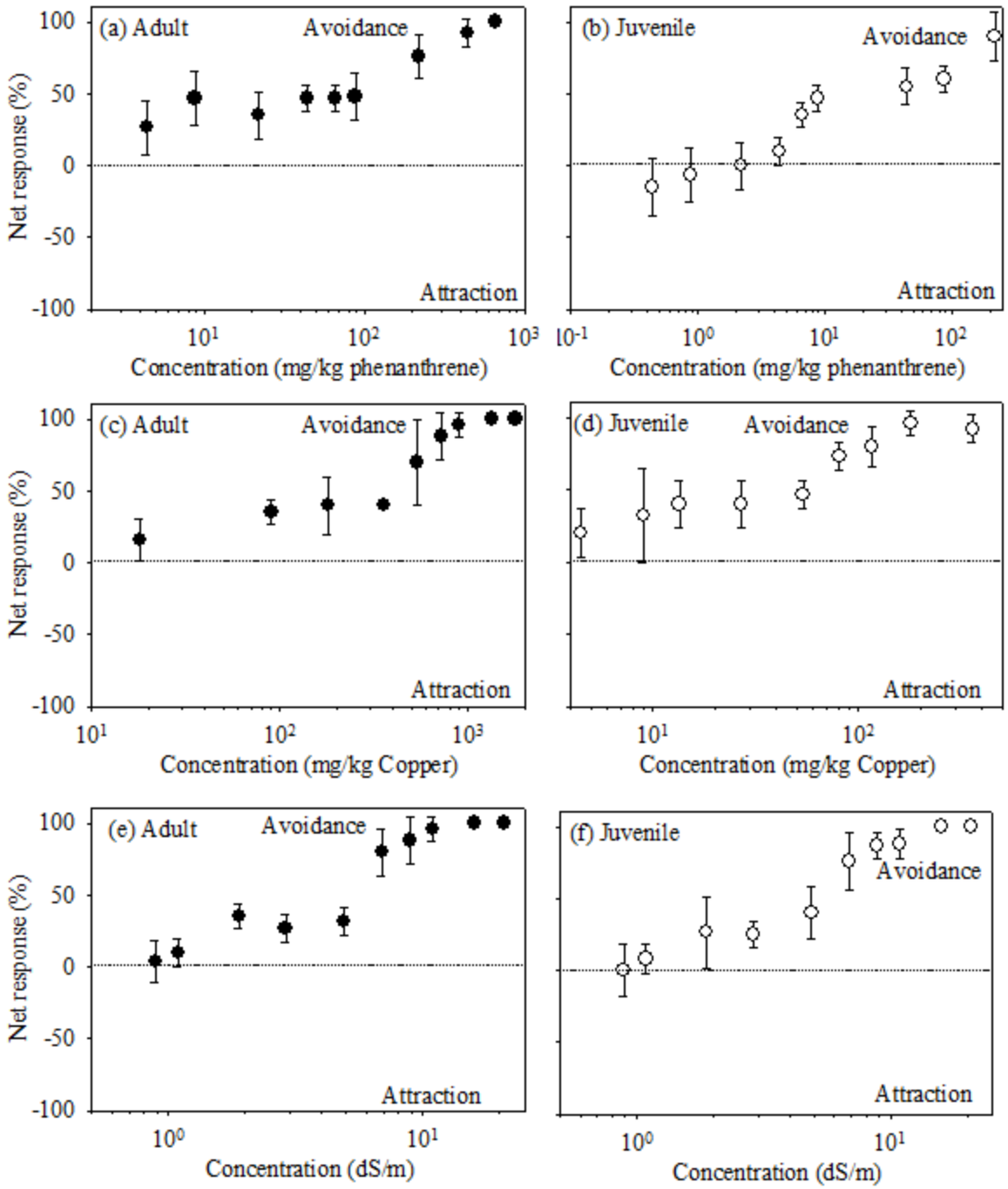

Figure 6-3. Avoidance net response of *Eisenia fetida* adults to phenanthrene (a), juveniles to phenanthrene (b), adults to copper (c), juveniles to copper (d), adults to sodium chloride (e) and juveniles to sodium chloride (f) contaminated soils. Sodium chloride concentration expressed as electrical conductivity (EC).

Table 6-1. The 25th percentile effective concentration values, 50th percentile effective concentration values with 95% confidence intervals, coefficient of determination and dose response curve slope emergence for soil invertebrate (*Eisenia fetida, Enchytraeus crypticus* and *Folsomia candida*) exposed to soil spiked with phenanthrene, copper and chloride.

Species	Life Stage	Contaminant (units)	EC25	EC50	Lower 95% CI	Upper 95% CI	Slope	r^2
Eisenia fetida	Juvenile	Electrical conductivity (dS/m)	2.9	4.6*	3.9	5.4	1.9	0.84
Eisenia fetida	Adult		4.9	6.4	5.7	7.2	3.7	0.77
Enchytraeus crypticus	Juvenile		8.1	10	9.6	11	4.1	0.81
Enchytraeus crypticus	Adult		5.4	8.9	7.7	10	1.9	0.87
Folsomia candida	Juvenile		4.3	10*	8.1	13	1.1	0.78
Folsomia candida	Adult		1.9	3.9	3.0	5.0	1.0	0.82
Eisenia fetida	Juvenile	Copper (mg/kg)	31	56*	46	69	1.2	0.81
Eisenia fetida	Adult		96	217	155	306	1.2	0.79
Enchytraeus crypticus	Juvenile		94	197*	161	243	1.3	0.74
Enchytraeus crypticus	Adult		417	813	687	961	1.6	0.82
Folsomia candida	Juvenile		194	667	508	878	0.85	0.73
Folsomia candida	Adult		181	516	373	716	0.99	0.73
Eisenia fetida	Juvenile	Phenanthrene (mg/kg)	5.3	27	18	39	0.68	0.83
Eisenia fetida	Adult		8.0	42	26	68	0.66	0.72
Enchytraeus crypticus	Juvenile				Non-avoidance			
Enchytraeus crypticus	Adult				Non-avoidance			
Folsomia candida	Juvenile				Non-avoidance			
Folsomia candida	Adult				Non-avoidance			

* indicates statistically different (α=0.05) between juvenile and adult; mg/kg= mg total petroleum contaminant per kg soil; dS/m=deciSiemen per metre; CI=confidence interval; EC=effective concentration; r^2=coefficient of determination; all values are actual; non-avoidance indicates the organisms did not show a strong attraction or avoidance

The avoidance response of juvenile organism's did not consistently display a greater sensitivity to contaminants than the adult's response (Figure 6-4). Out of the nine tests, in three tests the juvenile's avoidance response was more sensitive than adults. The adult *E. fetida* avoided sodium chloride (EC50=6.4dS/m) at a significantly higher concentration than the juveniles (EC50 =4.6 dS/m). The adult *E. crypticus* and adult *E. fetida* EC50 avoidance response (813 and 217 mg/kg, respectively) was significantly greater than the corresponding response for the juveniles (EC50=197 and 56 mg/kg, respectively) to copper-contaminated soils (both $p < 0.0001$). In one of the nine tests, the juvenile avoidance response EC50 was greater than the adult's response EC50 to contaminated soil. The EC50 (10 dS/m) for avoidance response of juvenile *F. candida* to sodium chloride-contaminated soils was significantly greater than the adult's avoidance response EC50 (3.9 dS/m) ($p <0.0001$) (Table 6-1). Three tests reported no differences between the juvenile and adult avoidance response to contaminated soil. No statistical difference ($p<0.05$) between the avoidance response of juvenile and adult life stages was observed in: *E. crypticus* exposed to sodium chloride, *F. candida* exposed to copper and *E. fetida* exposed to phenanthrene-contaminated soils.

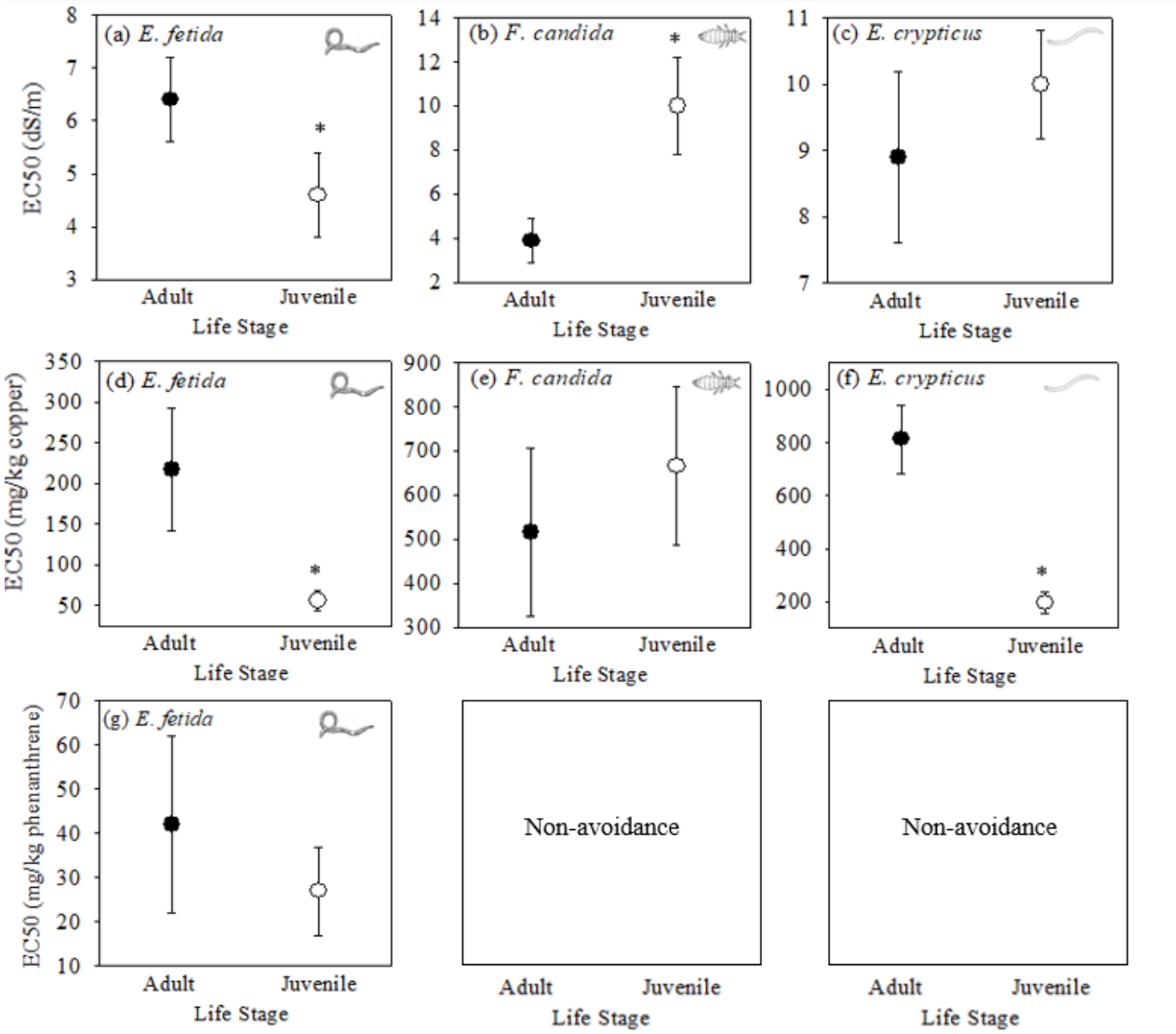

Figure 6-4. Effective concentration 50 (EC50) with 95% confidence intervals for the adult and juvenile soil invertebrate avoidance response for: (a) *Eisenia fetida* exposed to sodium chloride, (b) *Folsomia candida* exposed to sodium chloride, (c) *Enchytraeus crytpicus* exposed to chloride, (d) *Eisenia fetida* exposed to copper, (e) *Folsomia candida* exposed to copper, (f) *Enchytraeus crytpicus* exposed to copper and (g) *Eisenia fetida* avoiding phenanthrene. All concentrations represent actual concentrations. Sodium chloride concentration expressed as electrical conductivity. Asterisks indicates the juvenile's avoidance response is statistically different (α=0.05) than the adult's avoidance response.

The avoidance response EC50 values determined adult and juvenile soil invertebrates were compared to lethal and sublethal concentrations from literature (Table 6-2). For improved

comparison, test species and test soils most similar to our tests were included such as similar species and soil organic matter. The adult and juvenile avoidance response EC50 values were greater than the corresponding reproduction EC50 values for every species, with a few exceptions. The avoidance response EC50 was less than the reproduction EC50 for the following tests: *E. fetida* adult and juvenile copper, *F. candida* adult and juvenile copper, and *E. crypticus* juvenile response to copper. With respect to lethality, the juvenile avoidance response of all test species to sodium chloride was greater than the mortality EC50 for *E. fetida.* For the remaining species and contaminants, the juvenile avoidance response EC50 was less than the corresponding adult mortality EC50.

Table 6-2. Comparison of lethality, reproduction and growth 50th percentile effective concentrations from literature with the avoidance responses of *Folsomia candida, Enchytraeus crypticus* and *Eisenia fetida* exposed to sodium chloride, copper sulfate and phenanthrene. If reported in original source, 95% confidence intervals indicated in brackets.

Species	Contaminant	Units	Adult Avoidance EC50	Juvenile Avoidance EC50	Reproduction EC50	Source	Mortality LC50	Source
Folsomia candida	Sodium chloride	dS/m	3.9	10	0.52-1.03[F]	[a]	>1.62 [F]	[a]
Enchytraeus crypticus			8.9	10	0.52-1.03 [F] (*E. doerjesi*)	[a]	>1.62 [F]	[a]
Eisenia fetida			6.4	4.6	1.7 [A]	[b]	2.02 [A] (1.98-2.07)	[b]
Folsomia candida	Copper sulfate	mg/kg	516	667	75 [A] (624-905)	[c]	1810 [A] (1179-2783)	[c]
Enchytraeus crypticus			813	197	305 [A] (235-374) (*E. albidus*)	[d]	>320 [A] (*E. albidus*)	[e]
Eisenia fetida			216	56	53.3 [A] (32.5186)	[f]	555 [A] (460-678)	[f]
Folsomia candida	Phenanthrene	mg/kg	Non -avoidance		257 [L] (201-313)	[g]	366 [L] (337-394)	[g]
Enchytraeus crypticus			Non -avoidance		559 [L] (538-581)	[g]	2109 [L] (1437-2780)	[g]
Eisenia fetida			42	27	94 [F] (64-125) (*E. veneta* Growth)	[h]	134 [F]	[h]

mg/kg= mg total petroleum contaminant per kg soil; dS/m=deciSiemen per metre; EC=effective concentration; [F]=indicates field soil, [A]=indicates OECD artificial soil; [L]=indicates LUFA 2.2. soil; [a] Owojori et al 2009; [b] Owojori and Reinecke 2014; [c] Greenslade et al 2003; [d] Lock and Janssen 2002; [e] Amorim et al 2005c;[f] Spurgeon et al 1994; [g] Droge et al 2006; [h] Sverdup et al 2002

6.6 Discussion

Inconsistent patterns between the avoidance response of juvenile and adult soil invertebrates was observed with each test species and contaminant. In three of our nine tests, the avoidance response of the juveniles was a more sensitive endpoint than the corresponding adult's response. From one test, *F. candida* exposed to sodium chloride, the juveniles were less sensitive than the adults in terms of their avoidance response. No differences in the avoidance response between life stages occurred in three tests and non-avoidance was observed by both life stages in two tests. The rationale for this observed range in life stage differences in avoidance responses to contaminated soils is unclear as limited literature is available in this area. Of the available literature about chemosensing in juveniles of our test species, studies with earthworms indicate juveniles contain less sensory nerves than adults (West 1978; Moment and Johnson 1970), suggesting that juveniles may fail to detect and avoid contaminants at the same concentrations as adults. Although this study observed this between life stages of *F. candida* exposed to salts, this is not consistent with our findings for the earthworm *E. fetida*. This study found the avoidance response of juvenile *E. fetida* occurred at either lower or equivalent concentrations than the corresponding adult's net response to contaminants. Additional research is needed to better understand the variations in life stage avoidance responses to contaminated soils.

Avoidance behaviors of organism influence the population size and subsequent community structure, ultimately impacting soil quality and ecosystem services. Comparing the avoidance response of both life stages to the corresponding adult mortality and reproduction inhibition endpoints provides insights into how populations may change in the field. Since soil invertebrate toxicity literature strongly reports that juvenile soil invertebrates' lethality occurs at concentrations less than adults (Heckmann et al 2005; Spurgeon et al 1996; van Gestel et al 1991; Bicho et al 2015a; Kjaer et al 1998), this study used the adult reproduction inhibition endpoint as a surrogate indicator for juvenile lethality. Although likely an overestimation, no literature is available for juvenile lethality of out test species and contaminants. For five tests (all species exposed to sodium chloride, and *F. candida* and *E. fetida* exposed to copper), the juvenile avoidance response occurred at a concentration greater than the adult reproduction inhibition endpoint. This suggests that under longer exposure periods juvenile mortality may occur, depleting the population of juveniles. In combination with reduced production of juveniles

from adults, populations of organisms surrounding these contaminated soils may diminish, reducing soil quality as well as supplying less individuals to repopulate the contaminated soils in the future (Aldaya et al 2006; Aruajo et al 2016b). A more extreme population decline, and potential localized extinction, may occur in contaminated soils that organisms cannot detect, as this study observed in the non-avoidance of both life stages of *F. candida* and *E. crypticus* to phenanthrene. Both life stages of *F. candida* and *E. crypticus* failed to avoid phenanthrene at concentrations known to cause sublethal effects on reproduction (257 and 559 mg/kg, respectively) and mortality (366 and 2,109 mg/kg, respectively) (Droge et al 2006). Since both *F. candida* and *E. crypticus* live in close contact with soil pore water, non-avoidance likely occurred from lack of exposure to phenanthrene due to its limited water solubility. Previous studies with a hydrophobic petroleum hydrocarbon (Gainer et al 2018), also attributed non-avoidance to lack of exposure for *E. crypticus*. Other studies with Collembola support that contact with soil pore water likely influences their avoidance response (Fountain and Hopkin 2004; Filser et al 2000). Although it may appear that another pattern is obvious from our study: that both life stages of *F. candida* and *E. crypticus* avoid water soluble contaminants like sodium chloride and copper, this is not true across other contaminants. Other studies with water soluble contaminants reported non-avoidance of *F. candida* to cadmium (Greenslade et al 2003) and *E. crypticus* to boric acid (Bicho et al 2015b). Since our study was limited to three contaminants from different classes, additional research on more contaminants and contaminant groups would improve our understanding of soil invertebrate avoidance responses. Soils impacted by contaminants that organisms do not avoid exhibit a higher risk of population decline and greater deterioration in the long term than contaminants organisms detect and avoid (Bicho et al 2015; Rosa et al 2012). This study suggests non-avoidance behavior of soil invertebrates to a contaminant should continue to be treated with additional caution when conducting risk assessments, triggering a discussion on the long-term potential consequences to populations, especially for contaminants whose toxicity does not decrease with time.

