Investigations Into the Toxicity and Toxicokinetics of Individual and Binary Mixtures of CCME Petroleum Hydrocarbon Distillates in Soil

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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ABSTRACT

The Canada-wide Standards for Petroleum Hydrocarbons (PHC CWS) in soils are remedial standards based on four petroleum distillates (Fraction 1 [F1; ECN C6-C10], Fraction 2 [F2; ECN >C10-C16], Fraction 3 [F3; ECN >C16-C34], and Fraction 4 [F4; ECN >C34-C50]). Knowledge gaps regarding petroleum toxicity to soil organisms were identified including concerns that the ecological values for F3 were overly conservative, possibly due to differences in toxicity between the low and high boiling point constituents of this distillate, and unexpected less-than-concentration-additive toxicity of binary mixtures of distillates to earthworms. An understanding of petroleum toxicokinetics with soil organisms was also needed to interpret toxicity results.

Toxicity studies with one plant and two invertebrate (earthworm and collembolan) species were conducted with F3 and two subfractions of F3, F3a (ECN >C16-C22) and F3b (ECN >C22-C34), to determine if the toxicities of F3a and F3b were sufficiently different to recommend regulating the two separately. The difference in toxicities between the two was generally within the range of variability noted for the toxicity tests and thus it was not recommended to regulate the two separately.

The toxicity data indicated that the exposure duration of standard test methods may be insufficient for determining the toxicity of higher distillate ranges. Toxicokinetic studies conducted with earthworms and F2, F3a, and F3b confirmed that standard test durations generally were not of sufficient duration to attain maximum body residues with F3b and sometimes F3a. Internal exposure scenarios also differed among distillates, with various accumulation curves noted and attributed to differences in loss of distillate from the soil and changes in bioavailability. Aromatics were disproportionally accumulated by earthworms relative to the ratio of aromatics to aliphatics in soil, suggesting that aromatics were the main contributors to earthworm toxicity.

Toxicity and toxicokinetic studies with binary combinations of F2, F3a, and/or F3b and earthworms demonstrated that, on a soil concentration basis, toxicity was less-than-additive. Toxicokinetics indicated that this was due to a decrease in the bioavailability of distillates when a second distillate was present presumably as a non-aqueous phase liquid. However, on an internal tissue concentration basis, results were closer in agreement with concentration-addition.

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DEDICATION

To my parents:

Leo McCann (1939-2008)
Gisèle McCann (1936-2009)

Your love, support, and belief in me were always there. You may not have always understood what I was doing but you took pride in my efforts and accomplishments. I miss you. This thesis is dedicated to you.

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LIST OF ABBREVIATIONS

BCF	bioconcentration factor
BSAF	biota-soil accumulation factor
CCME	Canadian Council of Ministers of the Environment
CSPC	critical separate phase concentration
d	day
ECN	equivalent carbon number
GC-FID	gas chromatography-flame ionization detector
GC-MS	gas chromatography-mass spectrometry
K_{oc}	organic carbon partition coefficient
K_{sw}	soil-water partition coefficient
min	minute
NAPL	non-aqueous phase liquid
OC	organic carbon
OM	organic matter
PHC	petroleum hydrocarbons
PHC CWS	Canada-wide Standards for Petroleum Hydrocarbons in soils
SIM	selective ion monitoring
TPHCWG	Total Petroleum Hydrocarbon Criteria Working Group
wt.	weight

Chapter 1 GENERAL INTRODUCTION

1.1. OVERVIEW OF PETROLEUM CONTAMINATION AND THE CANADA-WIDE STANDARDS FOR PETROLEUM HYDROCARBONS IN SOILS

Petroleum (i.e., crude oil) is a variable and complex mixture of thousands of individual compounds, the majority of which are hydrocarbons (i.e., compounds consisting of hydrogen and carbon atoms). Minor constituents include S-, N-, and O-containing compounds and metals, particularly nickel and vanadium (ESG 2003; Wang and Fingas 2006; Weisman 1998). The hydrocarbon constituents of crude oil (i.e., petroleum hydrocarbons (PHCs)) can be broadly divided into two chemical classes, aliphatics and aromatics. Aliphatics include alkanes (straight, branched, and cyclic), alkenes, and alkynes (if present; unsaturated aliphatics are not generally found in crude oil but are formed during refining processes). Aromatics are compounds containing one or more benzene rings. Within the aromatic group, alkylated compounds predominate (Potter and Simmons 1998). The non-hydrocarbon constituents are primarily found within the higher boiling point fractions of crude oil (Weisman 1998) and are part of the resins and asphaltenes chemical classes. Resins consist of polar compounds such as heterocyclic S-, O-, and N-containing compounds, phenols, acids, alcohols, and monoaromatic steroids (Wang and Fingas 2006). Asphaltenes are polar compounds comprising condensed polycyclic aromatic ring systems of 6 to 15 rings carrying alkyl side-chains of varying lengths (CCME 2008; Wang and Fingas 2006). Crude oil is derived from organic material that has been converted over millions of years and under differing geological conditions (Wang and Fingas 2006), resulting in high compositional variability amongst crude oils from different sources or reservoirs (Wang and Fingas 2006; Wang et al. 2003). For example, Sockeye crude oil consists of 49.2% saturates (i.e., alkanes and cycloalkanes), 17.2% aromatics, and 18.5% asphaltenes (with minor concentrations of other chemical classes) while South Louisiana crude oil consists of 80.8% saturates, 12.6% aromatics, and only 0.8% asphaltenes (Wang et al. 2003).

Crude oil is refined into various petroleum products with different boiling point ranges mainly via distillation (Weisman 1998). Other refining processes, such as cracking (converting long chain alkanes into smaller alkanes, alkenes, and hydrogen) or reforming (converting aliphatics into aromatics), as well as the removal of undesirable or addition of desirable components, can alter the composition of petroleum products (Potter and Simmons 1998). Dependent on the source of crude oil and refining process used, the chemical composition of the same refined petroleum product will differ (Wang and Fingas 2006).

The potential for environmental contamination with petroleum hydrocarbons is high. Approximately 286,000 tonnes of petroleum (crude oil and petroleum products) is used daily in Canada (Wang and Fingas 2006). Between the years 1984 and 1995 (the most recent time period for spills publically reported by the Environmental Emergencies Branch, Environment Canada) approximately 1,200-3,400 petroleum spills (to all media) were reported each year in Canada, with total yearly quantities ranging from approximately 10,000 to 120,000 tonnes. Nearly 70% of all petroleum spills were to land ((EC 1998) http://www.ec.gc.ca/Publications/default.asp?lang=En&xml=32A2B99E-4EFF-9B56-6442AA9BDEF2). The Canadian Council of Ministers of the Environment (CCME) estimates that there are over 250,000 potential or actual terrestrial sites contaminated with PHCs in Canada (CCME 2008). Soil contamination can occur at petroleum extraction, refining and distribution facilities, during transport, through accidental spills, and by the deliberate addition of product to soil for petroleum bioremediation at landfarming facilities. The remediation and management of these contaminated sites are an important concern and a challenge for stakeholders (e.g., scientists, site managers and land owners).

Prior to 2000, Canadian regulatory approaches and guidelines for the remediation of PHC contaminated soils varied greatly across jurisdictions, comprising a mixture of generic and site-specific derived values based on widely differing analytical methodologies and based on differing degrees of scientific rationale. This led to inconsistency in the remediation and management of PHC contaminated sites across the country. In the late 1990s, the CCME recognized that there was a need for consistent, scientifically-based standards for PHCs in soils that were protective of both human and ecological receptors. In May 2001, the Canada-wide Standards for Petroleum Hydrocarbons (PHC CWS) in soils were accepted and endorsed by the CCME. The PHC CWS is a three-tiered remedial standard that is protective of both human and ecological health. The standards cover two soil types (coarse and fine textured), two depths (surface and subsoil), and were developed for four generic land-use scenarios (agriculture, residential/parkland, commercial, and industrial) (CCME 2008). A standardized analytical method for petroleum hydrocarbons was also developed for the PHC CWS (CCME 2001b).