Strong avoidance of sodium chloride and copper by all three test species in our study is indicative of potential absence of these species in field soils where contamination triggers avoidance. The absence of three species, like *E. crypticus, F. candida* and *E. fetida*, may lead to an accumulation of organic matter and reduced soil quality (Aldaya et al 2006; Rosa et al 2012;

Gillet and Ponge 2002). Furthermore, the strong avoidance response to these substances indicates their potential use as a reference substance for avoidance toxicity tests. Due to the non-avoidance response observed in *E. crypticus* and *F. candida* to boric acid, there is currently not a commonly used reference toxicant for avoidance tests (Amorim et al 2008; Bicho et al 2015; Amorim et al 2012). Characteristics of ideal reference substances include: sensitive response of substance for desired test, stable and resistant to degradation, obtainable for purchase, water soluble, minimal human health risks, available and cost-effective analytical determination and accessible disposal options (Owojori et al 2014; ISO 2008; ISO 2011; Amorim et al 2012; Princz et al 2017). Based on these factors, this study suggests sodium chloride as a reference substance for avoidance toxicity tests since it meets the majority of the ideal characteristics.

Our findings on the adult avoidance responses for each species are consistent with existing literature on the same or similar contaminants. Numerous studies on the avoidance response of adult earthworms confirm they avoid copper both as a single contaminant (Loureiro et al 2005; Lukkari et al 2005; Demuynck et al 2016) or in mixtures with other metals (Natal da Luz et al 2004; Bengtsson et al 1983). Loureiro et al (2005) reported an *E. fetida* copper (as copper sulfate) avoidance response EC50 of 46 mg/kg in natural LUFA 2.2 soil, slightly lower than the 95% confidence intervals reported (155 to 306 mg/kg), with the differences partially due to the reduced organic matter content of LUFA 2.2 soil compared to the OECD artificial soil this study tested. Other studies support the avoidance response this study observed in adult *Folsomia candida* and *E. crypticus* both as a single contaminant (Amorim et al 2008a; Amorim et al 2008b; Greenslade et al 2003) and in metal mixtures in a range of soils (Natal da Luz et al 2004; Natal da Luz et al 2009; Cesar et al 2015; Dai et al 2018; Lock et al 2002). The avoidance response of earthworms to sodium chloride-contaminated soils agrees with findings from a similar species avoidance response tested by Owojori and Reinecke (2009a). Owojori et al (2009b) reported an *E. fetida* avoidance response to sodium chloride in field soils with an EC50 of 0.56 dS/m (95% confidence interval range 0.44 to 0.71 dS/m), lower than our EC50 of 6.4 dS/m (95% confidence interval range of 5.7 to 7.2 dS/m). Currently no comparable avoidance studies with sodium chloride have been conducted with *F. candida* or *E. crypticus*, however, in similar species, another study (Owojori and Reinecke 2009a) found absolute cessation of reproduction at 1.62 and 1.21 dS/m, respectively. Limited literature for avoidance of soil

invertebrates to phenanthrene only contaminated soil exist. However, other researchers found non avoidance behavior with *F. candida* exposed to naphthalene in solution (Greenslade et al 2003), *E. crypticus* non-avoidance was observed in another study on soils contaminated with low water solubility petroleum hydrocarbon-contaminated (Gainer et al 2019a) and *E. fetida* avoided mixtures of petroleum hydrocarbon and polycyclic aromatic hydrocarbon (Gainer et al 2019a; Hentati et al 2013).

7. Synthesis, Conclusions and Future Directions

Elevated petroleum hydrocarbons in soils remains a global environmental contamination issue. For PHC-contaminated soils in Canada, dominated by F2 and F3 PHCs, remediation guidelines are often limited by the guideline protective of soil-dwelling organisms. Currently, the only option for modifying the F2 and F3 PHC exposure pathway protective of soil-dwelling organisms is by conducting an SSRA.

Site-specific ecological risk assessments for F2 and F3 PHC-contaminated soils provides numerous advantages, highlighted throughout this thesis, such as the ability to assess persistence as well as toxic effects to soil receptors. Within this thesis, numerous research topics surrounding toxicity to soil ecological receptors as well as persistence were integrated into a SSRA for a medium PHC product (lubricating oil) both in standardized test soils and a range of Canadian soils. More specifically the objectives included:

- assessing the persistence of PHC contamination from a lubricating oil across a range of Canadian soils;
- determine the toxic effects and mixture toxicity of PHC-contaminated soils to a range of taxonomically diverse soil invertebrates and plant species;
- evaluating the behavioural response and mixture toxicity of adult soil invertebrates to PHC-contaminated soils; and,
- investigating differences in behavioral responses between life stages of common soil invertebrates test species.

Fundamental research questions addressing the objectives included:

- Why do toxic and behavioral responses to PHC contamination vary across test species?
- Why does toxic effects from PHC-contaminated soils to soil invertebrates vary across field soils? Can we predict individual PHC fraction toxicity endpoints using data from testing with a mixture and available literature for the individual PHC fraction toxicity?
- Why do different life stages of soil invertebrates display varying behavioral responses to contaminated soils?

7.1 Petroleum Hydrocarbon Persistence and Toxicity (Manucripts 1 to 3)

7.1.1. Synthesis and Conclusions

Petroleum hydrocarbon-contaminated soils caused toxic effects in a range of standardized soil toxicity test species and test types. Soil invertebrates with a sensitive response in mortality to PHC-contaminated soils were identified as those with diets including soil, large body size, permeable cuticle, low lipid content, lack of ability to moult and no maternal transfer. Soil invertebrate species with sensitive reproductive responses to PHC-contaminated soils possess life history traits indicative of long-life spans with small clutch sizes. Differences across plant species responses to PHC-contaminated soils were attributed to seed size, life cycle duration, root lipid content as well as biotransformation enzyme activities. Soil invertebrates with a tolerant avoidance response to PHC-contaminated soils was attributed to either lack of exposure (*E. crytpicus*) or trophic position (*H. aculeifer*). The main finding from Manuscript 2 concluded soil invertebrate avoidance of PHC-contaminated soil occurred at similar soil concentrations as the average growth measurements for plant species sensitive to petroleum hydrocarbon - contaminated soils. Another interesting finding was how the avoidance response of soil invertebrates is not always as sensitive as reproduction, a trend seen in other studies in the literature. In Manuscript 3, a major conclusion was to incorporate organic carbon normalization into soil PHC toxicity data and SSD construction to provide a higher level of protection to soil-dwelling organisms and reduce variation in toxicity data. This chapter also concluded that the ecological relevance of toxicity test species included in SSD construction for protection of soil-dwelling organisms does not change the Canadian soil guidelines. In regard to mixture toxicity in Manuscript 1 and 2, it was demonstrated how theoretical assumptions about mechanisms of action in mixture toxicity produce similar outcomes as both concentration addition and independent action mixture toxicity models applied to soil invertebrate toxicity data, plant toxicity data and soil invertebrate avoidance behaviour could not discriminate between mixture toxicity reference models, and both concentration addition and independent action fit the models.

7.1.2. Future Directions

A common theme across both Manuscript 1 and 2 involved exploring why some species are sensitive and some are tolerant to PHC-contaminated soils. A trait-based approach aided the discussion on interspecies variations in soil invertebrate and plant responses to PHC contaminated soils, however, it was solely qualitative due to lack of available literature. To quantify a trait-based approach for soil invertebrate ecotoxicity, like those observed in aquatic toxicity literature for pesticides, additional research is required. Trait based approaches are useful for understanding and predicting the toxic responses of organisms. I suggest the following areas as future directions to advance soil invertebrate literature for use in trait-based approaches to PHCs and other contaminants:

- Determine baseline information for biotransformation enzymes within standardized soil invertebrates and plant toxicity test species such as catalase, cytochrome P450 enzymes, aldehyde and alcohol dehydrogenase, glutathione-S transferases, superoxide dismutase and amino acids transferases.
- Quantification of key detoxification proteins such as metallothionein within standardized soil invertebrate toxicity test species.
- Quantify the lipid content of standardized soil invertebrate toxicity test species and the lipid content of root surfaces of standardized plant toxicity test species since limited data is available for all these species and lipid content is important to predicting PHC toxicity to organisms.
- In general, obtain more information on the toxicokinetics of soil invertebrates for a range of contaminants such as the elimination rate. More specifically future research on the excretion of contaminants through moulting or maternal transfer.
- Quantify the exposure occurring in soil invertebrates residing in contaminated soils by exploring whether surrogate materials, like silicon or Tenex beads, designed with a similar shape and surface area to volume ratio as soil invertebrates can estimate exposure.

As evidenced in Manuscript 3, soil organic matter content influences toxicity of PHCs to soil invertebrates. Not only does organic matter influence soil invertebrate toxicity but also the habitat quality of soils. Many routes of future research are possible by focusing on the role of

organic matter in PHC soil ecotoxicity. Additional testing in a variety of field soils and field soils with higher organic matter contents is recommended, as our study was limited to a certain range of soil properties. For instance, the study in Manuscript 3 included many soils with low organic matter contents, with a maximum level of approximately 5%. In addition, an area of future research entails studying the influence of organic matter composition and type on soil invertebrate toxicity in PHC-contaminated soils. For instance, does the ratio of active to resistant potions of organic matter influence PHC toxicity to soil dwelling receptors? Other types of soil organic matter to explore include lignin, humus, fulvic acid, polysaccharides or glomalin. Another finding in this thesis was how organic carbon normalization is a promising approach to incorporate into site-specific ecological risk assessments, particularly in low organic matter soils where toxic effects on soil invertebrates increase. Follow up studies are required to assess what level of protection is provided when using the site-specific guideline derived using organic carbon provides. For instance, assess the level of protection offered to soil invertebrates in field soils at concentrations representing both currently approach and organic carbon normalization approach. This could be through assessing mortality, reproduction and avoidance response.

Avoidance tests with soil invertebrates provide unique information about how organism interact with contaminants in their environment. However, there are some unresolved issues with the test. There is currently no guidance on how to quantify non-avoidance or attraction behaviour response in avoidance test and incorporate into an SSD. This is especially concerning because non-avoidance and attraction behaviour in field settings potentially impacts local populations more than avoidance. Further research on how to include non-avoidance or attraction behaviour responses to avoidance test is required. Within ecological risk assessments, perhaps these types of behaviors trigger higher tier toxicity testing requirements or application of safety factors to data obtained from SSDs.

Uncertainty exists within soil ecotoxicity literature on the mechanisms in which soil invertebrates detect PHCs and many other contaminants. Existing literature outside toxicology highlights the important role of volatiles compounds in attracting both herbivores and carnivore terrestrial invertebrates. Further research is warranted on which specific compounds soils

organisms are attracted to in PHC contaminated soils. In addition, since a range of avoidance responses across soil invertebrate species was observed in Manuscript 2, additional research is required into how all these organisms individually detect PHC. Specifically, whether they all rely heavily on volatiles olfactory sensing within soil pore space or does detection of PHC also occur through gustatory cells and soil pore water.

A discovery of Manuscript 2 was how long-lived coniferous species from the boreal forest appear to be tolerant to PHC-contaminated soils, compared to agronomic and vegetable species. However, it is unclear if this trend continues to other long-lived boreal forest species with standardized protocols such as birch. Further research on other and all long-lived boreal forest tree species is recommended due to their importance to northern Canadian ecoregions as well as limited representation in the available literature. Furthermore, tolerance to contamination is an ideal characteristic of plants suitable for phytoremediation of contaminated soils. Not only are the conferous trees species assessed in this thesis tolerant to PHC-contaminated soils, they are also native species to Canada, in important feature for phytoremediation of certain sites. Further research both within laboratory and field settings assessing their potential for phytoremediation is recommended.

7.2 Avoidance Response of Juvenile Soil Invertebrates to Contaminated Soils (Manuscript 4)

7.2.1. Synthesis and Conclusions

Existing soil invertebrate avoidance response literature mostly reflects adults' responses. In Manuscript 4, the study concluded the avoidance behaviour of juvenile soil invertebrates was not consistently more sensitive to contaminants than adults. Depending on the contaminant, the juvenile avoidance response was more sensitive, less sensitive and the same as the adult response. Another key finding from Manuscript 4 is that sodium chloride is a potential reference toxicant for soil invertebrate avoidance assays, a current gap in the soil avoidance literature.

7.2.2. Future Directions

This study opened many opportunities for future research as there is a lack of literature surrounding numerous aspects of this Manuscript. Additional information is needed on how each test organism detects contaminants and in which body parts. For instance, does *F. candida* detect copper through olfactory or gustatory cells? In addition, future research opportunities exist in testing the behavior of juvenile stages of other standardized soil invertebrate species such as mites and isopods. Testing the juvenile avoidance response with many other contaminants will also likely aid our understanding of how soil invertebrates detect contaminants in their environment. In this study, the avoidance response in only one soil type was assessed. An area of future studies related to this Manuscript could repeat this work but in soils with varying properties. Notably, soils with a range of organic matter contents would produce interesting results due to the influence of organic matter on exposure of organisms to contaminants. The influence of mixtures of contaminants on juvenile soil invertebrates behaviour is another possible future area of research related to Manuscript 4.

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9. Appendix A: Manuscript 1 Supplemental Material

Table A- 1. Summary of parameters utilized to estimate the critical body residue for species exposed to petroleum hydrocarbon contaminated soils for 28 days.

Description	Units	Value	Source
Molecular weight	g/mol	235	-
Henrys law constant (H)	unitless	61305	derived based on aromatic and aliphatic composition and CCME (2008)
log octanol water coefficient (k_{ow}) $=k_{oc}/0.41$	L/kg	12.4	-
log octanol carbon coefficient (log k_{oc})	L/kg	12	-
octanol carbon coefficient (k_{oc})	L/kg	1.07E+12	derived based on aromatic and aliphatic composition and CCME (2008)
Soil bulk density (p)	g/cm^3	2.4	-
Artificial soil organic matter (OM) content	%	6.2	Princz et al 2010
Artificial soil organic carbon (OC) content (=0.56* OM)	%	3.4	-
elimination rate (k)	1/d	-0.1955	Cermak et al 2013 (arithmetic average between aliphatics and aromatics)
	Lipid content (wet weight)		
Lumbricus terrestris	%	0.012	Kraus et al 2000
Eisenia fetida	%	0.018	Wagman et al 2001
Folsomia candida	%	0.085	Holsmstrup et al 2002
Hypoaspis aculeifer	%	0.13	Convey (1992)
Oppia nitens	%	0.13	Convey (1992)[a]
Enchytraeid crypticus	%	0.047	Rodriguez and Verdonschot (2001)

[a] no available literature found with lipid content of predatory mites, assumed same as oribatid mites

Table A- 2. Summary of caclultaed fugacity values utilized to estimate the critical body residue for species exposed to petroleum hydrocarbon contaminated soils for 28 days.

Description	Units	Value	Source
Water fugacity capacity (Z_{water}) =1/H	mol/m^3Pa	1.63E-05	-
Soil fugacity capacity (Z_{soil}) = OC*p*K_{oc}*Z_{water}	mol/m^3Pa	1.46E+08	-
Organism fugacity capacity ($Z_{organism}$) =(lipid content*k_{ow})/H			
Lumbricus terrestris	mol/m^3Pa	5.12E+05	-
Eisenia fetida	mol/m^3Pa	7.68E+05	-
Folsomia candida	mol/m^3Pa	3.63E+06	-
Hypoaspis aculeifer	mol/m^3Pa	5.55E+06	-
Oppia nitens	mol/m^3Pa	5.55E+06	-
Enchytraeid crypticus	mol/m^3Pa	2.00E+06	-
Concentration in organism ($C_{organism}$) =C_{soil}*($Z_{organism}$/Z_{soil})+$C_{soil\ water}$ ($Z_{organism}$/Z_{water})			
Lumbricus terrestris	mmol/kg	0.05	-
Eisenia fetida	mmol/kg	0.11	-
Folsomia candida	mmol/kg	0.53	-
Hypoaspis aculeifer	mmol/kg	1.40	-
Oppia nitens	mmol/kg	1.94	-
Enchytraeid crypticus	mmol/kg	21.8	-
Concentration in organism following first order elimination after 28 days ($C_{organism}$) =C_0*e^{-kt}			
Lumbricus terrestris	mmol/kg	0.00020	-
Eisenia fetida	mmol/kg	0.00047	-
Folsomia candida	mmol/kg	0.00223	-
Hypoaspis aculeifer	mmol/kg	0.00588	-
Oppia nitens	mmol/kg	0.00813	-
Enchytraeid crypticus	mmol/kg	0.0913	-

Table A- 3. Summary of individual toxicity endpoint values utilized for predicted mixture response from independent action and concentration additon, estimated from arithmetic average of literature values or regulations.

Organism	Test type	F2 LC/ EC50	F3 LC/ EC50	Rationale	Source
Enchytraeid crypticus	reproduction	260	2100	ESSD50, no single distillates toxicity tests available	CCME 2008
Eisena fetida	mortality	451	3830	arithmetic average from CWS CCME and literature	CCME 2008, Erlacher et al 2013, Cermak et al 2013, Cermak et al 2010; ESG 2003
Lumbricus terrestris	mortality	451	3830	arithmetic average from CWS CCME and literature	
Folsomia candida	reproduction	485	2652	no single distillates toxicity tests available for F3, used half of the F3 value for mortality (Cermak et al 2010)	Cermak et al 2013, Cermak et al 2010
Folsomia candida	mortality	970	5305	no single distillates toxicity tests available for F2, used twice F2 value for reproduction (Cermak et al 2010)	
Hypoaspis aculeifer	reproduction	260	2100	ESSD50, no single distillates toxicity tests available	CCME 2008
Hypoaspis aculeifer	mortality	520	4200	double the CWS CCME ESSD50 to account for higher EC values for mortality; no single distillates toxicity tests available	
Oppia nitens	reproduction	260	2100	ESSD50, no single distillates toxicity tests available	
Oppia nitens	mortality	520	4200	double the CWS CCME ESSD50 to account for higher EC values for mortality; no single distillates toxicity tests available	

ESSD50= 50th percentile of estimated species sensitivty distribution of the soil invertebrate data used by CCME (2008);

Table A- 4. Summary of individual F2 PHC literature values or regulations utilized used in averages (Table S-3) for predicting mixture response from independent action and concentration additon.

Test Species	Test type	LC/IC 50	LC/IC 25 or 20	Reference	PHC single distillates or mixture
Dendrobaena hortensis	mortalityality	1000	nd	Erlacher et al 2013	distillates
Eisenia andrei	7 d mortality	580	448	CCME (2008))	distillates
Eisenia andrei	14 d mortality	580	402	CCME (2008)	distillates
Eisenia andrei	60 d repro	161	115		distillates
Eisenia andrei	60 d progeny wet weight	194	138	CCME (2008); ESG (2003)	distillates
Eisenia andrei	60 d progeny dry weight	191	132		distillates
Eisenia andrei	14 d mortality	237	211	CCME (2008)	distillates
Eisenia andrei	14 d mortality	270	240	CCME (2008)	distillates
Eisenia andrei	14 d mortality	303	287	CCME (2008)	distillates
Eisenia andrei	mortality	320	nd	Cermak et al 2013	distillates
Eisenia andrei	mortality	380	nd	Cermak et al 2013	distillates
Eisenia andrei	mortality-14d	390	350	Cermak et al 2010	distillates
Onychiurus folsomi	reproduction	485	211	CCME (2008)	distillates

Table A- 5. Summary of individual F3 PHC literature values or regulations utilized used in averages (Table S-3) for predicting mixture response from independent action and concentration additon.

Species	endpoint	LC/IC 50	LC/IC 25 or 20	Reference	PHC single distillates or mixture
Dendrobaena hortensis	mortality	5596	4846	Erlacher et al 2013	Distillates (F3)
Eisenia andrei	mortality-28d	8630	6780	Cermak et al 2010	Distillates (F3)
Eisenia andrei	reproductio n	740	350	Cermak et al 2010	Distillates (F3)
Eisenia andrei	juv growth	830	230	Cermak et al 2010	Distillates (F3)
Eisenia andrei	14 d mortality	2500	2500	CCME (2008)	Distillates (F3)
Eisenia andrei	14 d mortality	2500	1000	CCME (2008)	Distillates (F3)
Field invertebrates	chronic	3100	3100	CCME (2008)	crude oil
Field invertebrates	chronic	2500	1300	CCME (2008)	crude oil
Field invertebrates	chronic	3400	2300	CCME (2008)	crude oil
Onychiurus folsomi	7 d mortality	8280	3230	Cermak et al 2010	Distillates (F3)
Onychiurus folsomi	35 d mortality	2330	nd	Cermak et al 2010	Distillates (F3)
Eisenia andrei	mortality	2490	nd	Cermak et al 2013	Distillates (F3a)
Eisenia andrei	mortality	3120	nd	Cermak et al 2013	Distillates (F3a)
Eisenia andrei	mortality	2440	nd	Cermak et al 2013	Distillates (F3a)
Eisenia andrei	mortality	3360	nd	Cermak et al 2013	Distillates (F3a)

nd=not determined

Table A- 6. Summary of key traits and metrics used explaining the interspecies sensitivity in mortality and reproduction observed in standardized laboratory species exposed to PHC contaminated soils, adapted from Van Straalen (1994) and Rubach et al 2011). LT represents *Lumbricus terrestris,* HA represent *Hypoaspis aculeifer*, FC represent *Folsomia candida*, ON represents *Oppia nitens,* EF represents *Eisenia feida* and EC represents *Enchytraeid crypticus*.

Main Trait Category	Sub category	Trait	Options	EF	LT	FC	ON	HA	EC
External exposure		soil ingestion	yes/no	yes	yes	no	no	no	no
		body size	small (<0.5 cm), large (>0.5 cm)	large	large	small	small	small	small
	Dermal Absorption	permeability/gaps of integument	low/high	high	high	high	low	low	high
	Bioaccumulation	Lipid content	%	1.8	1.2	8.5	13	13	4.7
		Molting	yes/no	no	no	yes	no	no	no
		Maternal transfer	yes/no	no	no	yes	yes	yes	yes
	Internal distribution	Integument sequestration	yes/no	yes	yes	yes	no	no	yes
Demography	Life history characteristics	Life span	short (< 6 months), long (>6 months)	long	long	short	long	long	long
		Clutch size[a]	juvenile:adult	1.5	1.5	10	5	5	2.50

[a]for a standardized approached used ratio of mean juvenile production to adults from test validity criteria in negative controls

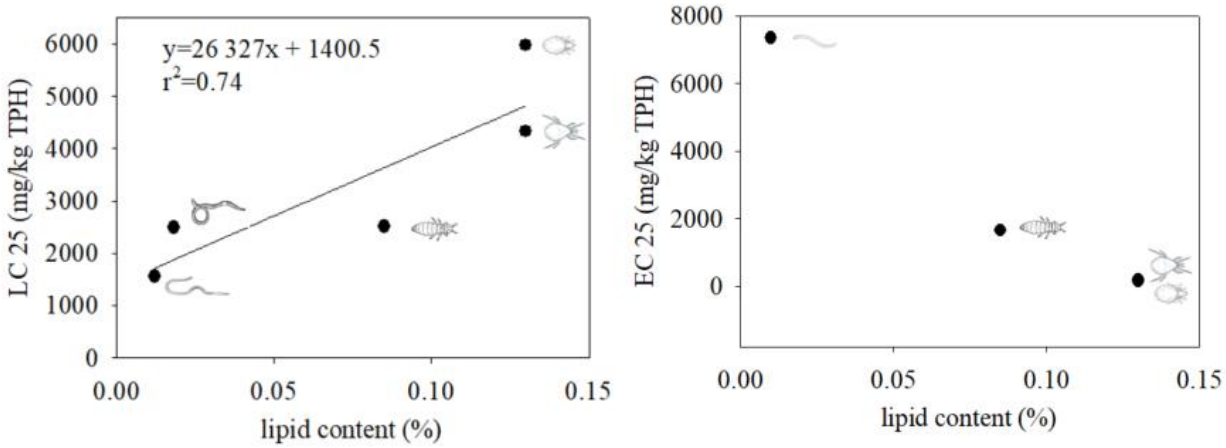

Figure A-1. Linear regression between organism lipid content for *Lumbricus terrestris, Eisenia fetida, Folsomia candida, Hypoaspis aculeifer* and *Oppia nitens,* and lethal concentration (LC) or effective concentration (EC) 25 from exposure to a petroleum hydrocarbon mixture contaminated soils.

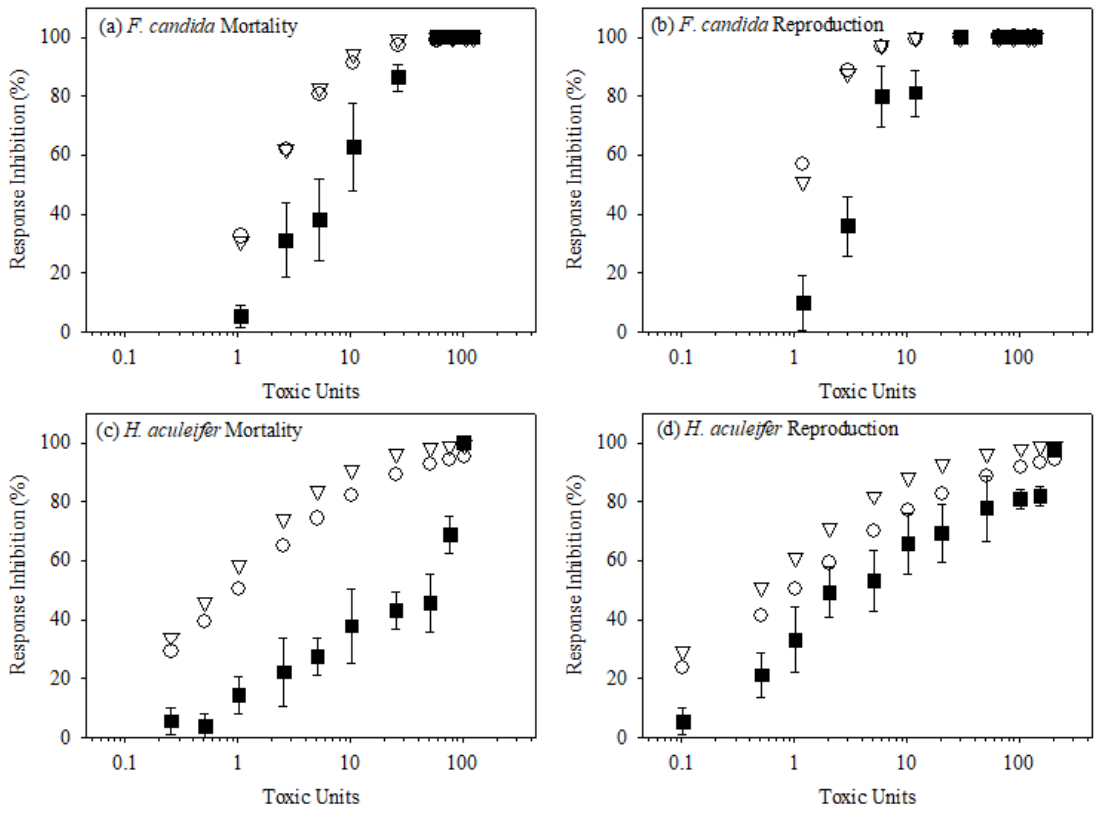

Figure A-2. *Folsomia candida* dose response data for mortality (a) and reproduction (b), and *Hypoaspis acculeifer* dose response data for mortality (c) and reproduction (d), as a function of toxic units, exposed to petroleum hydrocarbon mixture contaminated soils for 28 days. Solid squares represent average observed responses from the mixture with standard deviation intervals, open circles represent predicted concentration addition mixture toxicity from and open triangles represent predicted independent action mixture toxicity.

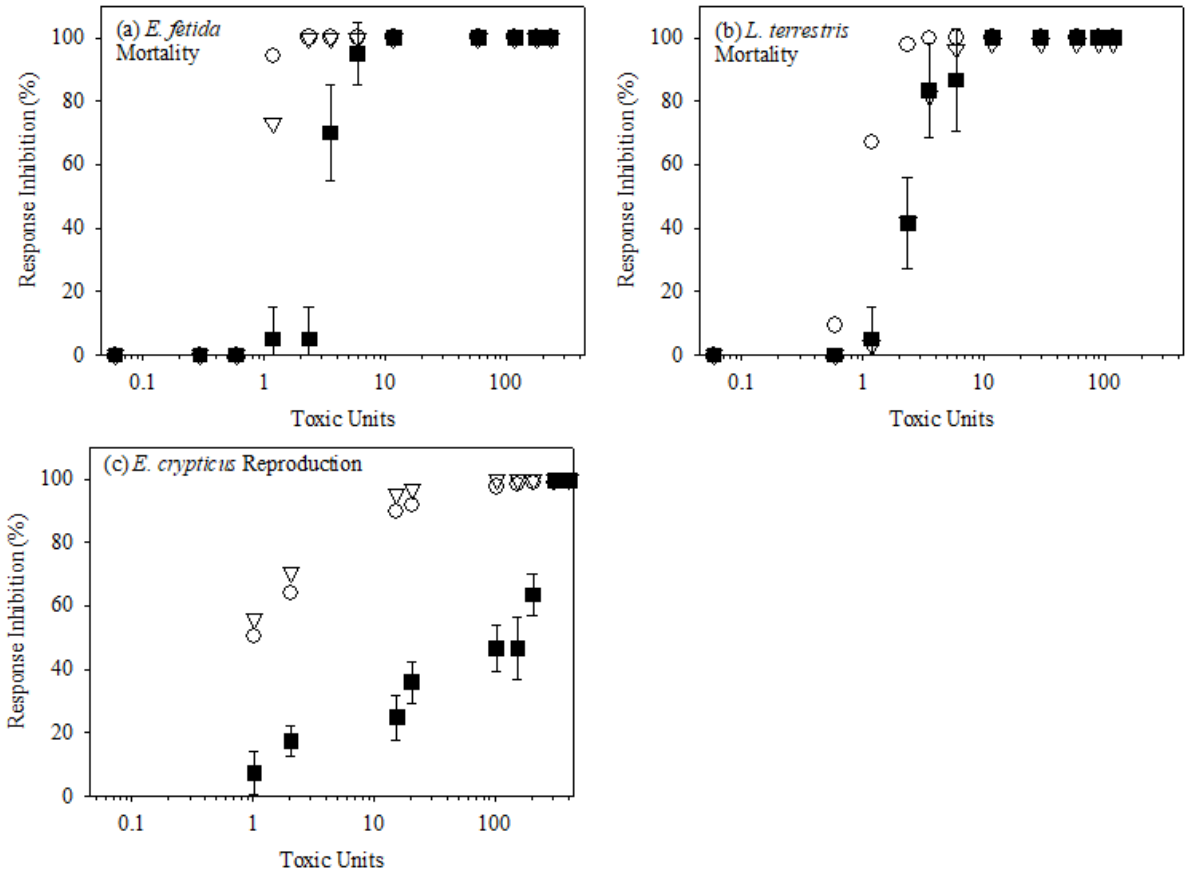

Figure A-3. *Eisena fetida* dose response data for mortality (a), *Lumbricus terrestris* dose response data for mortality (b) and *Enchytreaus crypticus* dose response data for reproduction (c) as a function of toxic units exposed to petroleum hydrocarbon mixture contaminated soils. Solid squares represent average observed responses from the mixture with standard deviation intervals, open circles represent predicted concentration addition mixture toxicity and open triangles represent predicted independent action mixture toxicity.

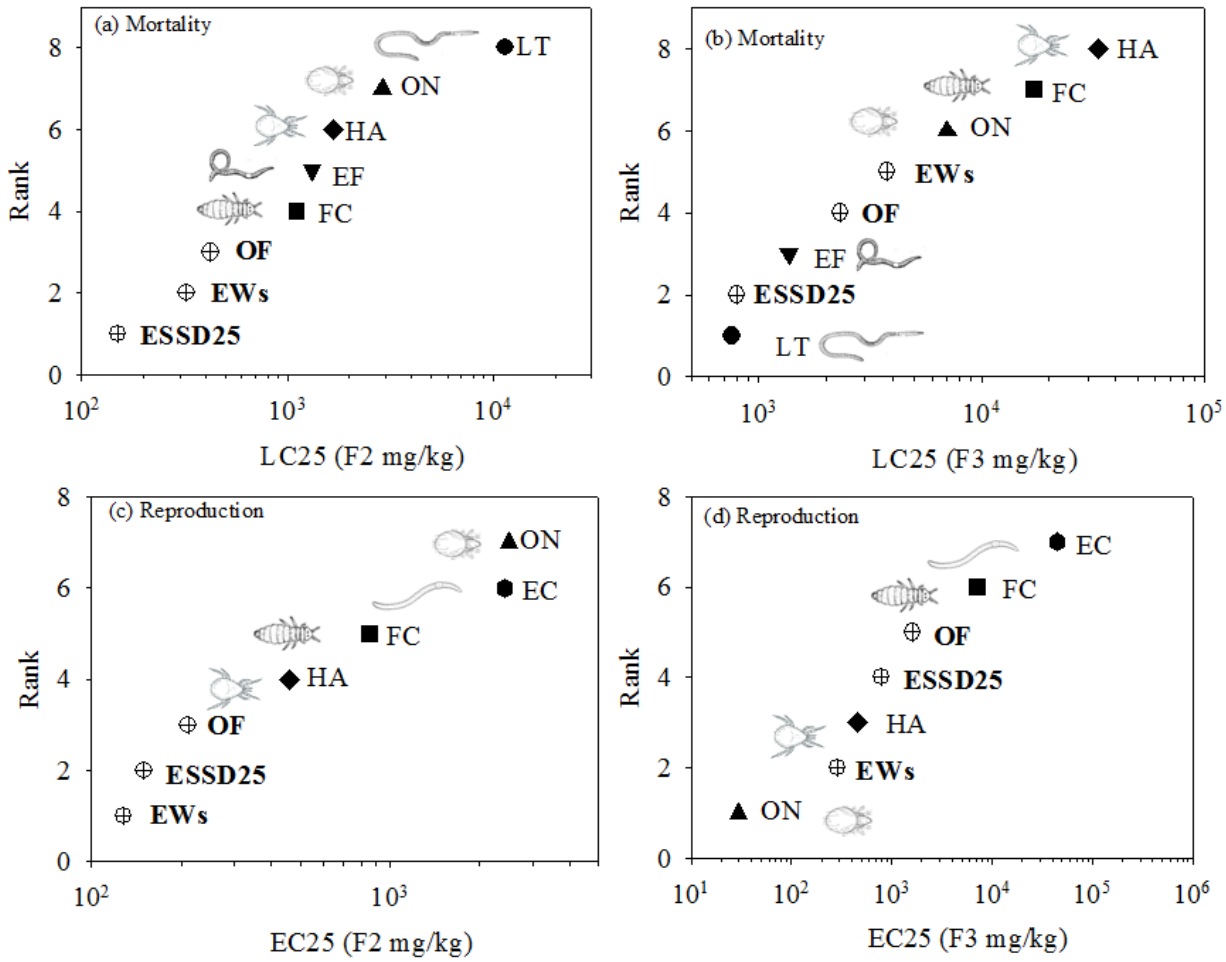

Figure A-4. Rank of individual petroleum hydrocarbon F2 (a, c) and F3 (b. d) lethal concentration (LC) for mortality and effective concentration (EC) 25 for reproduction predicted from an optimized independent action model fit as function of LC or EC25 predicted from an optimized independent action model fit, from most sensitive (1) to least. Empty circles with crosses and bold font indicates value from average of available literature or regulatory soil invertebrate ecological soil contact value for single F2 distillates. Solid circles represent *Lumbricus terrestris* (LT), solid diamonds represent *Hypoaspis aculeifer* (HA), solid squares represent *Folsomia candida* (FC), solid triangles represent *Oppia nitens* (ON), inverted solid triangles represent *Eisenia feida* (EF) and solid hexagons represent *Enchytraeus crypticus* (EC). ESSD25 represents the 25th percentile of estimated species sensitivity distribution of the soil invertebrate data used by CCME (2008) and EW represents average toxicity value for earthworms from literature.