Soils containing PHCs can be contaminated with hundreds of thousands of individual compounds covering a large range of physical-chemical properties that will affect their fate and distribution in the environment as well as their toxicity. This can complicate the assessment of risk

at a site. To address this problem, some jurisdictions have developed criteria based on groupings of petroleum constituents with similar environmental fate, behavior, and toxicity. These groupings are based on boiling point ranges, given as equivalent carbon number (ECN) ranges. ECN normalizes the carbon number of a compound, based on its boiling point, to that of the *n*-alkanes so that compounds with the same boiling point will have the same ECN (Gustafson et al. 1997). Jurisdictions that have taken such an approach include the Massachusetts Department of Environmental Protection (Massachusetts Department of Environmental Protection 2003), the Total Petroleum Hydrocarbon Criteria Working Group (TPHCWG) in the United States (Edwards et al. 1997; Gustafson et al. 1997; Potter and Simmons 1998; Vorhees et al. 1999; Weisman 1998), and the Canadian Atlantic provinces (through the Partnership in Risk-Based Corrective Action Implementation (PIRI) initiative (CCME 2008)). The Netherlands are considering using such an approach for the derivation of risk limits for sediments and possibly other media (Verbruggen et al. 2008). In addition, the European oil company organization for environment, health and safety, Conservation of Clean Air and Water in Europe (CONCAWE), has developed a model, PETROTOX, that utilizes petroleum groupings to predict concentrations and toxicity in aquatic environments (http://concawe.be/content/default.asp?PageID=778).

The PHC CWS has also taken a similar approach and defines four carbon-fractions (Fraction 1 (F1; ECN C6-C10], Fraction 2 [F2; ECN >C10-C16], Fraction 3 [F3; ECN >C16-C34], and Fraction 4 [F4; ECN >C34-C50]) for which standards have been established (CCME 2008). Unlike the others (e.g., TPHCWG (Edwards et al. 1997; Gustafson et al. 1997; Potter and Simmons 1998; Weisman 1998) and Massachusetts Department of Environmental Protection (Massachusetts Department of Environmental Protection 2003)), the PHC CWS does not regulate aliphatic and aromatic hydrocarbons separately, but rather assumes a 4:1 ratio of aliphatics to aromatics for petroleum (CCME 2008). However, also unlike the others ((Edwards et al. 1997; Gustafson et al. 1997; Massachusetts Department of Environmental Protection 2003; Potter and Simmons 1998; Vorhees et al. 1999; Weisman 1998), the PHC CWS derived environmental standards for each hydrocarbon fraction in addition to those derived for the protection of human health (CCME 2008).

In the PHC CWS, Tier 1 standards are generic national levels protective of human health and the environment which can be adjusted in Tier 2 to reflect unique site characteristics, while Tier 3 levels are developed from a site-specific risk assessment (CCME 2008). When the PHC CWS was first published, Tier 1 standards for F3 and F4 for all land-use scenarios were driven for the most

part by the value derived for soil contact by ecological receptors. Vapour inhalation (human health) and/or groundwater contamination (potable and/or for the protection of aquatic life) were more sensitive exposure pathways for F1 and F2, followed by ecological receptors (CCME 2000). Therefore, at sites where neither the vapour inhalation nor groundwater pathway was applicable for F1 or F2, the default value for remediation would become that derived for ecological receptors. In many cases, thus, the ecological receptors were driving the remediation levels and therefore, it was important to ensure that the values for this exposure pathway were accurate and scientifically-sound.

The ecological direct contact values for the PHC CWS in 2001 were derived, to a large extent, from studies on the toxicity of the CCME petroleum distillates (F2, F3, and F4) to plants and soil invertebrates conducted by ESG International (now Stantec Consulting Ltd.) (CCME 2000; ESG 2003). These toxicity tests were conducted with a variety of plant species, two earthworm species, and one collembolan species with two different soil types using a light, sweet Alberta crude from the Federated Pipelines in Alberta, Canada (ESG 2003).

The CCME recognized that there were significant data gaps in the knowledge of PHC contamination, including the effects of petroleum hydrocarbons on ecological receptors. While the state-of-knowledge was considered sufficient to derive Tier 1 standards in 2001, a provision was included in the PHC CWS to review new data in 2005 to determine if revisions to the criteria were required.

Following the publication of the PHC CWS in 2001, several gaps in our knowledge regarding PHC soil contamination and toxicity to soil organisms were identified. Questions regarding the appropriateness of the PHC CWS Tier 1 standards for Fraction 3 with regard to the protection of ecological receptors have arisen (CCME 2000; Ecological Criteria Advisory Sub Group 2006 (http://www.ccme.ca/assets/pdf/phc_5_yr_rvw_eco_dscg_1.0_e.pdf); Murphy and Charrois 2003) as well as concerns regarding the relationships derived for adjusting nominal to measured concentrations in ecological effect studies for some distillates (Ecological Criteria Advisory Sub Group 2006). The PHC CWS are also based on the total amount of petroleum hydrocarbons in the soil within the specified ECN ranges, assuming a constant 4:1 aliphatic:aromatic ratio. However, there are differences in the toxicity of aliphatic and aromatic compounds (Feuston et al. 1994; Khan et al. 1986; Payne et al. 1995; van Overbeek and Blondeau 1954; Vandervaart Cook 2002), potential

for differential accumulation of aliphatics and aromatics (Anderson 1973; Lee et al. 1972; Stegeman and Teal 1973), and variability in the ratio of these two chemical classes within petroleum products (Wang et al. 2003), leading to questions regarding how possible differential accumulation of the aliphatic and aromatic compounds by soil organisms might affect toxicity and thus risk. As well, the PHC CWS were developed considering exposure to individual ECN ranges only and not exposure to two or more distillates simultaneously. Additive or non-additive toxic interactions between different distillates may affect the applicability of the standards.

To address these questions, a research project was initiated in 2002 to investigate the toxicity and toxicokinetics of both individual and binary combinations of crude oil distillates, including subfractions of F3, by soil organisms as discussed below.

1.2. APPROPRIATENESS OF THE PHC CWS TIER 1 F3 STANDARDS FOR THE PROTECTION OF ECOLOGICAL RECEPTORS

Since 2001, it has been noted that it is often difficult to remediate soils to levels at or below the PHC CWS for F3 (Ecological Criteria Advisory Sub Group 2006) and some investigators have found that soils above the F3 soil criteria were not toxic to ecological receptors (Murphy and Charrois 2003). In 2001, the F3 PHC CWS value for fine-textured, agricultural soils was 800 mg/kg (CCME 2000). While publically-available data are limited, the concentrations of F3 at some Canadian sites have been reported in the 860-7,500 mg/kg range ((Murphy and Charrois 2003), http://www.esaa-events.com/remtech/2003/default.htm; (Harris and Jackson 2004), http://www.esaa-events.com/remtech/2004/default.htm; (Bédard 2006), http://www.esaa-events.com/remtech/2006/default.htm). The ECN range for F3 is very broad (ECN >C16-C34) in relation to other CCME distillate fractions and compared to PHC groupings used by others (Gustafson et al. 1997; Massachusetts Department of Environmental Protection 2003) and includes constituents with widely varying ranges of environmental fate and toxicity (CCME 2000). Differences in the environmental fate and toxicity between the lower molecular weight (e.g., lower boiling point) constituents and the higher molecular weight (e.g., higher boiling point) constituents of F3 might result in non-toxic soils exceeding the PHC CWS. Studies have indicated a higher toxicity of lower molecular weight PHCs (Dorn et al. 1998; Efroymson et al. 2004; Salanitro et al. 1997; Trapp et al. 2001; Van Gestel et al. 2001) compared to those of higher molecular weight (Dorn et al. 1998; Efroymson et al. 2004; Salanitro et al. 1997; Van Gestel et al. 2001), with one

study attributing the toxicity of a contaminated sediment to constituents in the C16-C22 range ((Scholten et al. 1997), http://www.rivm.nl/bibliotheek/rapporten/601501021.pdf). Constituents within the lower end of the boiling point range of F3 (aliphatics <C24 and aromatics ≤4 rings) are degraded much more readily than those in the upper range (Bartha 1986; Englert et al. 1993; Jonker et al. 2006). Thus, over time or with remediation, there is a depletion of the more toxic, lower boiling point constituents with a concurrent proportional enrichment in less toxic, higher boiling constituents in the soil. The lack of toxicity observed at some bioremediated sites with elevated concentrations of F3 might be due to this preferential loss of lower boiling point constituents from the soils, with the higher boiling point constituents of F3 being the main contributors to the concentrations above the PHC CWS.

The following hypothesis was tested in this investigation (Chapter 2):

Hypothesis: the toxicity of the lower molecular weight constituents of F3 is sufficiently different from the toxicity of the higher molecular weight constituents to recommend separate regulation of the two subfractions under the PHC CWS.