Equations

Hillslope Equation

$$y = \text{bottom} + \frac{top-bottom}{1+10^{(logEC50-X)\,slope}} \quad \text{[A-1]}$$

Where,

top=maximum response

bottom=minimum response

Fugacity Equations

$$C_{organism} = \left[C_{soil}\left(\frac{Z_{organisms}}{Z_{soil}}\right) + C_{soil\ water}\left(\frac{Z_{organism}}{Z_{water}}\right)\right] \quad \text{[A-2]}$$

$$C_t = C_0 e^{-kt} \quad \text{[A-3]}$$

Mixture Toxicity Equations

$$\frac{x_{F2}}{EC50_{F2}\left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{F2}}}} + \frac{x_{F3}}{EC50_{F3}\left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{F3}}}} = 1 \quad \text{[A-4]}$$

$$y = 100\left(\frac{1}{1+\left(\frac{x_{F2}}{EC50_{F2}}\right)^{\beta_{F2}}}\right)\left(\frac{1}{1+\left(\frac{x_{F3}}{EC50_{F3}}\right)^{\beta_{F3}}}\right) \quad \text{[A-5]}$$

where,

y=inhibition dose response

x_{Fi} = concentration of individual petroleum hydrocarbon fraction

$\beta_{Fi=}$ individual F2 and F3 slopes (Table 2)

Equation for deriving EC25 from EC50

$$EC_{25} = \left(EC_{50}\frac{maximum\ response-25}{25}\right)^{\frac{1}{slope}} \quad \text{[A-6]}$$

10. Appendix B: Manuscript 2 Supplemental Material

Table B- 1. The chamber dimensions, amount of soil per side, duration and counting methods for soil invertebrate avoidance tests.

Test Species	Container dimensions (cm)	Amount of Soil per Side (g dw)	Duration (hr)	Specimen Extraction	Protocol Source
Eisenia fetida	l=20 w=12 h=5	250	48	Manual counting	ISO 2008
Folsomia candida	d=7 h=6	30	48	Flotation with black ink	ISO 2011
Enchytraeus crypticus	d=8.5 h=4	20	48	Preserve and stain with 70% alcohol and Bengal red; Manual count by wet sieving	Amorim et al 2008 Kobeticova et al 2010
Oppia nitens	d=4.5 cm h=5 cm	15	24	Heat extracted with modified Tullgren apparatus over two days (room temperature for 24 hours then 25˚C for 24 hours)	Owojori et al 2011
Hypoaspsi aculeifer	d=5.5 cm h=7 cm	10	48	Heat extracted with modified Tullgren apparatus over three days (room temperature for 24 hours, 25˚C for 24 hours and 27˚C for 24 hours)	Owojori et al 2014

l=length; w=width; h-height; d=diameter; dw=dry weight

Table B- 2. Summary of individual toxicity endpoint values utilized for predicted mixture response from independent action and concentration additon, estimated from arithmetic average of literature values or regulations.

Species	Endpoint	PHC	Average EC25/20	Average EC50	Rationale
Ms	RL	F2	239	613	used available data for same species
Ms	root dw	F2	495	1689	used available data for same species
Ms	shoot dw	F2	204	452	used available data for same species
Ms	SL	F2	404.5	894	used available data for same species
Ms	Em	F2	2463	4926	used available data for same species
Ms	RL	F3	920	6300	used available data for same species
Ms	root dw	F3	1100	5500	used available data for same species
Ms	shoot dw	F3	620	2300	used available data for same species
Ms	SL	F3	620	8300	used available data for same species
Ms	Em	F3	43750	87500	no species specific data available; used EL emergence as only F3 emergence data available
El	root dw	F2	176	452	used available data for same species
El	shoot dw	F2	249	1039	used available data for same species
El	RL	F2	190	765	used available data for same species
El	SL	F2	869.5	2455	used available data for same species
El	Em	F2	1913	3826	used available data for same species
El	root dw	F3	627.5	2595	used available data for same species
El	shoot dw	F3	515	2145	used available data for same species
El	RL	F3	6340	21515	used available data for same species
El	SL	F3	1760	13250	used available data for same species
El	Em	F3	63455.5	87500	no species specific data available; used EL emergence as only F3 emergence data available
Ls/Rs/Pb/Pg	root dw	F2	364.0	965.1	no species specific data available; used average of all plants (EL, MS, Hv) with available data for F2 and F3 (except F3 emergence, used EL)
Ls/Rs/Pb/Pg	shoot dw	F2	237.3	719.6	no species specific data available; used average of all plants (EL, MS, Hv) with available data for F2 and F3 (except F3 emergence, used EL)

Ls/Rs/Pb/Pg	RL	F2	307.1	868.8	no species specific data available; used average of all plants (EL, MS, Hv) with available data for F2 and F3 (except F3 emergence, used EL)
Ls/Rs/Pb/Pg	SL	F2	631.4	1584.8	no species specific data available; used average of all plants (EL, MS, Hv) with available data for F2 and F3 (except F3 emergence, used EL)
Ls/Rs/Pb/Pg	Em	F2	3243.3	6486.7	no species specific data available; used average of all plants (EL, MS, Hv) with available data for F2 and F3 (except F3 emergence, used EL)
Ls/Rs/Pb/Pg	root dw	F3	3909.2	14398.3	no species specific data available; used average of all plants (EL, MS, Hv) with available data for F2 and F3 (except F3 emergence, used EL)
Ls/Rs/Pb/Pg	shoot dw	F3	16611.7	19248.3	no species specific data available; used average of all plants (EL, MS, Hv) with available data for F2 and F3 (except F3 emergence, used EL)
Ls/Rs/Pb/Pg	RL	F3	2460.0	10338.3	no species specific data available; used average of all plants (EL, MS, Hv) with available data for F2 and F3 (except F3 emergence, used EL)
Ls/Rs/Pb/Pg	SL	F3	2026.7	16383.3	no species specific data available; used average of all plants (EL, MS, Hv) with available data for F2 and F3 (except F3 emergence, used EL)
Ls/Rs/Pb/Pg	Em	F3	43750	87500	no species specific data available; used average of all plants (EL, MS, Hv) with available data for F2 and F3 (except F3 emergence, used EL)

Ms=*Medicago sativa;* El=*Elymus lanceolatus*; Ls/Rs/Pb/Pg= *Lactuca sativa/ Raphanus sativus/ Pinus banksiana/ Picea glauca;;* Hv=*Hordeum vulgare*; Em=emergence; RL=root length; SL=shoot length; dw=dry weight

Table B- 3. Summary of individual F2 PHC literature values or regulations utilized used in averages (Table S-1) for predicting mixture response from independent action and concentration additon.

Species	Endpoint	PHC type	EC25/20	EC50	reference
El	Em	F2	1186		Agnell et al 2012
El	SL	F2	647		Agnell et al 2012
El	RL	F2	295		Agnell et al 2012
El	SD	F2	198		Agnell et al 2012
El	RD	F2	290		Agnell et al 2012
Ms	Em	F2	966		Agnell et al 2012
Ms	SL	F2	354		Agnell et al 2012
Ms	RL	F2	257		Agnell et al 2012
Ms	SD	F2	264		Agnell et al 2012
Ms	RD	F2	226		Agnell et al 2012
Hv	Em	F2	6748		Agnell et al 2012
Hv	SL	F2	1199		Agnell et al 2012
Hv	RL	F2	389		Agnell et al 2012
Hv	SD	F2	262		Agnell et al 2012
Hv	RD	F2	493		Agnell et al 2012
Ms	SL	F2	455	894	CCME 2008/ESG2003
Ms	RL	F2	221	613	CCME 2008/ESG2003
Ms	shoot dw	F2	145	452	CCME 2008/ESG2003
Ms	root dw	F2	765	1689	CCME 2008/ESG2003
Hv	SL	F2	445	1362	CCME 2008/ESG2003
Hv	RL	F2	630	1501	CCME 2008/ESG2003
Hv	shoot dw	F2	320	845	CCME 2008/ESG2003
Hv	root dw	F2	392	828	CCME 2008/ESG2003
El	SL	F2	1092	2455	CCME 2008/ESG2003
El	RL	F2	85	765	CCME 2008/ESG2003
El	shoot dw	F2	300	1039	CCME 2008/ESG2003
El	root dw	F2	62	452	CCME 2008/ESG2003
Hv	SL	F2	382	1369	CCME 2008

Hv	RL	F2	653	1320	CCME 2008
Hv	shoot dw	F2	250	663	CCME 2008
Hv	root dw	F2	356	858	CCME 2008
Hv	SL	F2	455	1485	CCME 2008
Hv	RL	F2	297	864	CCME 2008
Hv	shoot dw	F2	198	495	CCME 2008
Hv	root dw	F2	300	577	CCME 2008
Ms	Em	F2	3960 LOEC		CCME 2008/ESG2003
Hv	Em	F2	3960 LOEC		CCME 2008/ESG2003
El	Em	F2	2640 LOEC		CCME 2008/ESG2003

Ms=*Medicago sativa;* El=*Elymus lanceolatus*; Hv=*Hordeum vulgare;* Em=emergence; RL=root length; SL=shoot length; dw=dry weight

Table B- 4. Summary of individual F3 PHC literature values or regulations utilized used in averages (Table S-1) for predicting mixture response from independent action and concentration additon.

Species	Endpoint	PHC type	EC25/20	EC50	reference
El	SL	F3	3190	13800	Cermak et al 2010
El	SD	F3	980	2890	Cermak et al 2010
El	RL	F3	8380	35730	Cermak et al 2010
El	RD	F3	1075	4300	Cermak et al 2010
El	Em	F3	38870-48630	LOAEC	Cermak et al 2010
Ms	SL	F3	620	8300	CCME 2008
Ms	RL	F3	920	6300	CCME 2008
Ms	shoot dw	F3	620	2300	CCME 2008
Ms	root dw	F3	1100	5500	CCME 2008
Hv	SL	F3	3700	27600	CCME 2008
Hv	RL	F3	120	3200	CCME 2008
Hv	shoot dw	F3	48700	53300	CCME 2008
Hv	root dw	F3	10000	35100	CCME 2008
El	SL	F3	330	12700	CCME 2008
El	RL	F3	4300	7300	CCME 2008
El	shoot dw	F3	50	1400	CCME 2008
El	root dw	F3	180	890	CCME 2008

Ms=*Medicago sativa;* El=*Elymus lanceolatus*; Hv=*Hordeum vulgare;* Em=emergence; RL=root length; SL=shoot length; dw=dry weight

Table B- 5. The sum of squares from perfect model fitting to reference models, individual PHC fraction toxicity values from perfect model fit to reference models and average residuals between observed response in plants (*Raphanus sativus, Elymus laceolatus, Medicago sativa, Pinus banksiana, Lactuca sativa* and *Picea glauca)* exposed to PHC contaminated soils and expected response from imperfect model fit to reference models.

Species	Endpoint	Perfect Model Fit Sum of Squares		Predicted Individual PHC EC25				Average Residuals between Observed and Expected	
				CA		IA			
		CA	IA	F2	F3	F2	F3	CA	IA
Raphanus sativus	Emergence	4,575	4,144	200,380	102,435	70,995	87,972	-42	-38
	Root length	2,950	5,769	2,553	3,108	1,156	102,691	-15	-20
	Shoot length	3,722	2,478	4,767	7,804	95,415	2,580	-13	-21
	Root dry mass	3,400	7,189	790	11,997	698	8,201	-8	-10
	Shoot dry mass	3,211	3,351	13,329	89,771	10,481	78,998	-33	-33
Elymus lanceolatus	Emergence	7,504	7,318	73,291	22,427	11,173	21,947	-10	-10
	Root length	12,934	2,902	5,526	22,472	3,884	22,406	-17	-19
	Shoot length	3,940	2,902	38,263	168	1,692	3,479	4	-3
	Root dry mass	7,422	5,682	235	22,241	11,530	243	-8	-12
	Shoot dry mass	4,868	4,972	192	23,327	1,215	1,214	-2	-8
Medicago sativa	Emergence	na	na	na	na	na	na	na	na
	Root length	13,100	4,158	110	102	387	443	3	-8
	Shoot length	3,415	3,453	158	135	738	749	0	-8
	Root dry mass	4,668	4,844	214	7,657	1,600	458	-6	-10
	Shoot dry mass	2,497	2,515	0	1,890	0	4,433	-6	-9
Lactuca sativa	Emergence	na	na	na	na	na	na	na	na
	Root length	3,101	3,206	312	36,805	327	34,157	-16	-22
	Shoot length	5,282	5,517	136	141	237	233	8	1

	Root dry mass	5,579	5,246	228	226	1.09E+11	173	10	10
	Shoot dry mass	6,068	6,468	103	105	163	162	5	0
Pinus banksiana	Emergence	4,427	2,240	39,789	62,450	27,355	401,003	-17	-17
	Root length	6,574	3,393	39,370	7,716	5,010	33,861	-33	-36
	Shoot length	na	na	na	na	na	na	na	na
	Root dry mass	5,091	4,770	3,052	28,026	4,750	31,064	-23	-26
	Shoot dry mass	5,806	5,564	37,064	5,958	2,738	28,439	-32	-34
Picea glauca	Emergence	3,133	2,860	25,606	26,426	14,235	1.21E+14	-11	-10
	Root length	24,005	3,281	11,856	34,810	36,703	14,889	-26	-22
	Shoot length	2,043	6,812	27,990	26,911	21,607	21,334	-23	-23
	Root dry mass	5,555	6,206	2,017	22,486	7,424	2,978	-12	-11
	Shoot dry mass	5,472	5,686	6,568	20,237	4,567	20,048	-21	-21

CA=concentration addition; IA=independent action; na=not applicable

Table B- 6. The sum of squares from perfect model fitting to reference models, individual PHC fraction toxicity values from perfect model fit to reference models and average residuals between observed response in soil invertebrates (*Eisenia fetida, Folsomia candida, Oppia nitens* and *Hypoaspis aculeifer)* avoidance to PHC contaminated soils and expected response from imperfect model fit to reference models.

Organism	Predicted Individual PHC EC25							
	Optimized Model Fit Sum of Squares		F2		F3		Average Residuals between Observed and Expected	
	CA	IA	CA	IA	CA	IA	CA	IA
Eisena fetida	31,899	21,876	3,588	814	578	1,665	-25.9	-27.8
Folsomia candida	10,505	20,860	446	0	4,380	1,781	-26.4	-27
Oppia nitens	8,481	8,509	730	648	1,346	1,108	2.3	2.3
Hypoaspis aculeifer	40,285	78,501	266	nd	1,377	nd	-52.9	-53.2

CA=concentration addition; IA=independent action; na=not applicable; nd=not determined, value too large

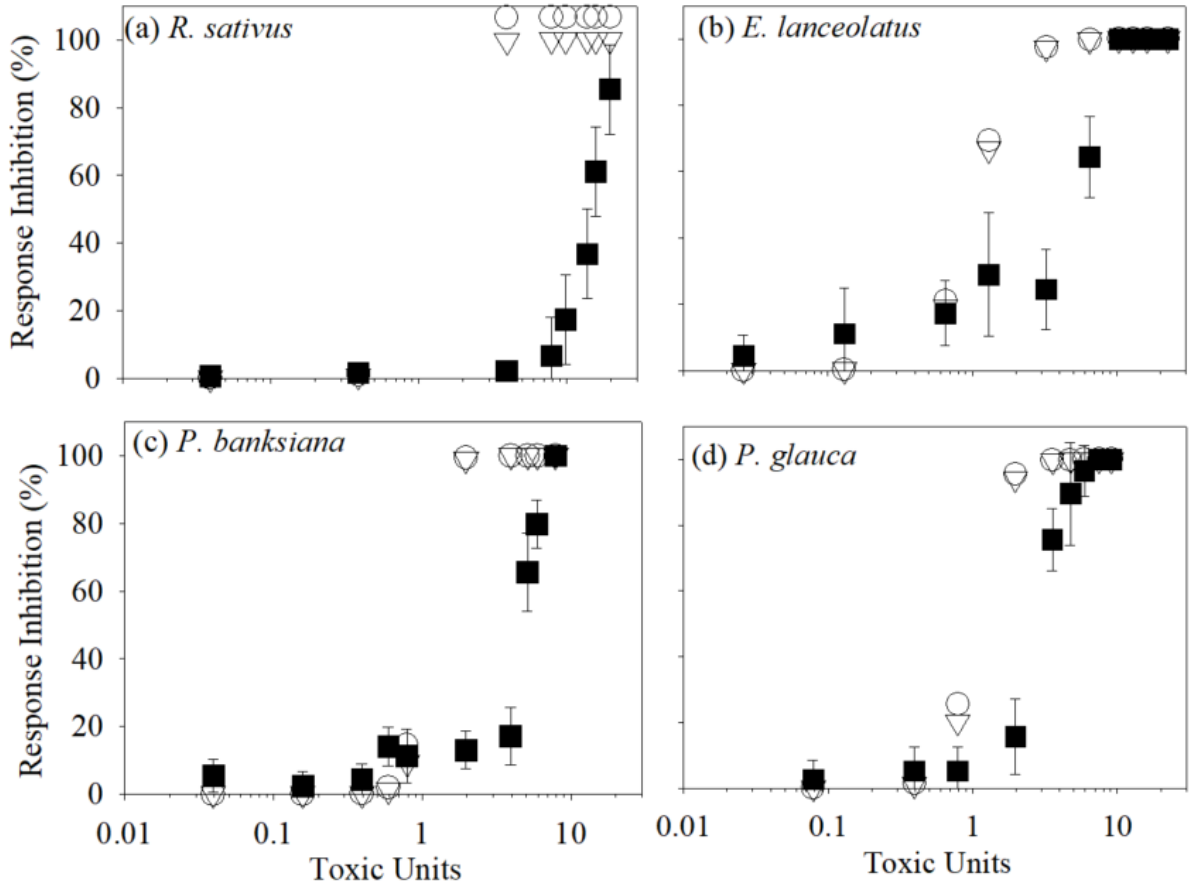

Figure B-1. Inhibition of emergence response data for (a) *Raphanus sativus,*(b) *Elymus laceolatus,* (c) *Pinus banksiana* and (d) *Picea glauca.* exposed to petroleum hydrocarbon mixture contaminated soils as a function of toxic units. Solid squares represent average observed responses from the mixture with standard deviation intervals, open circles represent imperfect predicted concentration addition mixture toxicity and open triangles represent imperfect predicted concentration addition mixture toxicity.

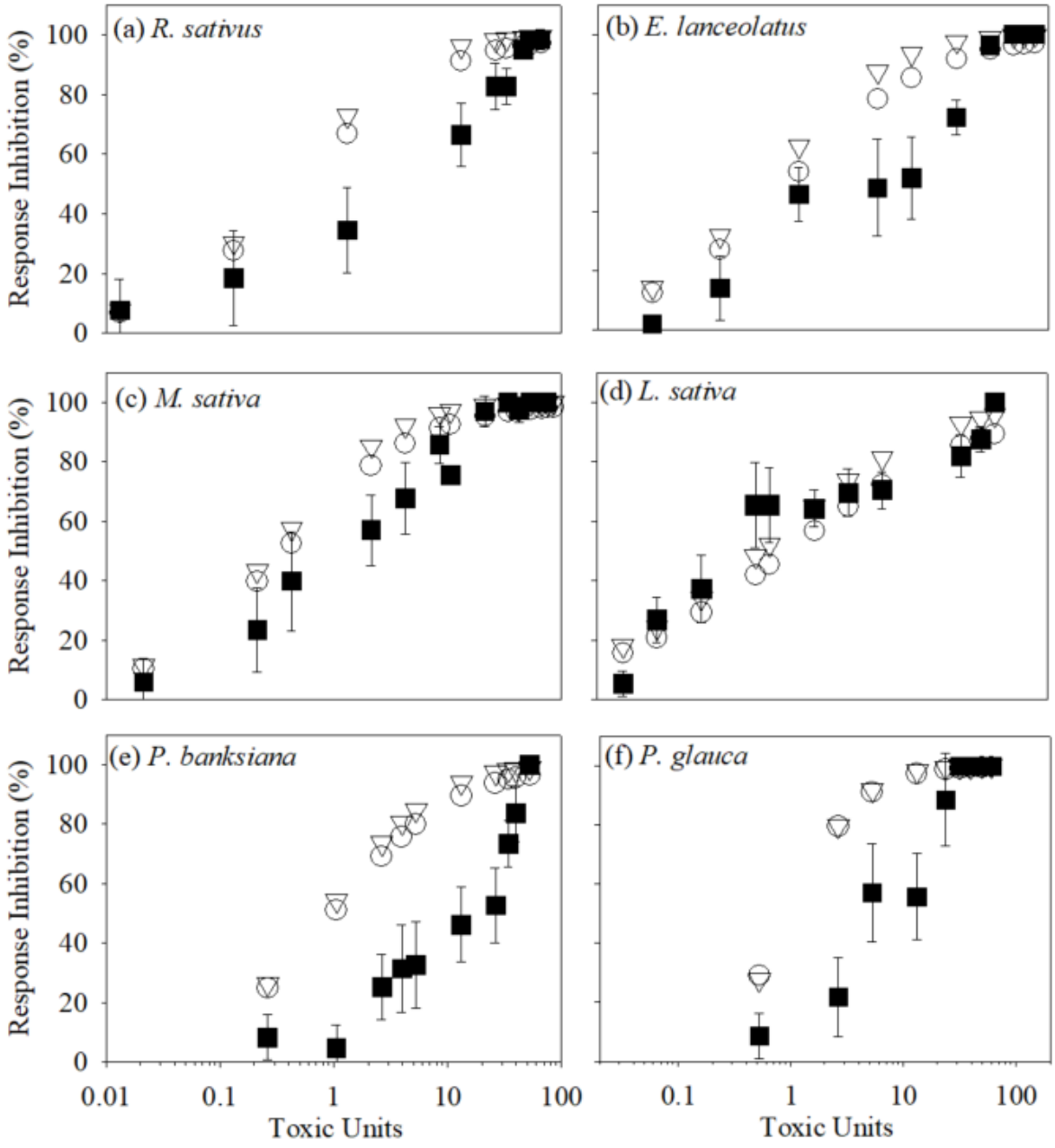

Figure B-2. Inhibition of root dry mass dose response data for (a) *Raphanus sativus,*(b) *Elymus laceolatus,* (c) *Medicago sativa,* (d) *Pinus banksiana,* (e) *Lactuca sativa* and (f) *Picea glauca.* exposed to petroleum hydrocarbon mixture contaminated soils as a function of toxic units. Solid squares represent average observed responses from the mixture with standard deviation intervals, open circles represent imperfect predicted concentration addition mixture toxicity and open triangles represent imperfect predicted concentration addition mixture toxicity.

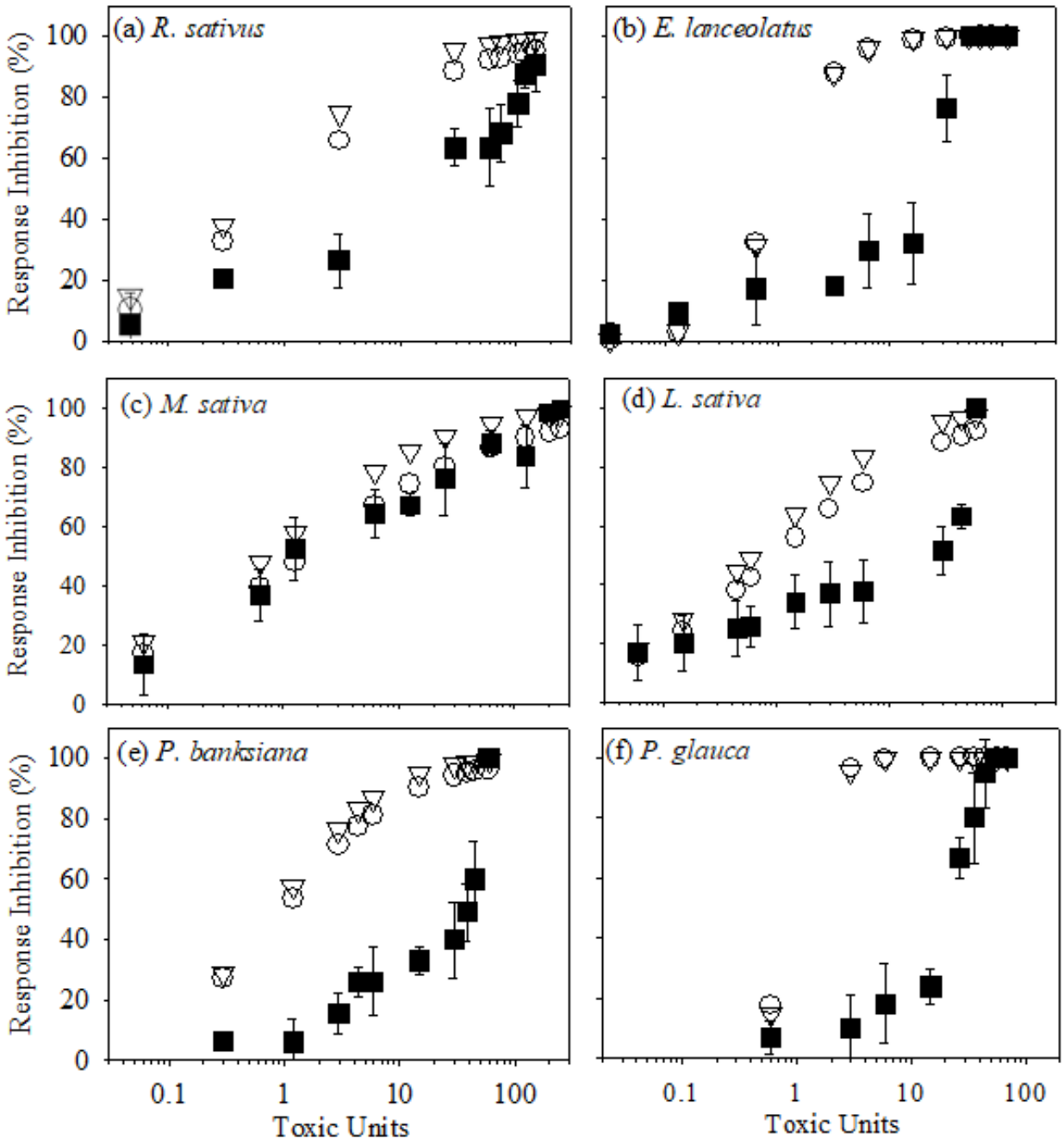

Figure B-3. Inhibition of root length dose response data for (a) *Raphanus sativus,*(b) *Elymus laceolatus,* (c) *Medicago sativa,* (d) *Pinus banksiana,* (e) *Lactuca sativa* and (f) *Picea glauca.* exposed to petroleum hydrocarbon mixture contaminated soils as a function of toxic units. Solid squares represent average observed responses from the mixture with standard deviation intervals, open circles represent imperfect predicted concentration addition mixture toxicity and open triangles represent imperfect predicted concentration addition mixture toxicity.

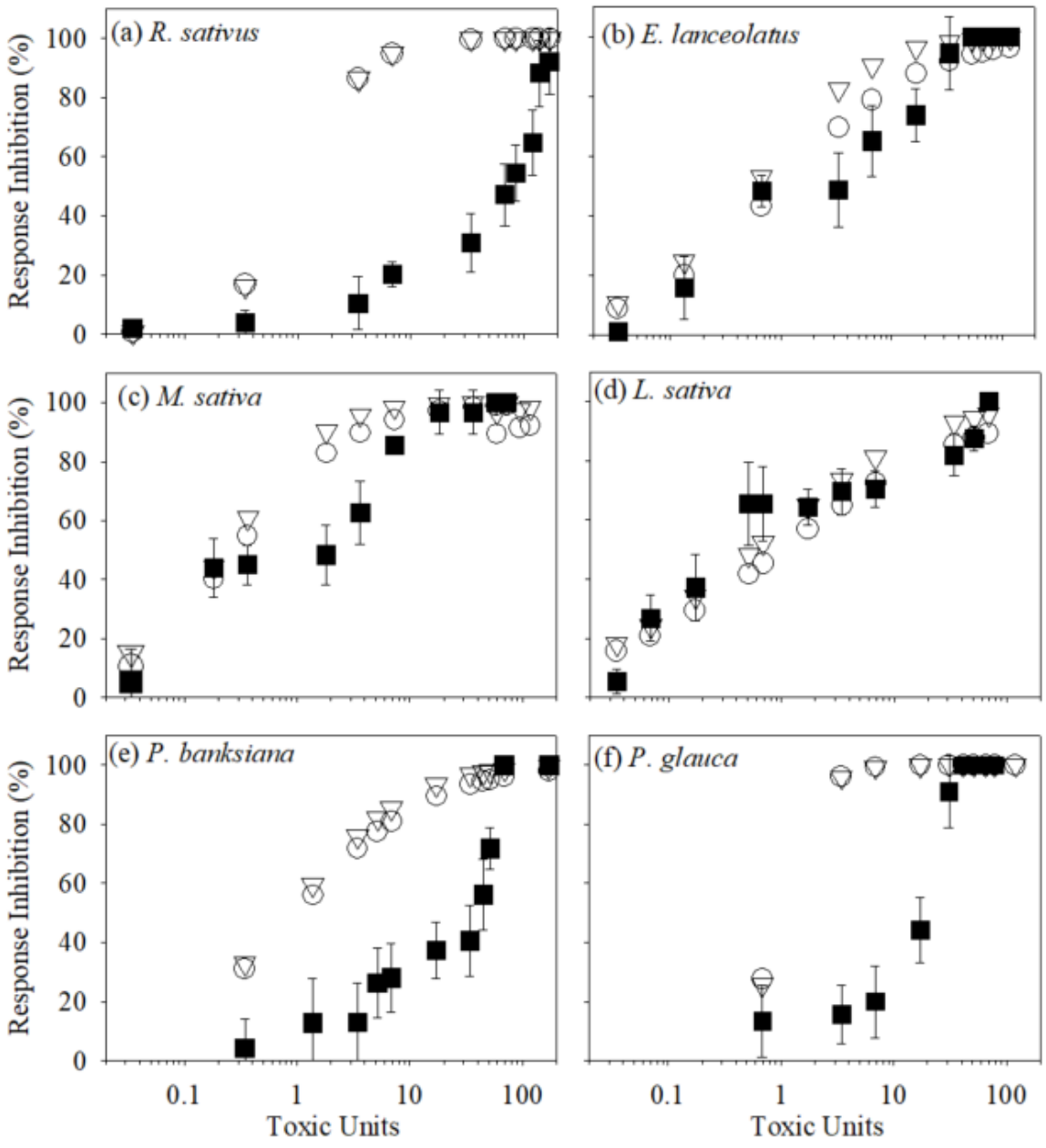

Figure B-4. Inhibition of shoot dry mass response data for (a) *Raphanus sativus,*(b) *Elymus laceolatus,* (c) *Medicago sativa,* (d) *Pinus banksiana,* (e) *Lactuca sativa* and (f) *Picea glauca.* exposed to petroleum hydrocarbon mixture contaminated soils as a function of toxic units. Solid squares represent average observed responses from the mixture with standard deviation intervals, open circles represent imperfect predicted concentration addition mixture toxicity and open triangles represent imperfect predicted concentration addition mixture toxicity.

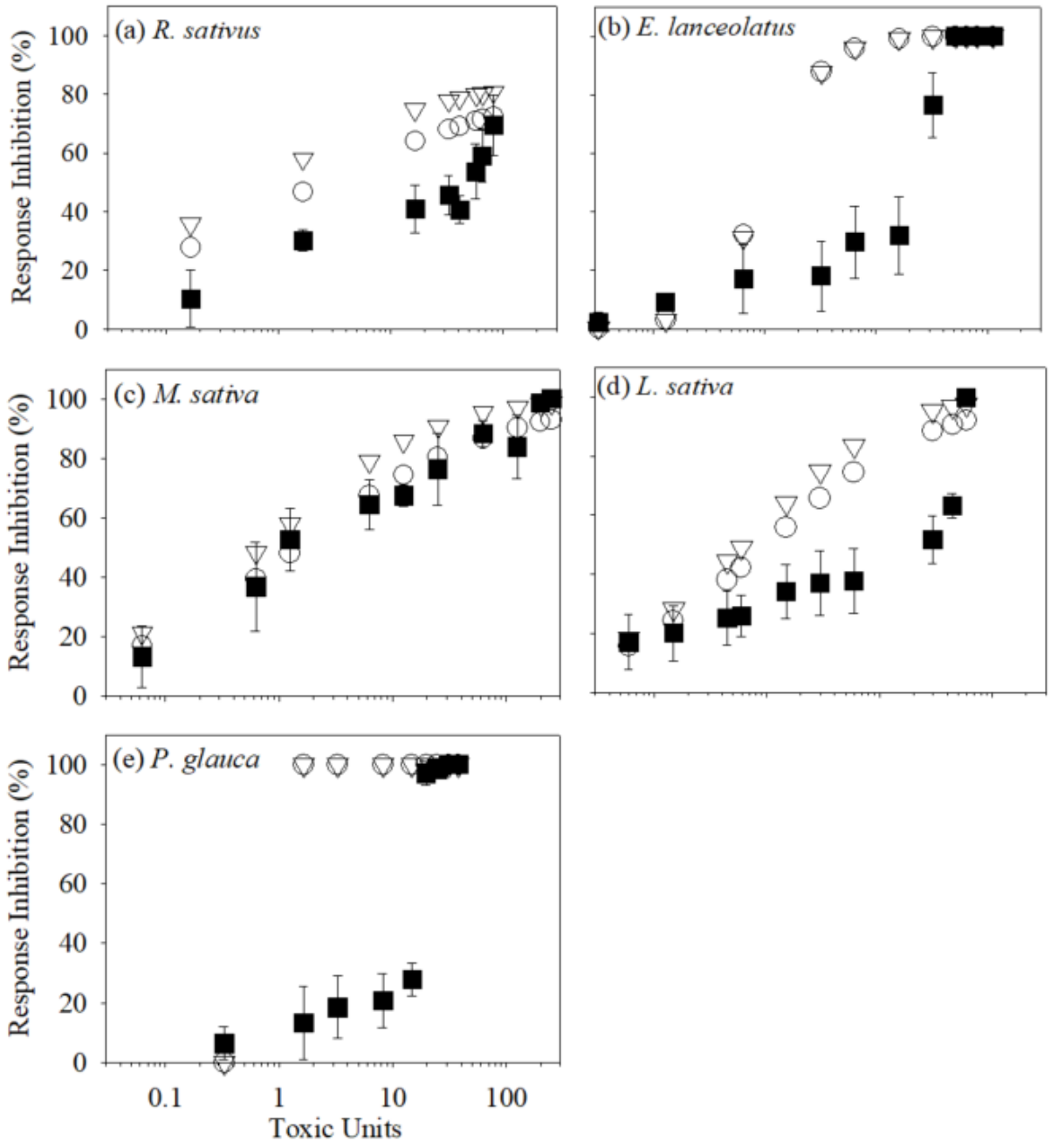

Figure B-5. Inhibition of shoot length dose response data for (a) *Raphanus sativus,*(b) *Elymus laceolatus,* (c) *Medicago sativa,* (d) *Lactuca sativa* and (E) *Picea glauca.* exposed to petroleum hydrocarbon mixture contaminated soils as a function of toxic units. Solid squares represent average observed responses from the mixture with standard deviation intervals, open circles represent imperfect predicted concentration addition mixture toxicity and open triangles represent imperfect predicted concentration addition mixture toxicity.