To investigate this, the following objectives needed to be fulfilled:

1) Fractionate F3 into two subfractions, F3a (ECN >C16-C22) and F3b (ECN >C22-C34), and characterize these subfractions.
2) Determine the toxicity of F3, F3a, and F3b in lethal and/or chronic toxicity tests with three species of soil organisms (one plant (*Elymus lanceolatus*) and two invertebrates (*Eisenia andrei* and *Orthonychiurus folsomi*)) known to be sensitive to F3 toxicity. Extended exposure durations were used in the earthworm lethality tests due to concerns that standard 14-d exposure durations (EC 2004) were not sufficient to determine threshold effect concentrations (defined as the concentration at which there is no increase in observed effects with indefinite exposure (Persoone et al. 1990)), especially with the highly hydrophobic, higher ECN distillates.
3) Determine the toxicity of F2 to earthworms, to provide data required for subsequent comparisons and studies.
4) Determine the relationship between nominal and measured concentrations for each distillate tested using select test concentrations from select tests. Additional test concentrations were prepared and analyzed with F2 to more fully characterize the

variability in measured concentration with this distillate, as the variability within and between soil treatments was expected to be higher due to the volatility and degradability of its constituents and due to the small volumes of distillate used in the preparation of test soils.

The results of Objective 4 are of interest to the CCME as the Ecological Criteria Advisory Sub Group of the CCME Soil Quality Guidelines Task Group noted in its review of the 2001 PHC CWS ecological direct soil contact guidance (Ecological Criteria Advisory Sub Group 2006) that low analytical recovery in the ESG study (ESG 2003) may lead to higher guideline values. Concerns were raised specifically with regard to F3 (Ecological Criteria Advisory Sub Group 2006) but similar concerns may also apply to F2.

1.3. INVESTIGATIONS INTO THE TOXICOKINETICS OF INDIVIDUAL DISTILLATES WITH THE EARTHWORM, *EISENIA ANDREI*

The internal concentration of contaminant, the tissue or body residue, is generally considered to be the more appropriate measure of exposure and toxicity than external exposure concentrations with toxicity occurring once a critical tissue residue concentration is achieved (Escher and Hermens 2002; McCarty et al. 1992). Mayer and Reichenberg (2006) note, however, that hydrophobic compounds require longer exposure durations to achieve tissue concentrations sufficient to elicit toxicity due to the slow accumulation kinetics of the compounds, and that acute test durations are often insufficient for toxicity to be manifested. From the data available, it appears that toxicity increases with increased test duration for some distillates and organisms (ESG 2003), suggesting that a threshold effect concentration was likely not reached during the duration of the test for every distillate. Therefore, different toxicokinetics are expected for each distillate. It is inappropriate, though, to compare toxicity values from fixed duration tests for chemicals that have different toxicokinetics (Alda Álvarez et al. 2006). Tests of sufficient duration to achieve threshold effect concentrations, however, implicitly take into consideration differences in toxicokinetics allowing for valid comparisons between toxicity values, and are thus more appropriate to use (McCarty et al. 2011; Sprague 1969). Furthermore, loss of lower ECN constituents of some distillates is expected over the course of the test duration leading to dissimilar exposure scenarios for each distillate. Thus, in order to effectively interpret the results of toxicity

tests with petroleum distillates, an understanding of the loss of PHCs from the test unit, as well as the toxicokinetics of PHCs by organisms, is required.

In soils, exposure to and accumulation of organic chemicals by earthworms is mainly via passive diffusion through the skin from the pore water (Belfroid et al. 1996), at least for compounds with log K_{ow} of approximately less than five (Belfroid et al. 1996; Jager et al. 2003b). At its simplest, exposure and uptake can be viewed as a partitioning between soil, pore water and the earthworm (di Toro et al. 1991; Jager 1998; Sijm et al. 2000). When this partitioning is in equilibrium, a soil-water partition coefficient (K_{sw}) and a bioconcentration factor (BCF; equal to the ratio of the concentration within the organism to the concentration in the pore water at equilibrium) describe the partitioning between soil and pore water, and pore water and earthworm toxicant concentrations, respectively (Jager 1998; Sijm et al. 2000). This is the basis of equilibrium partitioning theory (Belfroid et al. 1996; di Toro et al. 1991). The biota-to-soil accumulation factor (BSAF) then describes the relationship between the concentration in the soil and the earthworm (Sijm et al. 2000). As neutral organic compounds partition mainly into soil organic carbon (OC) (Belfroid et al. 1996; di Toro et al. 1991) and the lipid phase of organisms (Belfroid et al. 1996), K_{sw} can be normalized to the fraction of organic carbon, which gives the organic carbon partitioning coefficient, K_{oc} ($K_{oc} = K_{sw}$/fraction OC), and the BCF and BSAF can be reported on a lipid and organic carbon (if applicable) basis.

In reality, soil systems and earthworm toxicokinetics are not that simple. Pore water may not be the only route of exposure (ingestion of soil particles and food may occur (Belfroid et al. 1996; Jager 1998)) and several elimination processes may simultaneously occur to reduce body burdens including excretion, biotransformation, reproduction, and growth (Jager 1998). Uptake via ingestion of soil/food becomes an important route of exposure for earthworms for contaminants with a log $K_{ow} > 5$ and dominates for those with log $K_{ow} > 6$ (Belfroid et al. 1996; Jager et al. 2003b).

Changes in bioavailability of the contaminant in the vicinity of earthworms may also affect accumulation. Bioavailability can be defined as the fraction of the bulk amount of chemical in soil accessible for uptake and assimilation by organisms (Alexander and Alexander 2000). The concentration of a contaminant in the pore water in the vicinity of earthworms may become depleted as the contaminant partitions into earthworms since the soil pore water compartment is small and not well mixed, especially if replenishment of pore water with the contaminant is limited

by slow desorption of the contaminant from the soil. This can be the case for some hydrophobic contaminants (Belfroid et al. 1996; Jager et al. 2005). In addition, microbial degradation of a contaminant in pore water can further deplete the bioavailable pool if desorption is rate-limiting (Jager et al. 2000). Movement of the contaminant into non-bioavailable sites in the soil matrix (i.e., sequestration) can also reduce the amount of contaminant available for accumulation over time (Landrum 1989; Ma et al. 1995) while loss of the bulk contaminant from the soil through volatilization, degradation, and/or leaching can reduce pore water concentrations as well (Muijs and Jonker 2010; Widianarko and Van Straalen 1996). The complex interaction between PHC contamination, soils, and earthworms, some of which change over time (i.e., bioavailable concentrations) will alter the toxicokinetics of the contaminants.

A study (Chapter 3) was initiated to determine the toxicokinetics for three PHC distillates (F2, F3a, and F3b) with the earthworm, *Eisenia andrei*, and to compare the results to available toxicity data (Chapter 2 (Cermak et al. 2010)). *E. andrei* was chosen as the model organism due to its sensitivity to petroleum intoxication (Chapter 2 (Cermak et al. 2010)), its exposure scenario to soil toxicants (oral ingestion and whole-body contact, as opposed to organ-specific (i.e., roots of plants)), and size which provided sufficient mass for the determination of tissue concentrations.

In addition, while the PHC CWS assume a 4:1 ratio of aliphatics to aromatics (CCME 2008), which is approximately true of some crude oils (Wang et al. 2003) and petroleum products (CCME 2008), it is not true of others. For example, fuel oil no. 2/diesel is only approximately 10% aromatics, while fuel oil no. 5 and heavy fuel oil range from approximately 41 to 47% aromatics (Wang et al. 2003). Other jurisdictions regulate aliphatic and aromatic PHCs separately (Edwards et al. 1997; Gustafson et al. 1997; Massachusetts Department of Environmental Protection 2003; Potter and Simmons 1998; Vorhees et al. 1999; Weisman 1998) and some studies indicate differences in the toxicity of aliphatic and aromatic PHCs (Feuston et al. 1994; Khan et al. 1986; Payne et al. 1995; van Overbeek and Blondeau 1954; Vandervaart Cook 2002) or their accumulation by organisms (Anderson 1973; Lee et al. 1972; Stegeman and Teal 1973). Therefore, toxicokinetics were determined for both aliphatic and aromatic constituents of each distillate separately in order to determine if it is reasonable to regulate each ECN range based on the concentration of petroleum hydrocarbons as a whole or whether further division into the concentration of aliphatics and aromatics for each distillate is advisable.

The following hypotheses were tested (Chapter 3):

Hypothesis: the toxicokinetics of PHC distillates F2, F3a, and F3b are not the same and therefore toxicity values for each distillate derived from fixed-duration tests are not directly comparable.

Hypothesis: the toxicokinetics of aliphatic and aromatic PHCs do not differ.