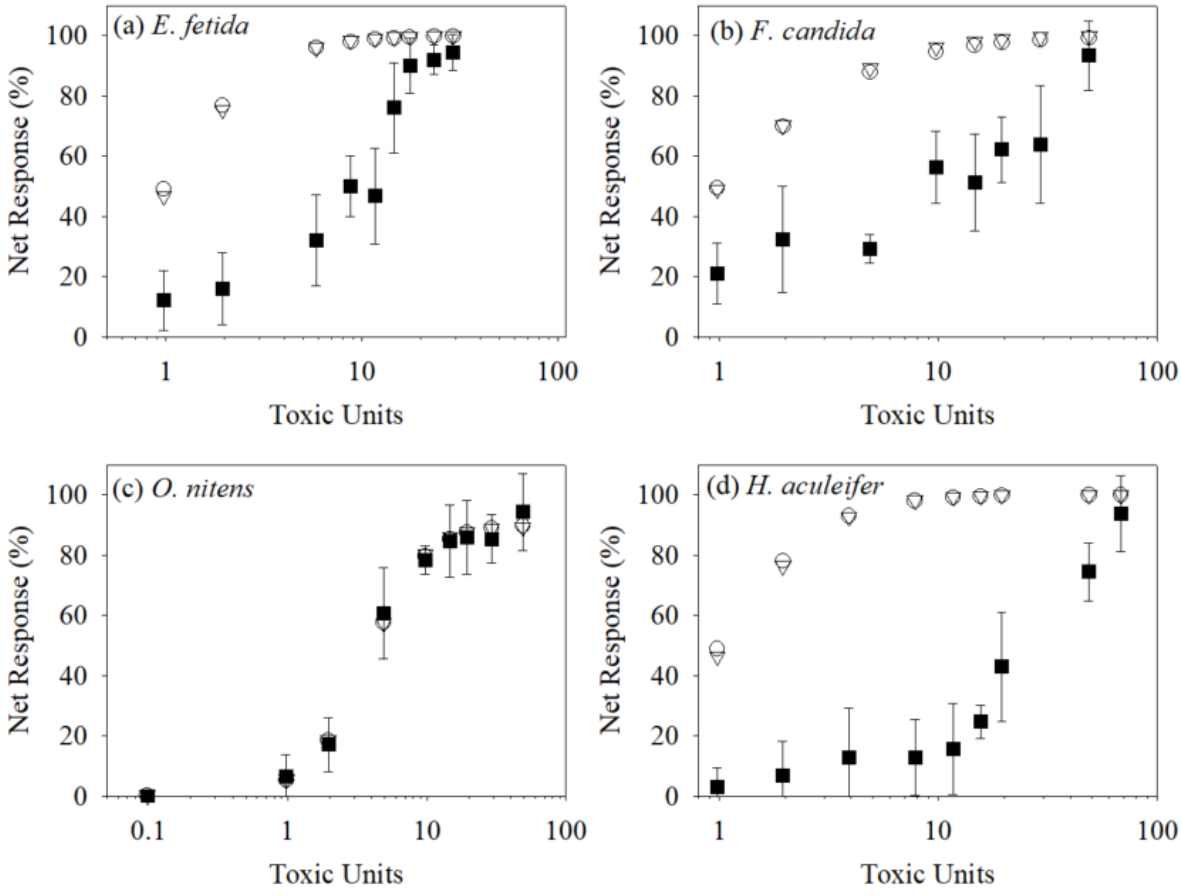

Figure B-6. Soil avoidance dose response data for (a) *Eisenia fetida,* (b) *Folsomia candida,* (c) *oppia nitens* and (d) *H. aculeifer* exposed to petroleum hydrocarbon mixture contaminated soils as a function of toxic units. Solid squares represent average observed responses from the mixture with standard deviation intervals, open circles represent imperfect predicted concentration addition mixture toxicity and open triangles represent imperfect predicted concentration addition mixture toxicity.

Equations

Hillslope Equation

$$y = \text{bottom} + \frac{top-bottom}{1+10^{(logEC50-X)\,slope}} \quad \text{[A-1]}$$

Mixture Toxicity Equations

$$\frac{x_{F2}}{EC50_{F2}\left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{F2}}}} + \frac{x_{F3}}{EC50_{F3}\left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{F3}}}} = 1 \quad \text{[A-2]}$$

$$y = 100\left(\frac{1}{1+\left(\frac{x_{F2}}{EC50_{F2}}\right)^{\beta_{F2}}}\right)\left(\frac{1}{1+\left(\frac{x_{F3}}{EC50_{F3}}\right)^{\beta_{F3}}}\right) \quad \text{[A-3]}$$

where,

y=inhibition dose response

x_{Fi} = concentration of individual petroleum hydrocarbon fraction

$\beta_{Fi=}$ individual F2 and F3 slopes (Table 2)

Equation for deriving EC25 from EC50

$$EC_{25} = \left(EC_{50}\frac{maximum\ response-25}{25}\right)^{\frac{1}{slope}} \quad \text{[A-4]}$$

11. Appendix C: Manuscript 3 Supplemental Material

Table C- 1. Aliphatic and aromatic percent composition of the lubricating oil used in toxicity tests and the Canada Wide Standards for Petroleum Hydrocarbons (CWS PHC) (CCME 2008) recommended composition for F2 and F3 petroleum hydrocarbons distillates for toxicity testing.

Fraction	Sub fraction	Branch type	% Composition (standard deviation)	
			Lubricating oil	CWS PHC
F2	>C10-12	aliphatic	8.6 (0.2)	36
		aromatic	2.0 (0.3)	9
		subtotal	11	45
	>C12-C16	aliphatic	78 (1.8)	44
		aromatic	12 (1.7)	11
		subtotal	90	55
		F2 total	100	100
F3	>C16-C21	aliphatic	53 (1.4)	56
		aromatic	20 (1.4)	14
		subtotal	73	70
	>C21-C34	aliphatic	22 (0.59)	24
		aromatic	5 (0.50)	6
		subtotal	27	30
		F3 total	100	100

CWS PHC=Canada Wide Standards for Petroleum Hydrocarbons (CCME 2008)

Table C- 2. Model fit of the general linear model using a Gaussian distribution and results for the influence of fertilizer application and cation exchange capacity on degradation rate constant.

Petroleum Hydrocarbon Fraction		Estimate	Standard error	T value	P value	
F2	intercept	0.024	0.0024	10.314	1.12E-09	***
	Fertilizer	-0.0056	0.0026	-2.12	0.04591	*
	CEC	-0.0005	0.00016	-3.44	0.00245	**
	AIC=-169; null deviance 0.0016 (23 df); residual deviance 0.00087 (21 df); pseudo R^2 =46%					
F3	intercept	0.022	0.0018	12	6.96E-11	***
	Fertilizer	-0.0052	0.002	-2.52	0.019	*
	CEC	-0.0004	0.00012	-3.554	0.001	**
	AIC=-181; null deviance 0.0010 (23 df); residual deviance 0.00053 (21 df); pseudo R^2 =47%					

CEC=cation exchange capacity; AIC= Akaike information criterion; df=degrees of freedom

Table C- 3. Summary of the first order degradation rate constants (k) (days^{-1}) for F2 and F3 Petroleum Hydrocarbon when fertilized (at a CNP ratio of 100:9:1) and unfertilized in field soils with a range of soil cation exchange capacities (CEC) (cmol/kg). Initial petroleum hydrocarbon concentrations for each soil was approximately 1400 mg/kg F2 and 1300 mg/kg F3.

Petroleum Hydrocarbon Fraction	Treatment	CEC (cmol/kg)	Average k (standard deviation) (days^{-1})	Average half life (standard deviation) (days)
F2	F	all soils	0.019 (0.0093)	45 (19)
	UF	all soils	0.015 (0.0067)	58 (28)
F3	F	all soils	0.018 (0.0072)	47 (19)
	UF	all soils	0.013 (0.0049)	60 (22)
F2	F and UF	Detection limit-15	0.021 (0.0098)	47 (24)
		18-28	0.012 (0.0025)	66 (19)
F3	F and UF	Detection limit-15	0.018 (0.0065)	43 (17)
		18-28	0.0099(0.0027)	74 (20)

Table C- 4. Summary of available literature on toxicity of medium petroleum hydrocarbons (diesel, motor oil and lubricating oil) to soil organisms and plants. The table includes a total of 43 individual endpoints from a total of four studies. For artificial soil, the total organic carbon content was the geometric mean of values from two studies (ESG 2003; Agnell et al 2012).

Species	Test Type	EC25 mg TPH/ kg soil	test soil fOM (%)	test soil fOC (%)	EC 25 OC normalized (mg/kg_{oc})	Source
Eisenia fetida	28 day mortality	3,880		2.3	168,696	Ramadass et al 2015
Eisenia fetida	28 day mortality	40,330		2.3	1,753,478	Ramadass et al 2015
Eisenia fetida	Avoidance	2,130		2.69	79,182	Gainer et al 2019
Folsomia candida	Avoidance	2,370		2.69	88,104	Gainer et al 2019
Hypoaspis aculeifer	Avoidance	7,110		2.69	264,312	Gainer et al 2019
Oppia nitens	Avoidance	1,140		2.69	42,379	Gainer et al 2019
Phaseolus vulgaris	fruit dry weight	6000	2.3	1	521,739	Chaineau et al 1997
Triticum aestivum	Height reduction, 40 days	3000	2.3	1	260,870	Chaineau et al 1997
Zea mays	Height reduction, 40 days	12000	2.3	1	1,043,478	Chaineau et al 1997
Zea mays	leaf dry weight	6000	2.3	1	521,739	Chaineau et al 1997
Eisenia fetida	mortality	2,493		2.69	92,677	Gainer et al 2018
Folsomia candida	mortality	2,514		2.69	93,457	Gainer et al 2018
Hypoaspis aculeifer	mortality	4,328		2.69	160,892	Gainer et al 2018
Lumbricus terrestris	mortality	1,558		2.69	57,918	Gainer et al 2018
Oppia nitens	mortality	5,982		2.69	222,379	Gainer et al 2018
Enchytraeus crypticus	reproduction	7,346		2.69	273,086	Gainer et al 2018
Folsomia candida	reproduction	1,658		2.69	61,636	Gainer et al 2018
Hypoaspis aculeifer	reproduction	206		2.69	7,658	Gainer et al 2018
Oppia nitens	reproduction	142		2.69	5,279	Gainer et al 2018
Elymus lanceolatus	Root dry mass	660		2.69	24,535	Gainer et al 2019
Lactuca sativa	Root dry mass	180		2.69	6,691	Gainer et al 2019
Medicago sativa	Root dry mass	550		2.69	20,446	Gainer et al 2019
Picea glauca	Root dry mass	5,080		2.69	188,848	Gainer et al 2019
Pinus banksiana	Root dry mass	6,330		2.69	235,316	Gainer et al 2019
Raphanus sativus	Root dry mass	2,020		2.69	75,093	Gainer et al 2019
Elymus lanceolatus	Root length	11,990		2.69	445,725	Gainer et al 2019
Lactuca sativa	Root length	840		2.69	31,227	Gainer et al 2019
Medicago sativa	Root length	140		2.69	5,204	Gainer et al 2019
Picea glauca	Root length	22,770		2.69	846,468	Gainer et al 2019

Species	Test Type	EC25 mg TPH/ kg soil	test soil fOM (%)	test soil fOC (%)	EC 25 OC normalized (mg/kg_{oc})	Source
Pinus banksiana	Root length	11,153		2.69	414,610	Gainer et al 2019
Raphanus sativus	Root length	2,910		2.69	108,178	Gainer et al 2019
Elymus lanceolatus	Shoot dry mass	490		2.69	18,216	Gainer et al 2019
Lactuca sativa	Shoot dry mass	80		2.69	2,974	Gainer et al 2019
Medicago sativa	Shoot dry mass	220		2.69	8,178	Gainer et al 2019
Picea glauca	Shoot dry mass	14,150		2.69	526,022	Gainer et al 2019
Pinus banksiana	Shoot dry mass	9,470		2.69	352,045	Gainer et al 2019
Raphanus sativus	Shoot dry mass	41,140		2.69	1,529,368	Gainer et al 2019
Elymus lanceolatus	Shoot length	550		2.69	20,446	Gainer et al 2019
Lactuca sativa	Shoot length	90		2.69	3,346	Gainer et al 2019
Medicago sativa	Shoot length	230		2.69	8,550	Gainer et al 2019
Picea glauca	Shoot length	44,540		2.69	1,655,762	Gainer et al 2019
Raphanus sativus	Shoot length	6,640		2.69	246,840	Gainer et al 2019
Zea mays	stem dry weight	6000	2.3	1	521,739	Chaineau et al 1997

Table C- 5. Summary of species geometric mean of toxicity endpoints from available literature on toxicity of medium petroleum hydrocarbons (diesel, motor oil and lubricating oil) to soil organisms and plants. Bold indicates species representative of the boreal forest regions. The table includes a total of 19 geometric means and the boreal forest region species includes 11 geometric means.

Species	number of data points	Geomean of OC normalized EC25 (mg TPH/kg$_{OC}$)	non-OC normalized geomean (mg/kg)
Eisenia fetida	4	301,526	7,307
Eisenia fetida	1	79,182	2,130
Elymus lanceolatus	4	44,924	1,208
Enchytraeus crypticus	1	164,709	7,346
Folsomia candida	2	75,897	2,042
Folsomia candida	1	88104	2370
Hypoaspis aculeifer	2	35,101	944
Hypoaspis aculeifer	1	264312	7110
Lactuca sativa	4	6,753	182
Lumbricus terrestris	1	34933	1558
Medicago sativa	4	9,288	250
Oppia nitens	2	97,079	2,611
Oppia nitens	1	42379	1140
Phaseolus vulgaris	1	521,739	6,000
Picea glauca	4	610,845	16,432
Pinus banksiana	3	325,059	8,744
Raphanus sativus	4	235,324	6,330
Triticum aestivum	1	260870	3000
Zea mays	3	657,350	7,560

Table C- 6. Summary of available literature on toxicity of F2 (>C10-C16) petroleum hydrocarbon fraction distillates to soil organisms and plants. The table includes a total of 90 individual endpoints from a total of eight studies. For artificial soil, the total organic carbon content was the geometric mean of values from two studies (ESG 2003; Agnell et al 2012).

Species	Test Type	EC25 (mg/kg F2)	test soil fOC (%)	OC normalized EC25 (mg F2/ kg_{oc})	Source
Dendrobaena hortensis	mortality	776	5.3	14642	Erlacher et al 2013
Eisena fetida	avoidance	3,588	2.69	133383	Gainer et al (2019)
Eisenia andrei	14 d mortality	402	6.48	6204	CCME (2008)
Eisenia andrei	14 d mortality	211	6.48	3256	CCME (2008)
Eisenia andrei	14 d mortality	240	6.48	3704	CCME (2008); ESG (2003)
Eisenia andrei	14 d mortality	287	6.48	4429	CCME (2008); ESG (2003)
Eisenia andrei	14 d mortality	448	6.48	6914	CCME (2008); ESG (2003)
Eisenia andrei	60 d progeny dry weight	132	6.48	2037	CCME (2008); ESG (2003)
Eisenia andrei	60 d progeny wet weight	138	6.48	2130	CCME (2008); ESG (2003)
Eisenia andrei	62 d repro	115	6.48	1775	CCME (2008); ESG (2003)
Eisenia andrei	juvenile dry mass	293	5.24	5592	Agnell et al (2012)
Eisenia andrei	juvenile wet mass	300	5.24	5725	Agnell et al (2012)
Eisenia andrei	mortality	320	5.8	5517	Cermak et al (2013)
Eisenia andrei	mortality	380	5.8	6552	Cermak et al (2013)
Eisenia andrei	mortality-14d	350	5.8	6034	Cermak et al (2010)
Eisenia andrei	reproduction	139	5.24	2653	Agnell et al (2012)
Eisenia fetida	mortality	2,466	2.69	91673	Gainer et al (20018)
Elymus lanceolatus	Root dry mass	62	6.48	957	CCME (2008); ESG (2003)
Elymus lanceolatus	Root dry mass	235	2.69	8736	Gainer et al (2019)
Elymus lanceolatus	Root dry mass	290	5.24	5534	Agnell et al (2012)
Elymus lanceolatus	Root length	85	6.48	1312	CCME (2008); ESG (2003)
Elymus lanceolatus	Root length	5,526	2.69	205428	Gainer et al (2019)
Elymus lanceolatus	Root length	295	5.24	5630	Agnell et al (2012)
Elymus lanceolatus	Shoot dry mass	300	6.48	4630	CCME (2008); ESG (2003)
Elymus lanceolatus	Shoot dry mass	192	2.69	7138	Gainer et al (2019)
Elymus lanceolatus	Shoot dry mass	198	5.24	3779	Agnell et al (2012)
Elymus lanceolatus	shoot length	1092	6.48	16852	CCME (2008); ESG (2003)
Elymus lanceolatus	Shoot length	38,263	2.69	1422416	Gainer et al (2019)
Elymus lanceolatus	shoot length	647	5.24	12347	Agnell et al (2012)
Enchytraeus crypticus	reproduction	2,714	2.69	100892	Gainer et al (2019)
Folsomia candida	mort	337	5.24	6431	Agnell et al (2012)
Folsomia candida	mortality	1,464	2.69	54424	Gainer et al (2018)
Folsomia candida	reproduction	1,687	2.69	62714	Gainer et al (2018)
Folsomia candida	reproduction	533	5.24	10172	Agnell et al (2012)

Species	Test Type	EC25 (mg/kg F2)	test soil fOC (%)	OC normalized EC25 (mg F2/ kg_{oc})	Source
Folsomia candida	avoidance	446	2.69	16580	Gainer et al (2019)
Hordeum vulgare	Root dry mass	128	6.48	1975	CCME (2008)
Hordeum vulgare	Root dry mass	300	6.48	4630	CCME (2008)
Hordeum vulgare	Root dry mass	356	6.48	5494	CCME (2008)
Hordeum vulgare	Root dry mass	392	6.48	6049	CCME (2008)
Hordeum vulgare	Root dry mass	493	5.24	9408	Agnell et al (2012)
Hordeum vulgare	Root length	171	6.48	2639	CCME (2008)
Hordeum vulgare	Root length	297	6.48	4583	CCME (2008)
Hordeum vulgare	Root length	653	6.48	10077	CCME (2008)
Hordeum vulgare	Root length	630	6.48	9722	CCME (2008)
Hordeum vulgare	Root length	389	5.24	7424	Agnell et al (2012)
Hordeum vulgare	Shoot dry mass	333	6.48	5139	CCME (2008)
Hordeum vulgare	Shoot dry mass	198	6.48	3056	CCME (2008)
Hordeum vulgare	Shoot dry mass	250	6.48	3858	CCME (2008)
Hordeum vulgare	Shoot dry mass	320	6.48	4938	CCME (2008)
Hordeum vulgare	Shoot dry mass	262	5.24	5000	Agnell et al (2012)
Hordeum vulgare	shoot length	765	6.48	11806	CCME (2008)
Hordeum vulgare	shoot length	455	6.48	7022	CCME (2008)
Hordeum vulgare	shoot length	382	6.48	5895	CCME (2008)
Hordeum vulgare	shoot length	445	6.48	6867	CCME (2008)
Hordeum vulgare	shoot length	1199	5.24	22882	Agnell et al (2012)
Hypoaspis aculeifer	avoidance	266.00	2.69	9888	Gainer et al (2019)
Hypoaspis aculeifer	mortality	39,269	2.69	1459814	Gainer et al (2018)
Hypoaspis aculeifer	reproduction	237	2.69	8810	Gainer et al (2018)
Lactuca sativa	Root dry mass	228	2.69	8476	Gainer et al (2019)
Lactuca sativa	Root length	312	2.69	11599	Gainer et al (2019)
Lactuca sativa	Shoot dry mass	103	2.69	3829	Gainer et al (2019)
Lactuca sativa	Shoot length	136	2.69	5056	Gainer et al (2019)
Lumbricus terrestris	mortality	1,593	2.69	59219	Gainer et al (2019)
Medicago sativa	root dry mass	765	6.48	11806	CCME (2008); ESG (2003)
Medicago sativa	Root dry mass	214	2.69	7955	Gainer et al (2019)
Medicago sativa	Root dry mass	226	5.24	4313	Agnell et al (2012)
Medicago sativa	Root length	221	6.48	3410	CCME (2008); ESG (2003)
Medicago sativa	Root length	110	2.69	4089	Gainer et al (2019)
Medicago sativa	Root length	257	5.24	4905	Agnell et al (2012)
Medicago sativa	shoot dry mass	145	6.48	2238	CCME (2008); ESG (2003)
Medicago sativa	Shoot dry mass	264	5.24	5038	Agnell et al (2012)
Medicago sativa	shoot length	455	6.48	7022	CCME (2008); ESG (2003)

Species	Test Type	EC25 (mg/kg F2)	test soil fOC (%)	OC normalized EC25 (mg F2/ kg_{oc})	Source
Medicago sativa	Shoot length	158	2.69	5874	Gainer et al (2019)
Medicago sativa	shoot length	354	5.24	6756	Agnell et al (2012)
Onychiurus folsomi	mort	211	6.48	3256	CCME (2008); ESG (2003)
Onychiurus folsomi	reproduction	211	6.48	3256	CCME (2008); ESG (2003)
Oppia nitens	avoidance	730	2.69	27138	Gainer et al (2019)
Oppia nitens	mortality	4,482	2.69	166617	Gainer et al (2018)
Oppia nitens	reproduction	32	2.69	1190	Gainer et al (2018)
Picea glauca	Root dry mass	2,017	2.69	74981	Gainer et al (2019)
Picea glauca	Root length	11,856	2.69	440743	Gainer et al (2019)
Picea glauca	Shoot dry mass	6,568	2.69	244164	Gainer et al (2019)
Picea glauca	Shoot length	27,990	2.69	1040520	Gainer et al (2019)
Pinus banksiana	Root dry mass	3,052	2.69	113457	Gainer et al (2019)
Pinus banksiana	Root length	39,370	2.69	1463569	Gainer et al (2019)
Pinus banksiana	Shoot dry mass	37,064	2.69	1377844	Gainer et al (2019)
Raphanus sativus	Root dry mass	790	2.69	29368	Gainer et al (2019)
Raphanus sativus	Root length	2,553	2.69	94907	Gainer et al (2019)
Raphanus sativus	Shoot dry mass	13,329	2.69	495502	Gainer et al (2019)
Raphanus sativus	Shoot length	4,767	2.69	177212	Gainer et al (2019)

Table C- 7. Summary of species geometric mean of toxicity endpoints from available literature on toxicity of F2 (>C10-C16) petroleum hydrocarbon fraction distillates to soil organisms and plants. Bold indicates species representative of the boreal forest regions. The table includes a total of 18 geometric means and the boreal forest region species includes includes 11 geometric means.

Species	# of data points	Geometric mean of OC normalized EC25 (mg F2/ kg oc)	Geometric mean of EC25 (mg/kg F2)
Onychiurus folsomi	2	3256	211
Eisena fetida/andrei	15	5120	293
Medicago sativa	11	5270	249
Hordeum vulgare	20	5930	368
Lactuca sativa	4	6605	178
Hypoaspis aculeifer	1	9888	266
Elymus lanceolatus	12	10827	488
Oppia nitens	2	14079	379
Dendrobaena hortensis	1	14642	776
Folsomia candida		16580	446
Oppia nitens	1	27138	730
Folsomia candida	3	32622	1096
Lumbricus terrestris	1	59219	1593
Enchytraeus crypticus	1	100892	2714
Hypoaspis aculeifer	2	113409	3051
Raphanus sativus	4	125077	3365
Eisena fetida	1	91673	2466
Picea glauca	4	302704	8143
Pinus banksiana	3	611620	16453

Table C- 8. Summary of available literature on toxicity of F3 (>C16-C34) petroleum hydrocarbon fraction distillates to soil organisms and plants. The table includes a total of 62 individual endpoints from a total of seven studies. For artificial soil, the total organic carbon content was the geometric mean of values from two studies (ESG 2003; Agnell et al 2012).

Species	Endpoint	EC25 (mg/kg F3)	test soil fOC (%)	OC normalized EC25 (mg F3/ kg_{oc})	Source
Dendrobaena hortensis	mortality	4846	5.3	91434	Erlacher et al 2013
Eisena fetida	avoidance	578	2.69	21487	Gainer et al (2019)
Eisenia andrei	mortality-28d	7435	5.8	128190	CCME (2008); Cermak (2005)
Eisenia andrei	# juveniles 60 d	540	5.8	9310	CCME (2008); Cermak (2005)
Eisenia andrei	mass juveniles 60 d	730	5.8	12586	CCME (2008); Cermak (2005)
Eisenia andrei	juv growth	230	5.8	3966	Cermak et al (2010)
Eisenia andrei	mortality-28d	6780	5.8	116897	Cermak et al (2010)
Eisenia andrei	reproduction	350	5.8	6034	Cermak et al (2010)
Eisenia andrei	mortality	2490	5.8	42931	Cermak et al (2013)
Eisenia andrei	mortality	3120	5.8	53793	Cermak et al (2013)
Eisenia andrei	mortality	2440	5.8	42069	Cermak et al (2013)
Eisenia andrei	mortality	3360	5.8	57931	Cermak et al (2013)
Eisenia fetida	mortality	2,533	2.69	94164	Gainer et al (2018)
Elymus lanceolatus	Root length	17,020	2.69	632714	CCME (2008); Cermak (2005)
Elymus lanceolatus	Root dry mass	1,280	2.69	47584	CCME (2008); Cermak (2005)
Elymus lanceolatus	Root length	4,550	2.69	169145	CCME (2008); Cermak (2005)
Elymus lanceolatus	Shoot dry mass	1,180	2.69	43866	CCME (2008); Cermak (2005)
Elymus lanceolatus	Shoot length	3450	2.69	128253	CCME (2008); Cermak (2005)
Elymus lanceolatus	Root dry mass	22,241	2.69	826803	Gainer et al (2019)
Elymus lanceolatus	Root length	22,472	2.69	835390	Gainer et al (2019)
Elymus lanceolatus	Shoot dry mass	23,327	2.69	867175	Gainer et al (2019)
Elymus lanceolatus	Shoot length	168	2.69	6245	Gainer et al (2019)
Enchytraeus crypticus	reproduction	67,184	2.69	2497546	Gainer et al (2019)
Folsomia candida	mortality	3,448	2.69	128178	Gainer et al (2019)
Folsomia candida	reproduction	1,631	2.69	60632	Gainer et al (2019)
Folsomia candida	avoidance	4,380	2.69	162825	Gainer et al (2019)
Hordeum vulgare	Root dry mass	3,280	2.69	121933	CCME (2008); Cermak (2005)
Hordeum vulgare	Root length	4960	2.69	184387	CCME (2008); Cermak (2005)
Hordeum vulgare	Shoot dry mass	4,900	2.69	182156	CCME (2008); Cermak (2005)
Hordeum vulgare	Shoot length	5650	2.69	210037	CCME (2008); Cermak (2005)
Hordeum vulgare	Root dry mass	9,290	2.69	345353	CCME (2008); Cermak (2005)
Hordeum vulgare	Root length	9930	2.69	369145	CCME (2008); Cermak (2005)
Hordeum vulgare	Shoot dry mass	5,560	2.69	206691	CCME (2008); Cermak (2005)

Species	Endpoint	EC25 (mg/kg F3)	test soil fOC (%)	OC normalized EC25 (mg F3/ kg_{oc})	Source
Hordeum vulgare	Shoot length	6470	2.69	240520	CCME (2008); Cermak (2005)
Hypoaspis aculeifer	mortality	1,927	2.69	71636	Gainer et al (2018)
Hypoaspis aculeifer	reproduction	234	2.69	8699	Gainer et al (2018)
Hypoaspis aculeifer	avoidance	1,377	2.69	51190	Gainer et al (2019)
Lactuca sativa	Root dry mass	226	2.69	8401	Gainer et al (2019)
Lactuca sativa	Root length	36,805	2.69	1368216	Gainer et al (2019)
Lactuca sativa	Shoot dry mass	105	2.69	3903	Gainer et al (2019)
Lactuca sativa	Shoot length	141	2.69	5242	Gainer et al (2019)
Lumbricus terrestris	mortality	1,545	2.69	57435	Gainer et al (2018)
Medicago sativa	Root dry mass	7,657	2.69	284647	Gainer et al (2019)
Medicago sativa	Root length	102	2.69	3792	Gainer et al (2019)
Medicago sativa	Shoot dry mass	1,890	2.69	70260	Gainer et al (2019)
Medicago sativa	Shoot length	135	2.69	5019	Gainer et al (2019)
Onychiurus folsomi	35 d repro	510	5.8	8793	CCME (2008); Cermak (2005)
Onychiurus folsomi	7 d mortality	3490	5.8	60172	CCME (2008); Cermak (2005)
Oppia nitens	mortality	10,907	2.69	405465	Gainer et al (2018)
Oppia nitens	reproduction	1,799	2.69	66877	Gainer et al (2018)
Oppia nitens	avoidance	1,346	2.69	50037	Gainer et al (2019)
Picea glauca	Root dry mass	22,486	2.69	835911	Gainer et al (2019)
Picea glauca	Root length	34,810	2.69	1294052	Gainer et al (2019)
Picea glauca	Shoot dry mass	20,237	2.69	752305	Gainer et al (2019)
Picea glauca	Shoot length	26,911	2.69	1000409	Gainer et al (2019)
Pinus banksiana	Root dry mass	28,026	2.69	1041859	Gainer et al (2019)
Pinus banksiana	Root length	7,716	2.69	286840	Gainer et al (2019)
Pinus banksiana	Shoot dry mass	5,958	2.69	221487	Gainer et al (2019)
Raphanus sativus	Root dry mass	11,997	2.69	445985	Gainer et al (2019)
Raphanus sativus	Root length	3,108	2.69	115539	Gainer et al (2019)
Raphanus sativus	Shoot dry mass	89,771	2.69	3337212	Gainer et al (2019)
Raphanus sativus	Shoot length	7,804	2.69	290112	Gainer et al (2019)

Table C- 9. Summary of species geometric mean of toxicity endpoints from available literature on toxicity of F2 (>C10-C16) petroleum hydrocarbon fraction distillates to soil organisms and plants. Bold indicates species representative of the boreal forest region. The table includes a total of 19 geometric means and the boreal forest region species includes 13 geometric means.

Species	# of data points	Geometric mean of OC normalized EC25 (mg F2/ kg_{oc})	Geometric mean of EC25 (mg/kg F2)
Onychiurus folsomi	2	23002	1334
Eisena fetida/andrei	11	30631	1657
Dendrobaena hortensis	1	91434	4846
Oppia nitens	1	50037	1346
Lumbricus terrestris	1	57435	1545
Lactuca sativa	4	22022	592
Medicago sativa	4	24838	668
Hordeum vulgare	8	219819	5913
Eisena fetida	1	21487	578
Folsomia candida	2	88157	2371
Elymus lanceolatus	9	168099	4522
Oppia nitens	2	164671	4430
Hypoaspis aculeifer	2	24963	672
Folsomia candida	1	162825	4380
Hypoaspis aculeifer	1	51190	1377
Enchytraeus crypticus	1	2497546	67184
Pinus banksiana	3	404513	10881
Raphanus sativus	4	472606	12713
Picea glauca	4	949884	25552

Table C- 10. Summary of goodness of fit for different SSD models (normal, logistic, extreme value and Gumbel) evaluated using the CCME SSD Master V 3.0. The models were evaluated using all test species and only test species representative of the boreal forest region species.

Petroleum Hydrocarbon Composition	Model	A^2			
		Actual concentration (mg/kg)		Actual concentration normalized to soil OC (mg/kg_{OC})	
		All species	Boreal forest species	All species	Boreal forest species
Total Petroleum Hydrocarbons	Normal	0.516	**0.256**	0.339	0.172
	Logistic	0.466	0.266	0.341	**0.17**
	Extreme Value	**0.379**	0.308	**0.267**	0.236
	Gumbel	1.439	0.398	0.833	0.384
F2	Normal	0.462	0.462	0.388	0.282
	Logistic	0.473	0.45	0.417	0.286
	Extreme Value	0.825	0.729	0.646	0.512
	Gumbel	**0.3**	**0.3**	**0.277**	**0.169**
F3	Normal	0.398	0.384	0.464	0.368
	Logistic	0.338	0.351	0.448	0.324
	Extreme Value	0.927	1.071	0.973	1.112
	Gumbel	**0.304**	**0.276**	**0.353**	**0.247**

A^2=Anderson-Darling statistic; Bold indicates lowest A^2 value indicating best model (lowest value is best model fit); OC=organic carbon

Table C- 11. Model fit of the general linear model and results for the influence of soil properties and contaminant level expressed as hazard concentrations on adult survival and juvenile production for *Folsomia candida* and *Oppia nitens*.

Species	Test	(blank)	Estimate	Standard Error	Z Value	P value	Model
Folsomia candida	Adult survival	Intercept	2.09	0.088	23.71	<0.001	quasi poisson
		HC25*OM	0.33	0.063	5.16	<0.001	
		HC50*OM	0.66	0.11	6.02	<0.001	
		null deviance 463 on 113 df; residual deviance 123 on 108 df; pseudo r^2=73%; AIC=410					
	Juvenile Production	Intercept	2.89	0.099	28.98	<0.001	negative binomial
		HC25*OM	1.57	0.14	11.35	<0.001	
		HC50*OM	1.28	0.21	5.98	<0.001	
		null deviance 1150 on 113 df; residual deviance 130 on 108 df; pseudo r^2= 88% ; AIC=507					
Oppia nitens	Adult survival	Intercept	2.55	0.081	31.73	<0.001	quasi poisson
		HC25*OM	0.31	0.063	4.81	<0.001	
		HC50*OM	0.53	0.13	4.09	<0.001	
		null deviance 736 on 115 df; residual deviance 146 on 110 df; pseudo r^2=75%; AIC=479					
	Juvenile Production	Intercept	2.39	0.07	36.48	<0.001	negative binomial
		HC25*OM	0.42	0.06	6.99	<0.001	
		HC50*OM	0.017	0.13	0.13	0.896	
		null deviance 719 on 116 df; residual deviance 93 on 111 df; pseudo r^2= 89% ; AIC=386					

OM=organic matter content; AIC= Akaiki information criterion; df=degrees of freedom

Table C- 12. The HC5 values for the carbon normalized medium PHC mixture data for two difference species assemblages.