To investigate this, the following objectives needed to be fulfilled:

1) Determine the accumulation and elimination kinetics of the aliphatic and aromatic fractions of each distillate at one treatment level with one species (earthworms) over extended exposure durations.
2) Determine the concentration of aliphatic and aromatic fractions of each distillate in soil over the exposure duration.
3) Interpret the toxicity results for each distillate considering the results of the toxicokinetic study and determine if it is valid to compare toxicity values derived for different distillates using the test system described in Chapter 2.

1.4. TOXICITY AND TOXICOKINETICS OF BINARY MIXTURES OF PETROLEUM DISTILLATES F2, F3A, AND F3B

The PHC CWS regulate petroleum contamination based on the concentration in the soil of petroleum hydrocarbons within distinct ECN ranges. However, contaminated sites will rarely, if ever, contain only petroleum hydrocarbons within one CCME PHC distillate range and, instead, are likely to contain hydrocarbons from two or more CCME PHC distillate ranges yet possible interactions affecting toxicity between distillates are not considered.

For the most part, petroleum hydrocarbons have a similar mode of action, non-polar narcosis, and thus their toxicities are expected to be concentration-additive (King et al. 1996). Narcosis is a reversible, non-specific disruption of membrane function due to the accumulation of toxicant into the lipid phase of the membrane (Van Wezel and Opperhuizen 1995). In concentration addition, components of a mixture do not interact with each other. However, as each component acts

through a similar mode of action to produce toxicity, each component contributes to the common response with the degree of contribution to the response only differing based on the relative toxicity or potency of each component of the mixture (Broderius et al. 1995). The toxicity of each component can be standardized to its effective concentration (e.g., EC*x* or LC*x*, where *x* is the percent response), providing a value expressed in toxic units (TU). Once each component is expressed as a TU, the mixture toxicity can then be expressed as the sum of the toxic units (Altenburger et al. 2000):

$$Toxicity\ of\ mixture\ (TU) = \sum_{i=1}^{n} \frac{c_i}{ECx_i}$$

Equation 1-1

where n is the number of mixture components; ECx_i is the effective concentration for the endpoint of concern of the ith mixture component (when the endpoint of concern is lethality, the effective concentration is given as LC*x*); x is the percent response for the endpoint of concern; and c_i is the concentration of the ith component in the mixture.

If a mixture is concentration additive, then the EC*x* of the mixture will occur at 1 TU. If the EC*x* of the mixture occurs at a value less than 1 TU, the response is greater-than-additive (more toxic than expected); if it is greater than 1 TU, the response is less-than-additive (less toxic than expected).

Though petroleum distillates are mixtures themselves, they are treated like individual entities in the PHC CWS with individual toxicity values. A previous study used the toxicity values for the individual distillates to investigate the toxicity of binary mixtures of CCME distillates F1, F2, and F3. The results of that study suggest that the toxicity of the mixtures was less-than-additive with the earthworm *Eisenia andrei* based on the toxic unit approach (ESG 2003). The toxic unit approach should hold for mixtures of distillates as well, as long as it is assumed that the proportion of each constituent within the distillate remains unchanged regardless of whether it is tested alone or with another distillate. As interactions between and among distillates at a contaminated site may have ramifications for remediation and regulations (ESG 2003), it was considered important to both verify these effects and to determine whether the observed effects were caused by external factors affecting exposure to the distillates or to true non-additive interactions occurring within the

organism. It was speculated that a temporal separation in internal exposure to different boiling point distillates due to differences in accumulation was cause for the observed less-than-additive toxicity (ESG 2003). A study was initiated to investigate this further through toxicity testing and toxicokinetic studies with binary combinations of distillates F2, F3a, and F3b.

The following hypothesis was tested (Chapter 4):

Hypothesis: the toxicities of binary combinations of petroleum distillates are concentration-additive.

In order to test this hypothesis, the following objectives needed to be fulfilled:

1) Determine the toxicities of binary combinations of consecutive petroleum distillates (F2F3a and F3aF3b) using the toxic unit approach.
2) Determine the toxicokinetics of binary combinations of F2F3a and F3aF3b for both aliphatic and aromatic fractions. The concentrations for each distillate will be the same as those used in Chapter 3 to allow for direct comparisons of the toxicokinetics from both studies (i.e., of distillates in binary combination and tested alone).
3) Interpret the results of the binary toxicity study in light of the observed toxicokinetics to determine if the combinations of petroleum distillates are concentration-additive.

Chapter 2 **TOXICITY OF PETROLEUM HYDROCARBON DISTILLATES TO SOIL ORGANISMS**

2.1. OVERVIEW

Canadian standards for petroleum hydrocarbons in soil are based on four distillate ranges (F1, C6-C10; F2, >C10-C16; F3, >C16-C34; and F4, >C34). Concerns have arisen that the ecological soil contact standards for F3 may be overly conservative. Oil distillates were prepared and characterized, and the toxicity of F3 and two subfractions, F3a (>C16-C23) and F3b (>C23-C34), to earthworms (*Eisenia andrei*), springtails (*Orthonychiurus folsomi*), and northern wheatgrass (*Elymus lanceolatus*), as well as the toxicity of F2 to earthworms, was determined. Clean soil was spiked with individual distillates and measured concentrations were determined for select tests. Results agree with previous studies with these distillates. Reported toxicities of crude and petroleum products to invertebrates were generally comparable to that of F3 and F3a. The decreasing order of toxicity was F3a > F3 > F3b with invertebrates, and F3a > F3b > F3 with plants. The toxicities of F3a and F3b were not sufficiently different to recommend regulating hydrocarbons based on these distillate ranges. The results also suggest that test durations may be insufficient for determining toxicity of higher distillate ranges, and that the selection of species and endpoints may significantly affect interpretation of toxicity test results.

2.2. INTRODUCTION

Crude oil is a variable and complex mixture of thousands of individual compounds, the majority of which are hydrocarbons. Minor constituents include S-, N-, and O-containing heterocyclics and metals, particularly nickel and vanadium. The hydrocarbons can be divided into two broad chemical classes comprising aliphatic and aromatic compounds. Aliphatics include all alkanes (straight, branched, and cyclic), alkenes and alkynes, while aromatics include compounds containing one or more aromatic benzene rings. In crude oil, alkynes are not present, and alkenes, if present, are minor constituents. Within the aromatic grouping, alkylated compounds predominate (Potter and Simmons 1998).

The potential for environmental contamination with petroleum hydrocarbons (PHCs) is high. It is estimated that Canada has more than 250,000 potential or actual terrestrial sites contaminated with PHCs ((CCME 2008); http://www.ccme.ca/assets/pdf/pn_1399_phc_sr_std_1.2_e.pdf). Soil contamination can occur at petroleum extraction, refining and distribution facilities, during transport, through accidental spills, and by the deliberate addition of product to soil at landfarming facilities for petroleum bioremediation.

The constituents of crude oil and petroleum products have widely ranging physico-chemical properties and toxicity, making it difficult to develop suitable soil criteria for PHC-contaminated soil on the basis of the total petroleum hydrocarbon concentration. To address this problem, some jurisdictions have developed criteria based on groupings of petroleum constituents with similar environmental fate, behaviour, and toxicity (CCME 2008; Gustafson et al. 1997). These groupings are based on boiling point ranges, given as equivalent carbon number (ECN) ranges. Equivalent carbon number normalizes the carbon number of a compound, based on its boiling point, to that of the *n*-alkanes so that compounds with the same boiling point will have the same ECN (Gustafson et al. 1997). The groups may (Gustafson et al. 1997), or may not (CCME 2008), be separated further into aliphatics and aromatics.

In 2001, the Canadian Council of Ministers of the Environment (CCME) endorsed the Canada-wide standards for petroleum hydrocarbons in soil (PHC CWS) (CCME 2008) for the protection of both human health and the environment to address the management of lands contaminated with petroleum hydrocarbons in Canada. The PHC CWS regulate petroleum hydrocarbons based on PHC groups consisting of four consecutive ECN ranges (F1, C6 to C10; F2, >C10 to C16; F3, >C16 to C34; and F4, >C34), and assume a 4:1 ratio of aliphatics to aromatics (CCME 2008). However, the ECN range for F3 is very broad, including constituents with widely varying ranges of environmental fate and toxicity. Differences in the fate and toxicity of low versus high molecular weight constituents within F3 might result in non-toxic soils whose F3 concentrations exceed the PHC CWS. Studies have demonstrated a higher toxicity of lower molecular weight PHC mixtures compared to those of higher molecular weight (Dorn et al. 1998; Salanitro et al. 1997; Van Gestel et al. 2001). Constituents within the lower end of the boiling point range of F3 (aliphatics <C24 and aromatics <4 rings) are degraded more readily (Englert et al. 1993) and have a greater potential for volatilization (Gustafson et al. 1997; National Research Council 1975) than those in the upper range. Thus, over time or with remediation, there is depletion in the soil of the lower molecular weight constituents within the lower boiling point range of F3 with a concurrent proportional enrichment of higher molecular weight constituents. The lack of toxicity observed at some bioremediated sites with concentrations of F3 above the F3 PHC CWS might be due to this preferential loss of lower molecular weight constituents. In such cases, the higher molecular weight constituents become the main contributors to the F3 concentrations above the PHC CWS. If this is true, then it might be preferable to regulate F3 PHC contamination in soil based on the concentrations of smaller ECN range subfractions.

The objective of the present study was to determine the toxicity of PHC CWS distillate F3 and two subfractions of F3, F3a (ECN >C16 to C23) and F3b (ECN >C23 to C34), to three soil organisms (*Elymus lanceolatus* [northern wheatgrass], *Eisenia andrei* [earthworm], and *Orthonychiurus folsomi* [collembola]) using a variety of endpoints. This was done to determine if there were sufficient differences between the toxicities of the low and high molecular weight subfractions of F3 to warrant regulation of F3 contamination based on the smaller ECN ranges. To relate and compare the results of this and subsequent studies to the data generated previously, clean soil, oil distillates, and test methodologies similar to those used in previous work (ESG 2003) were utilized. As well, for the assessment of the toxicity of F3a and F3b, three species previously demonstrated to be sensitive to F3 toxicity (ESG 2003) were used on the premise that differences in the toxicity of F3a and F3b would be readily identified with these species. In addition, test durations were extended for several earthworm lethality tests to determine if the test durations recommended by standard methods and used in the development of the PHC CWS and in site risk assessments were sufficiently long to determine toxicity. Data from F2 earthworm lethality tests conducted for subsequent studies are included for comparative purposes.

The results are discussed in relation to the PHC CWS, as well as with regard to implications for both soil toxicity testing and risk assessment of petroleum products and other high molecular weight organic compounds.

2.3. MATERIALS AND METHODS

2.3.1. Crude oil distillates

Crude oil was supplied by Federated Pipelines (Edmonton, AB, Canada) in December 1998, and stored at the Environmental Technology Centre, Environment Canada (Ottawa, ON, Canada) until the start of this study in 2003. Federated crude oil used for the present study was used previously in a study on the toxicity of CCME PHC CWS distillates (ESG 2003).

The oil was distilled into four boiling-point distillates by Imperial Oil (Sarnia, ON, Canada): PHC CWS Fractions 2 (F2; ECN >C10 to C16) and 3 (F3; ECN >C16 to C34) (CCME 2008), and two subfractions of Fraction 3, F3a (ECN >C16 to C23) and F3b (ECN >C23 to C34). A 15-theoretical plate column distillation method (ASTM 2001a) was used to distil F2 and F3a and a vacuum potstill

distillation method (ASTM 2001b) was used to distil F3b. F3 was prepared by combining appropriate proportions of F3a and F3b. The distillates were covered with a blanket of high-purity nitrogen in 2-L metal containers and stored at room temperature. Imperial Oil provided the physico-chemical properties of the distillates. Environment Canada (Ottawa, ON) quantified and provided an in depth characterization of the aliphatics and aromatics in each distillate, described below (Environment Canada fractionation and characterization). EnviroTest Laboratories (ETL, Edmonton, AB, Canada, now ALS Environmental Group) also quantified four chemical class fractions for each distillate, described below (ETL fractionation and characterization). The ETL characterization was done to demonstrate that both the ETL and Environment Canada fractionations produced comparable results; ETL conducted all further analysis of soils in the study.

2.3.2. Environment Canada fractionation and characterization

Each oil distillate was fractionated into aliphatic (normal-, branched-, and cyclo-alkanes) and aromatic (monoaromatic hydrocarbons, polyaromatic hydrocarbons [PAHs], and sulphur-containing heterocyclics) fractions by Environment Canada (and thereby termed EC aliphatics and EC aromatics) following Environment Canada Method 5.3/1.4/M (Wang 2002) with modifications. Briefly, for each oil distillate, 0.8 g was weighed and made up to 10 ml in either hexane or pentane. Surrogates (100 µl of 200 mg/L *o*-terphenyl in pentane and 100 µl of a mixture of d_{10}-phenanthrene (10 mg/L) and d_{12}-benz[a]anthracene (10 mg/L) in iso-octane) were added to a 200 µl sample of this mixture and quantitatively transferred to a prepared silica gel column (Wang et al. 1994) which had been pre-conditioned with 20 ml pentane. Aliphatics were eluted with pentane and aromatics with 1:1 (v/v) dichloromethane:pentane. Prior to analysis, each fraction was spiked with the internal standards 5-α-androstane and d_{14}-terphenyl.

The concentrations of *n*-alkanes and total aliphatics and aromatics in the distillate were determined by high-resolution capillary gas chromatography (GC) using a Hewlett Packard (HP) 5890 gas chromatograph equipped with a flame-ionization detector (FID) and HP 7673 autosampler (Agilent). A 30 m x 0.32 mm i.d. (0.1 µm film thickness) DB-5HT fused silica column (J&W Scientific) was used. The carrier gas was helium at 3.0 ml/min. The temperature regime was: 35°C for 4 min, ramp 25°C/min to 340°C and hold for 15 min, for a total run time of 31.2 min. The injector and detector temperatures were maintained at 290°C and 325°C, respectively. For analysis, a 1.0 µl subsample of the fraction was injected in splitless mode with a 1-min purge-off. System control and data acquisition for GC-FID analysis was achieved with Chemstation (Agilent).

Target parent PAHs and petroleum-associated alkylated-PAHs were determined using an Agilent 6890 gas chromatograph equipped with a 7683 autosampler and coupled with an HP 5973 mass selective detector (Agilent) in selective ion monitoring mode (SIM). A 30 m x 0.25 mm id (0.25 µm film thickness) HP-5MS fused silica column (Agilent) was used. The carrier gas was helium at 1.0 ml/min. The injector and detector temperatures were maintained at 290°C and 300°C, respectively. For analysis, 1.0 µl was injected in splitless mode with a 1-min purge-off. Two different temperature programs were used for the determination of unsubstituted (parent) and alkylated PAHs. For unsubstituted PAHs, the temperature program was 90°C for 1 min, ramp at 15°C/min to 160°C, then ramp 8°C/min to 290°C and hold 8 min. The total run time was 30 min. For alkylated-PAHs, the temperature program was 60°C for 2 min, ramp at 6°C/min to 300°C and hold for 13 min, for a total run time of 55 min. System control and data acquisition for all gas chromatography-mass spectrometry (GC-MS) analysis was achieved with ChemStation (Agilent).

The concentrations of benzene, toluene, ethylbenzene, xylene, and 13 other alkylbenzenes were determined following Environment Canada Method 5.2/1.4/M (Wang 2003). Deuterated d_{10}-ethylbenzene was used as an internal standard. The instrumentation and column described above for PAHs was used. The temperature program was 35°C for 2 min, ramp at 10°C/min to 300°C and hold for 10 min, for a total run time of 39 min.

2.3.3. ETL fractionation and characterization

Distillates F2, F3a, and F3b were fractionated, in triplicate, by EnviroTest Laboratories into four chemical class fractions: ETL aliphatics (normal-, branched-, and cyclo-alkanes, and monoaromatic hydrocarbons); ETL aromatics (PAHs, sulphur-containing heterocyclics, and polycyclic furans); ETL nitrogen-containing heterocyclics and ETL polars (hydroxy-PAHs) (Later et al. 1981). Though aromatic in nature, the monoaromatic hydrocarbons eluted with the aliphatic fraction in this fractionation method. Each distillate was chromatographed on a neutral alumina column, calibrated to the most appropriate deactivation level, for the separation of distillates into the four chemical class fractions, as described in Appendix A. The quantity of each chemical class fraction within the oil was determined gravimetrically by evaporating a 200-µl aliquot of each fraction to dryness.

2.3.4. Toxicity tests

2.3.4.1. Soil treatment preparation

A clean, clay loam Delacour Orthic Black Chernozem soil (29.9% sand, 42.4% silt, 27.7% clay; 10.0% organic matter, pH 6.1, conductivity 0.428 mmhos/cm, water-holding capacity of 77.5%) was used in all tests (see Appendix B). This soil is classified as fine-textured following the definition presented in the PHC CWS (CCME 2008).