Model	All species	Boreal forest species
Gumbel	18,750 (11,670-30 110)	19,925 (13,560-29,277)
Extreme value	5,120 (3,640-7,220)	5,400 (3,200-9,100)
Logistic	9,420 (6,530-13,600)	10,500 (7940-13900)

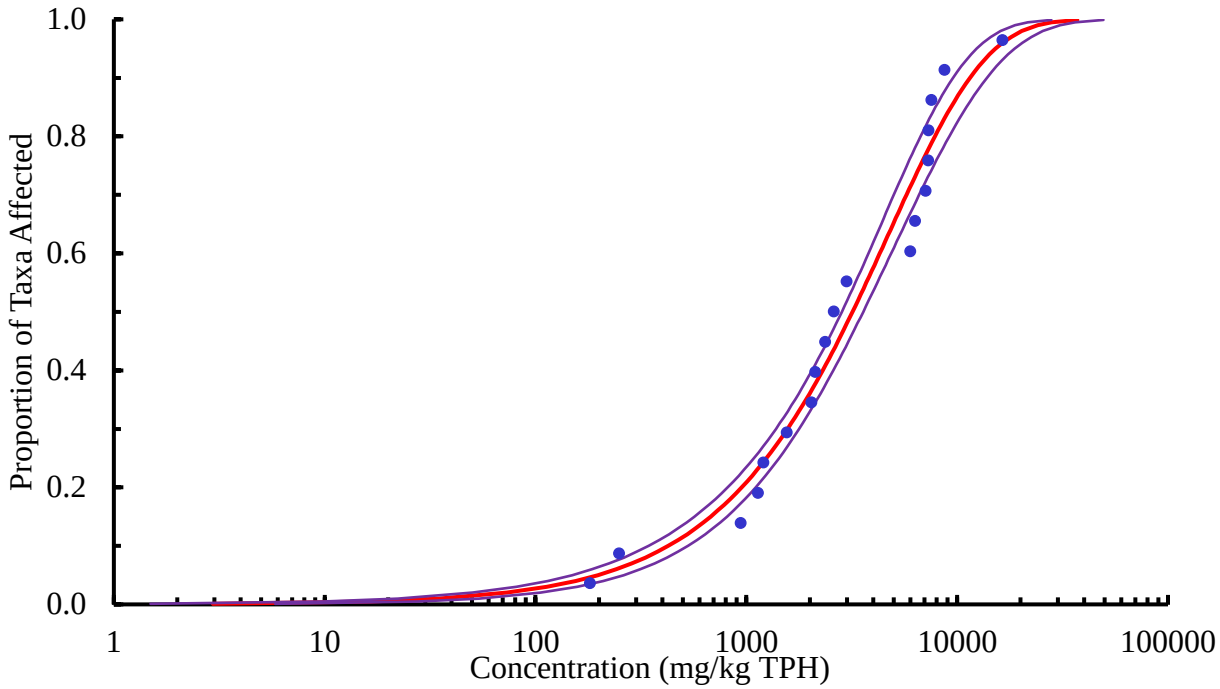

Figure C-1. Species sensitivity distribution for soil invertebrates and plants exposed to whole mixtures (total petroleum hydrocarbon (TPH)) normalized to soil organic carbon content in soil from all species in available literature. Red line indicates model fit and blue lines represent 95% confidence intervals.

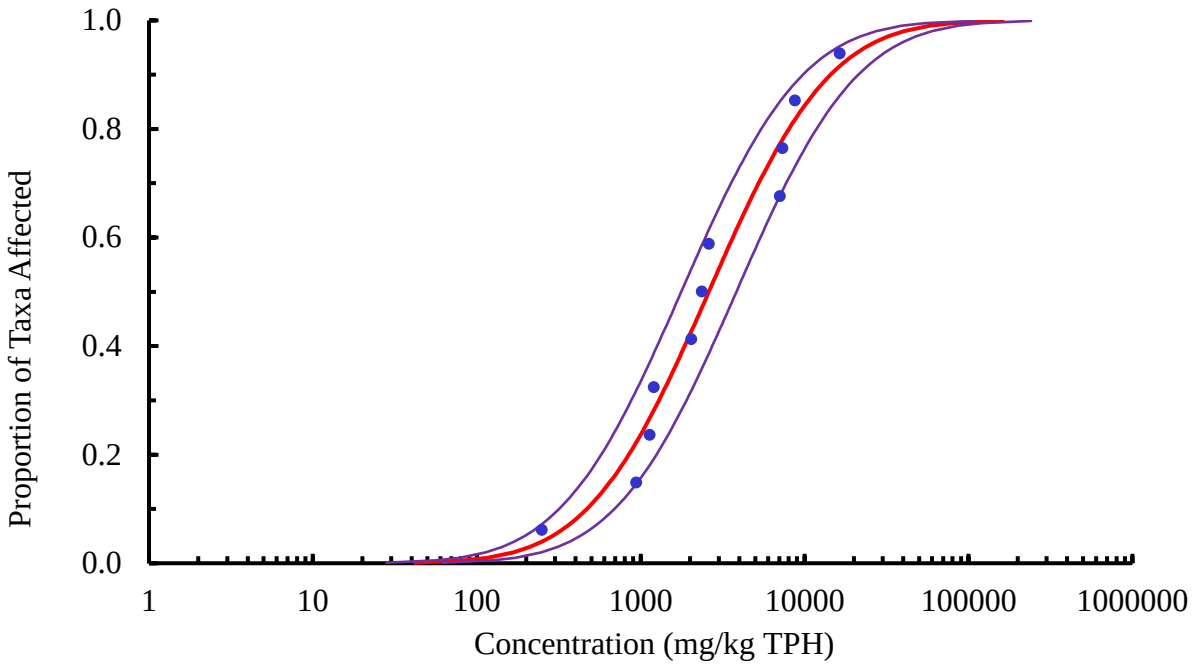

Figure C-2. Species sensitivity distribution for soil invertebrates and plants exposed to whole mixtures (total petroleum hydrocarbon (TPH)) normalized to soil organic carbon content in soil from select species representative of the boreal forest from available literature. Red line indicates model fit and blue lines represent 95% confidence intervals.

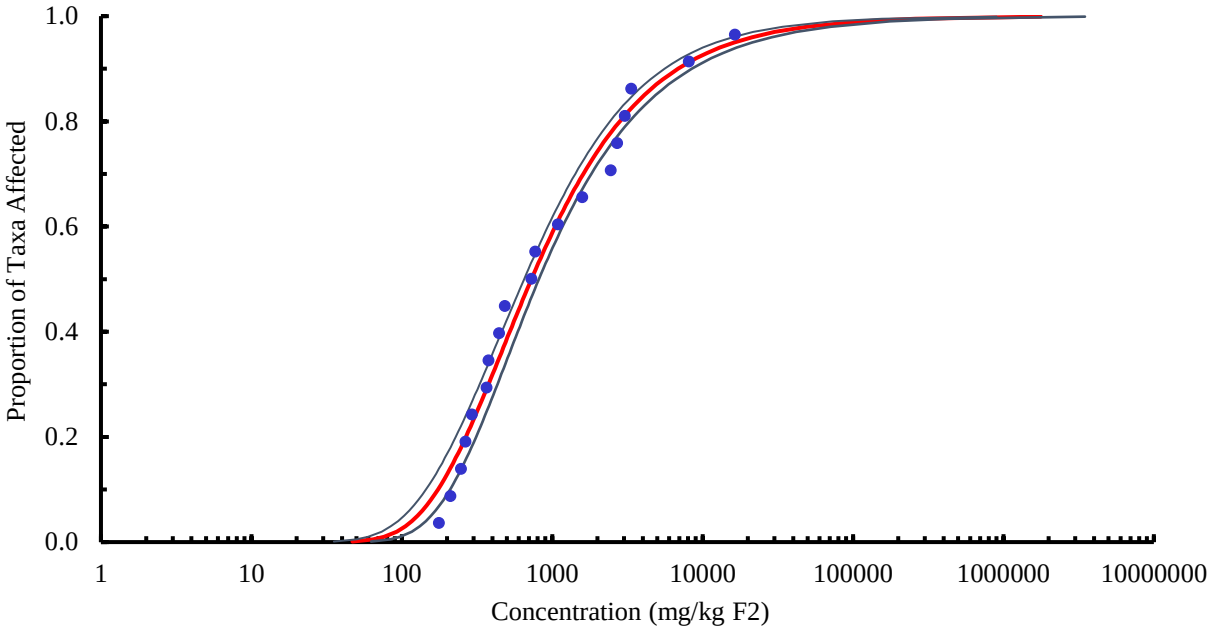

Figure C-3. Species sensitivity distribution for soil invertebrates and plants exposed to F2 petroleum hydrocarbon distillates, normalized to soil organic carbon content, from all species in available literature. Red line indicates model fit and blue lines represent 95% confidence intervals.

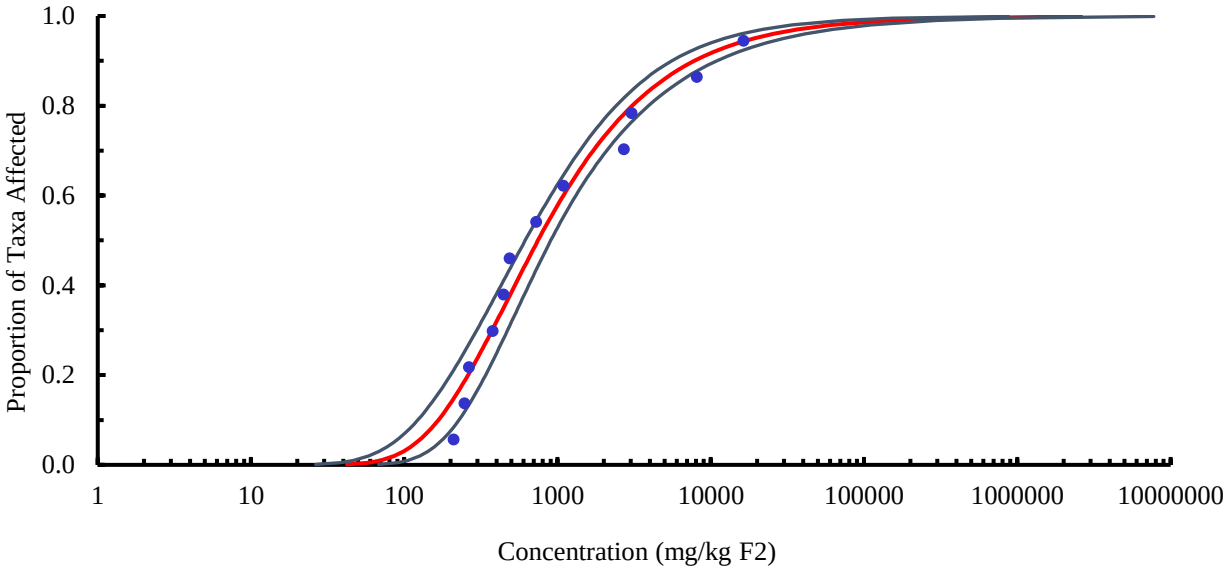

Figure C-4. Species sensitivity distribution for soil invertebrates and plants exposed to F2 petroleum hydrocarbon distillates, normalized to soil organic carbon content, select species representative of the boreal forest from available literature. Red line indicates model fit and blue lines represent 95% confidence intervals.

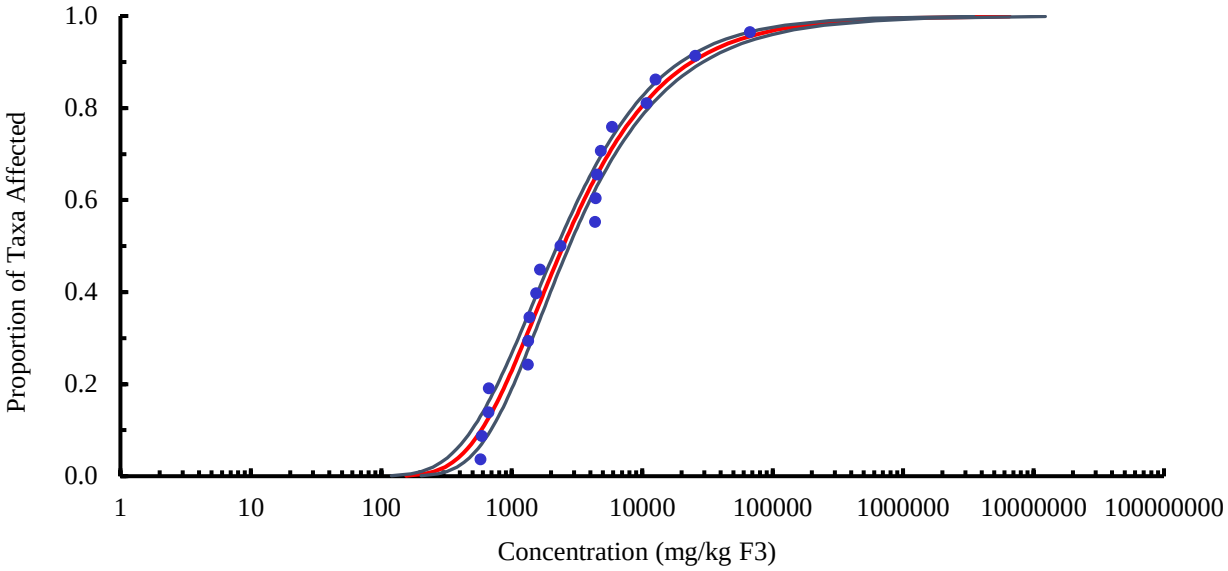

Figure C-5. Species sensitivity distribution for soil invertebrates and plants exposed to F3 petroleum hydrocarbon distillates, normalized to soil organic carbon content, from all species in available literature. Red line indicates model fit and blue lines represent 95% confidence intervals.

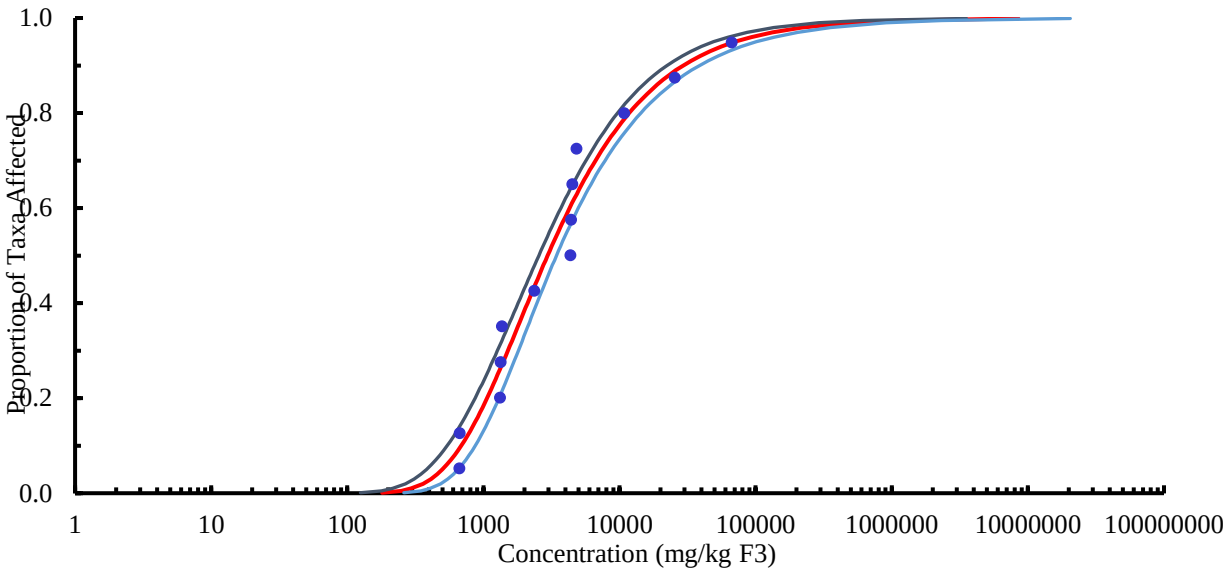

Figure C-6 Species. sensitivity distribution for soil invertebrates and plants exposed to F3 petroleum hydrocarbon distillates, normalized to soil organic carbon content, select species representative of the boral forest from available literature. Red line indicates model fit and blue lines represent 95% confidence intervals.

EQUATIONS

Organic carbon normalization

$$\frac{mg}{kg_{OC}} = \frac{\frac{mg}{kg}}{\frac{kg\ TOC}{kg}}$$ Equation [C-1]

Where,
mg/kg_{oc} = milligrams of chemical per kilogram of organic carbon
mg/kg = milligrams of chemical per kilogram of dry weight soil
kg TOC/kg = percent total organic carbon in dry weight expressed as a decimal (1% TOC=0.01)

12. Appendix D: Manuscript 4 Supplemental Material

Table D- 1. Summary of definitive test concentrations for sodium chloride, reported as electrical conductivity (dS/m).

Folsomia candida		*Enchytraeus crypticus*		*Eisenia fetida*	
juvenile	adult	juvenile	adult	juvenile	adult
0.9	0.9	0.9	0.9	0.9	0.9
1.1	1.1	1.1	1.1	1.1	1.1
1.4	1.9	1.9	1.9	1.9	1.9
1.9	2.9	2.9	2.4	2.9	2.9
2.4	5.9	4.9	2.9	4.9	4.9
2.9	10.9	6.9	4.9	6.9	6.9
5.9	15.9	8.9	8.9	8.9	8.9
10.9	20.9	10.9	12.9	10.9	10.9
15.9	25.9	12.9	16.9	15.9	15.9
20.9	30.9	14.9	20.9	20.9	20.9
		16.9			

Table D- 2. Summary of definitive test concentrations (actual) for copper (mg/kg).

Folsomia candida		*Enchytraeus crypticus*		*Eisenia fetida*	
juvenile	adult	juvenile	adult	juvenile	adult
0	0	0	0	0	0
9	18	9	18	4.5	18
18	90	18	90	9	90
45	180	45	135	13.5	180
90	360	90	180	27	360
135	720	135	360	54	540
180	900	180	720	81	720
270	1080	270	1080	117	900
360	1440	360	1440	180	1350
450	1800		1800	360	1800
900					
1800					

Table D- 3. Summary of definitive test concentrations (actual) for phenanthrene (mg/kg).

Folsomia candida		*Enchytraeus crypticus*		*Eisenia fetida*	
juvenile	adult	juvenile	adult	juvenile	adult
0	0	0	0	0	0
4.4	4.4	44	88	0.44	4.4
8.8	8.8	88	440	0.88	8.8
13.2	13.2	440	880	2.2	22
17.6	17.6	660	1760	4.4	44
22	22	880	3520	6.6	66
26.4	26.4	1320	4400	8.8	88
35.2	35.2	2640	5280	44	220
44	44	4400	7040	88	440
52.8	52.8	6600	8800	220	660
88	88				
220	440				
440	660				
616					
748					

Note: solvent controls were included in dual tests with negative controls.