All calculations for preparation of the test soils were determined on a soil dry-weight basis and made by amending (i.e., spiking) clean soil with the distillates following methods previously used (ESG 2003), and as described in Appendix C. Test soils were prepared either the day before (d-1; invertebrate tests (EC 2004; EC 2007)) or on the day of (d0; plant tests (EC 2005a)) test initiation and placed into sealed test units. All test organisms were introduced on d0 of the test.

2.3.4.2. Test organisms

Test organisms were northern wheatgrass, *Elymus lanceolatus* ([Scribn. & J.G. Sm.] Gould) var. Critana (synonyms: thickspike wheatgrass, formerly *Elytrigia dasystachyum*, formerly *Agropyron dasystachyum*), the earthworm *Eisenia andrei* (André), and the collembolan *Orthonychiurus folsomi* (Schäffer). The northern wheatgrass seed lot was 98.33% pure with a 93% germination rate. Seeds were stored in the dark at approximately 4°C. *E. andrei* and *O. folsomi* were cultured together in the dark at ambient temperature (16 to 25°C, typically >20°C). Culture media consisted of an organic triple-mix soil (baked for 3 h at 100°C to kill any indigenous organisms) and sieved *Sphagnum* spp. peat moss (2-mm mesh) in a 4:5 (v/v) ratio, respectively. The substrate pH was adjusted to between 6 and 7 with calcium carbonate (Fischer Scientific, ACS grade), and hydrated to approximately 75% (on a wet weight basis) with de-ionized water. Cultures were fed finely chopped clean vegetables and/or cooked oatmeal weekly.

Earthworms were used in all studies while plants and collembola were used only in toxicity studies with Fractions 3, 3a, and 3b.

2.3.4.3. *Toxicity tests with plants and invertebrates*

Toxicity tests with plants and invertebrates were initiated prior to the publication of the Environment Canada test methods for these species (EC 2004; EC 2005a; EC 2007). All test methods followed those previously used (ESG 2003), which generally followed the methods subsequently recommended by Environment Canada with minor deviations. Test conditions and methodology specific to these experiments are provided in Table 2-1. Test methods are described briefly herein and in more detail in Appendix C.

Two repetitions of the plant definitive toxicity test with each distillate were conducted. Measurement endpoints determined for each test unit included seedling emergence, average shoot and root length, and total shoot and total root dry biomass.

Acute lethality and reproduction toxicity tests (which included chronic lethality) were conducted with collembola. The acute lethality test was only conducted once with each distillate, while the reproduction test was conducted twice. On d0, adult collembola were transferred directly from the culture to the test units. Food was not provided during the acute test, but was provided weekly (three grains of dry yeast/test unit) during the reproduction test starting on day 7. Mortality was determined in the acute lethality test (day 7) and both adult mortality and the average number of progeny produced per treatment level were determined on day 35 of the reproduction test.

Table 2-1: Summary of experimental conditions for determining the toxicity of distillates F2, F3, F3a, and F3b to plants and/or invertebrates

Test	Duration	No. of treatments	No. of replicate/ treatment	No. of organisms/ test unit	Test unit	Amount soil at 33% moisture	Lighting and temperature regime
Eisenia andrei							
Mortality	28 d (21 d for one test with F2; 42 d for two tests with F3a)	7-8	3-4	5	500-ml glass mason jar with perforated aluminum foil lid[1]	300-375 g	Continuous fluorescent, 50-100 µmol/(m²·s) 22 ° C
Reproduction	56-63 d	10	10[2]	2	same as for mortality test	375 g	Continuous fluorescent, 50-100 µmol/(m²·s) 22 ° C
Orthonychiurus folsomi							
Mortality	7 d	6-7	3	10	125-ml glass mason jar with metal lid loosely placed	30 g	Continuous fluorescent, 50-100 µmol/(m²·s) 22 ° C
Reproduction	35 d	10	10	10	same as for mortality test	30 g	Continuous fluorescent, 50-100 µmol/(m²·s) 22 ° C

Table 2-1 continued

Test	Duration	No. of treatments	No. of replicate/ treatment	No. of organisms/ test unit	Test unit	Amount soil at 33% moisture	Lighting and temperature regime
Elymus lanceolatus							
Definitive growth	25 d	9-10	3-6[3]	5	1-L clear plastic tub with clear lid[4]	480 g	Fluorescent, 300-400 µmol/(m²·s) 16:8 hr day:night 24:16 ° C day:night

[1]lids were not perforated until day 7 for all tests with F2

[2]the test with F3b had ten replicates in the control and five lowest concentrations, eight replicates in the next two concentrations and six replicates in the highest concentration tested

[3]six replicates in the control, three replicates in the three highest concentrations and four replicates in all other concentrations tested

[4]lids were removed from all test units the day the first plants reached the level of the lid on one test unit

Both earthworm lethality and reproduction toxicity tests were conducted with earthworms. Three repetitions of the lethality test were conducted for F2, F3, and F3b, and six for F3a. Reproduction was only determined for F3, F3a, and F3b (2 repetitions per distillate). For all toxicity tests, sexually mature, fully clitellated, adult earthworms were selected directly from the culture on d0. The average weight of the earthworms was within the weight range recommended by Environment Canada (250 to 600 mg) (EC 2004), although a few individuals were slightly outside of this range. For most lethality tests, the duration was extended beyond the standard 14 d, acute test duration (EC 2004; ESG 2003). All lethality tests were 28 d, except for one repetition with F2 which was 21 d and two repetitions with F3a which were extended to 42 d. To allow for the extended exposure durations, earthworms were fed approximately 5 ml cooked oatmeal on days 14 and 28. Earthworms were not fed for the initial 14 d. Cumulative mortality was assessed every 7 d.

Earthworm reproduction tests were 56 or 63 d in duration as prescribed in (EC 2004). Food (~5 ml cooked oatmeal) was added to the test units every two weeks, starting on day 14. Adults were removed on day 28 or 35. If adult mortality occurred and the data allowed, a median lethal concentration (LC50) was determined. Twenty-eight days later, the average number of progeny produced and the average total dry weight of progeny per treatment level were determined.

2.3.5. Statistical analysis of toxicity data

Statistical analyses followed the recommendations of Environment Canada (EC 2005b). Non-linear regression analyses of non-quantal data were conducted using SYSTAT® (Systat Software) (Systat Software Inc. 2004b). In cases where the data set did not fit the assumptions of normality and equal variance, a non-parametric linear interpolation method (ICp statistical program, version 2.0, U.S. Environmental Protection Agency) (Norberg-King 1993) was used to estimate the toxicity values. Logit (WEST and Gulley 1996), non-trimmed Spearman-Karber (WEST and Gulley 1996), or binomial procedures were applied to the quantal data, where appropriate (EC 2005b). All soil concentrations were log-transformed prior to analysis. Control values, when used, were assigned a log value of 0.0001, considered to be a no-effect level. Both the 50 and 20% effect concentrations were estimated, if possible. Some statistical methods are not amenable to the determination of 20% effect concentrations (Spearman-Karber and binomial methods) and, for some tests, the 20% effect estimates were below the lowest concentration tested, resulting in little confidence in the extrapolated estimates of toxicity. In these cases, no 20% effect concentration is reported. All

analysis of variance (ANOVA) procedures and determinations of data variability were conducted using SYSTAT® (Systat Software Inc. 2004b). Data normality and homoscedasticity of residuals were determined by the Shapiro-Wilk normality test and Levene's test, respectively. No- and lowest-observable-adverse-effect-concentrations (NOAECs and LOAECs) for plant emergence were determined using a one-sided Dunnett's test ($\alpha = 0.05$) (Systat Software Inc. 2004b).

All toxicity data were analyzed on a nominal concentration basis. Results were adjusted to measured concentrations using equations that described the relationship between the analytically measured concentrations to the nominal concentrations of the distillate in soil. When toxicity tests were repeated, an average toxicity value for each endpoint was determined as the geometric mean of the individual endpoints (EC 2005b). Ninety-five percent confidence limits were only calculated if four or more replicates were available; in cases with less than four replicates, data were considered to be insufficient to reliably determine confidence limits.

2.3.6. Chemical analysis of soil

Soil subsamples (50 to 200 g) were collected on the day the test soils were prepared and sent to EnviroTest Laboratories for chemical analysis. All subsamples were placed into amber glass jars, frozen (-20°C) and shipped on dry ice. Fraction 2 soil subsamples were shipped the day they were collected via overnight courier, and extracted on the day of arrival at ETL to limit the potential loss of volatile components. Approximately 5 to 10 g of soil was extracted and analyzed by EnviroTest Laboratories for ETL aliphatic and ETL aromatic hydrocarbons as described in Appendix A. All extracts were analyzed by gas chromatography with a flame ionization detector following the CCME protocol (CCME 2001b); an additional standard (*n*C23) was used to allow the reporting of F3a and F3b.

All concentrations of ETL aliphatics and ETL aromatics were corrected for the analytical recovery, as determined by matrix spikes, in triplicate, with each distillate and clean soil. Results for each distillate were also corrected for the background concentration of each distillate in the control soil. Only hydrocarbons occurring within the boiling point range of the distillate that was tested, and those immediately before and after this range, were considered to be petrogenic and used in the determination of concentration for that distillate. One exception to this was F2, for which no hydrocarbons below the ECN of C10 were quantified. The analytical method was not

suitable for the determination of such light hydrocarbons. It is assumed that these hydrocarbons constitute a very small proportion of F2.

2.3.7. Nominal versus measured concentrations

One replicate soil subsample from each of three treatment levels (low, medium and high) plus the control soil, was collected on the day the test soils were prepared from a minimum of two toxicity tests for F2, F3, and F3a; subsamples were collected from one toxicity test for Fraction 3b. When analytical data were available from other types of tests using the same spiking methodology (i.e., toxicokinetic tests, Chapter 3), these data were included as well. The analytical results from all tests for each distillate were combined to determine the relationship between the measured and nominal concentrations using linear regression procedures in SigmaPlot® (Systat Software Inc. 2004a). The regressions were not forced through the origin in order to capture any systematic errors made during soil preparation that would lead to concentrations consistently over or under the desired concentration.

Because the volumes of F2 used in the tests were small and the probability of volatilization high, two tests were conducted to determine the variability in the measured concentration at various nominal concentrations. In one test, soil was spiked with F2 at a nominal concentration of 480 mg/kg dry weight and three replicate soil subsamples collected. In the second test, soils were spiked at three nominal concentrations (400, 800, and 1,200 mg/kg dry weight) and six replicate subsamples were collected from each concentration.

SYSTAT®11 (Systat Software Inc. 2004b) was used for the statistical analyses of the data. For these analyses, measured soil concentrations, as a percent of the nominal concentration, were used.

2.4. RESULTS AND DISCUSSION

2.4.1. Characterization of oil distillates

The distillation methods produced fairly clean cuts for most petroleum distillates. Both Environment Canada and EnviroTest Laboratories reported similar aromatic and aliphatic contents for each distillate, despite the fact that monoaromatic compounds fractionated out into different chemical class fractions. This is due to the small (~2%) amount of monoaromatics present in only

one distillate (Table 2-2). Based on the total aromatic and aliphatic content, as determined by Environment Canada, 87% F2, 96% F3, 88% F3a, and 90% F3b were within the desired boiling point ranges for each distillate. The assumption that constituents within the boiling point range <ECN C10 contributes minimally to the concentration of F2, and thus need not be measured, was verified as only 4% of F2 fell within this range. See Appendix D for additional information not previously published on the characterization of the distillates.

Each distillate had distinct ranges of physico-chemical properties (Table 2-2). The aliphatic fraction comprises approximately 80% and the aromatic fraction less than 20% of the distillates, with each containing very little N-containing heterocyclics (<1%) or polar material (≤2%). Therefore, it can be said that the distillates consist mainly of aliphatics and aromatics (86% F2, >97% F3, F3a, and F3b). The total aliphatic and aromatic content of F2 was likely lower due to the loss of volatile constituents during fractionation and quantification.

Figure 2-1 and Figure 2-2 illustrate the distribution of *n*-alkanes and aromatics, respectively, for distillates F2, F3, F3a, and F3b. Typical for petroleum (Potter and Simmons 1998), within a homologous series the concentration of alkylated PAHs is greater than that of the parent PAH (Figure 2-2). While several parent and alkylated PAHs were identified and quantified, these represented only a small proportion of the total aromatics within each distillate: 35.3, 10.2, 17.6, and 2.3% (w/w) for F2, F3, F3a, and F3b, respectively. It is apparent that the majority of aromatic compounds within each distillate were not identified. Only distillate F2 contained monoaromatics (monoaromatic hydrocarbons; sum of benzene and 18 alkylbenzenes, Table 2-2) at a concentration of approximately 2% (w/w), of which only approximately 4% (w/w) was benzene, toluene, ethylbenzene, and xylene. The presence of monoaromatic hydrocarbons in F2 represents the overlap from lower boiling point ranges into F2.

Table 2-2: Physico-chemical properties of crude oil distillates.

Property	Fraction 2	Fraction 3	Fraction 3a	Fraction 3b
Equivalent carbon number	>nC10-nC16	>nC16-nC34	>nC16-nC23	>nC23-nC34
Boiling point range (°C)	>174-287	>287-481	>287-380	>380-481
Physical state @ 25ºC	liquid	liquid	liquid	solid
Total EC aliphatics (%)[1]	80.3	82.2	83.6	81.6
Total EC aromatics (%)[1]	5.8	18.3	16.2	18.7
EC Monoaromatics (%)[1, 2]	1.97	0%	0%	0%
Total ETL aliphatics (%)[3]	80.6	ND	83.6	74.8
Total ETL aromatics (%)[3]	5.6	ND	14	19.9
Total ETL N-containing aromatics/heterocyclics (%)[3]	<1	ND	<1	<1
Total ETL polars (%)[3]	0.48	ND	1.2	2

[1]As determined by the Environment Canada (Ottawa, ON, Canada). Values are from one replicate. Values are percent by weight.

[2]Sum benzene, toluene, ethylbenzene, m-, o-, and p-xylene, isopropylbenzene, propylbenzene, 3- and 4-ethyltoluene, 1,3,5-trimethylbenzene, 2-ethyltoluene, 1,2,4-trimethylbenzene, 1,2,3-trimethylbenzene, isobutylbenzene, 1-methyl-2-isopropylbenzene, 1,2-dimethyl-4-ethylbenzene, amylbenzene, and n-hexylbenzene. Values are percent by weight.

[3]As determined by EnviroTest Laboratories (Edmonton, AB, Canada) (average of three replicates). Values are percent by weight.

ND not determined

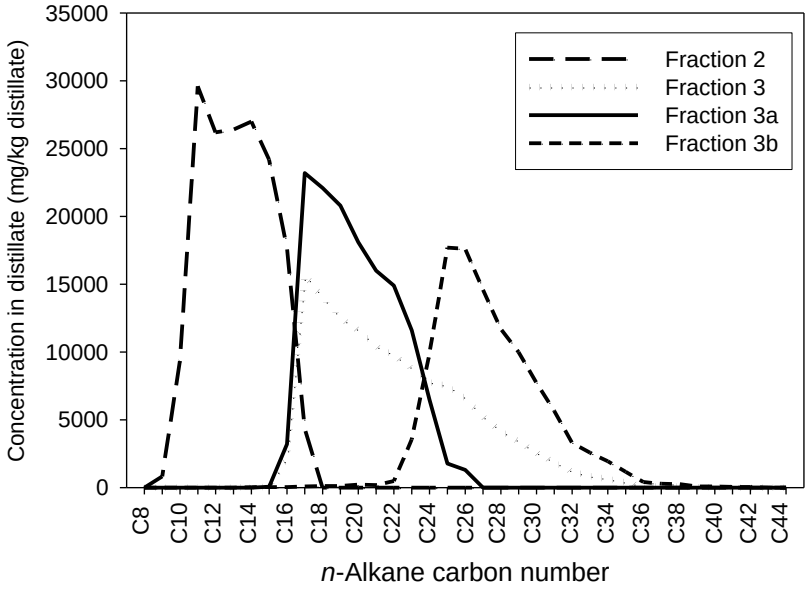

Figure 2-1: Distribution of *n*-alkanes in oil distillate Fractions 2, 3, 3a, and 3b as determined by GC-FID.

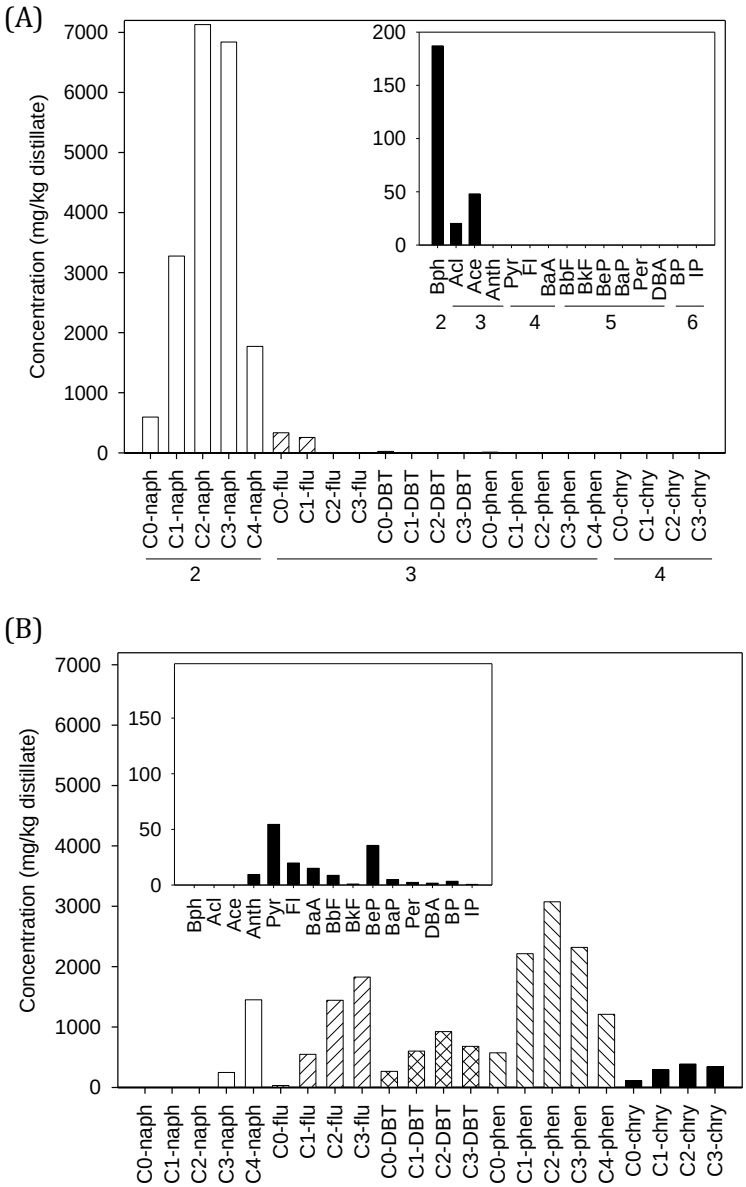

Figure 2-2: Distribution of parent and alkyl-aromatic compounds as determined by selective ion monitoring GC-MS in distillates F2 (A), F3 (B), F3a (C), and F3b (D). Compound names listed along the x-axis: 0- 4- alkylnaphthalene (C0- C4-naph); 0- 3-alkylfluorene (C0- C3-flu); 0- 3-alkyldibenzothiophene (C0- C3-DBT); 0- 3-alkylphenanthrene (C0-C3-phen); 0- 3-alkylchrysene (C0- C3-chry); biphenyl (Bph), acenaphthylene (Acl); acenaphthene (Ace); anthracene (Anth); pyrene (Pyr); fluoranthene (Fl); benz(a)anthracene (BaA); benzo(b)fluoranthene (BbF); benzo(k)fluoranthene (BkF); benzo(e)pyrene (BeP); Benzo(a)pyrene (BaP), perylene (Per); dibenzo(ah)anthracene (DBA); benzo(ghi)perylene (BP); and indeno(1,2,3-cd)pyrene (IP). Numbers below compound name in (A) indicate the number of rings. Compounds are listed in order of increasing equivalent carbon number for non-alkylated parent compounds.

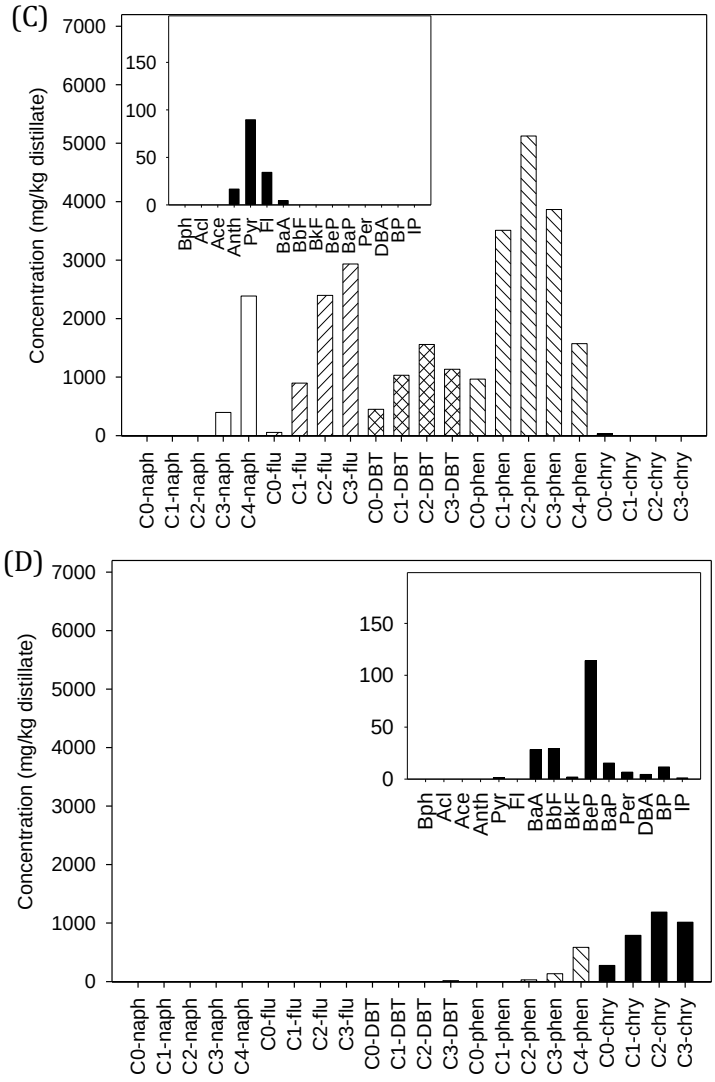

Figure 2-2 continued

Differences in the nature of each distillate will result in their different behaviour within the environment and during toxicity testing. The degree of volatilization and biodegradation of petroleum hydrocarbons will vary depending on the constituents in each distillate. The general order of degradation, from most to least easily degraded, is alkanes > isoalkanes > alkenes > monoaromatics > PAHs > high molecular weight cycloalkanes (Potter and Simmons 1998). Within the aliphatic and aromatic chemical class fractions, the lower molecular weight compounds (aliphatics <C24 and aromatics <4 rings) are most readily degraded (Englert et al. 1993), compounds associated mainly with F2, F3a, and part of F3. With regard to volatility, F2 is comprised of potentially volatile constituents (National Research Council 1975); a study with F2 noted a 55% loss of F2 due to volatilization within 24 h (Visser et al. 2003). Fraction 3a contains

some compounds with limited volatility and F3b contains compounds that are considered non-volatile (National Research Council 1975). Fraction 3 contains both volatile and non-volatile constituents.

Based on their propensity for volatilization and/or degradation, the loss of petroleum hydrocarbons is expected to decrease in the order F2 > F3 (F3a > F3b), with the quickest loss expected with the aliphatics. Very little, if any, loss is expected with F3b. Loss of constituents via volatilization during the preparation of test soils will affect the initial soil concentration during toxicity testing, while loss via volatilization and/or degradation during testing will result in changes in exposure concentrations during a test. While constant exposure concentrations are desired, this is not always achievable due to the static nature of soil toxicity tests, especially when contaminants are volatile and/or biodegradable. Therefore, differences in the behaviour of contaminants must be recognized when interpreting the results.

2.4.2. Soil treatment: Nominal versus measured concentrations

2.4.2.1. Relationship in test soils

Measured concentrations of soil were based on the total concentrations of ETL aliphatics and aromatics as these two chemical class fractions constituted the majority of the distillates. Since the relationships between the nominal and measured concentrations of these distillates were linear and highly correlated (r^2 = 0.81, 0.99, 0.94, and 0.99 for F2, F3, F3a, and F3b, respectively; see Appendix E) these regression equations were used to adjust all toxicity values from nominal to measured concentrations. These relationships were determined for concentration ranges covering all low (20%) and most high (50%) effect concentrations; some less sensitive endpoints (F3 root length; F3a shoot and root length; and F3b shoot and root length and *O. folsomi* lethality) had toxicity values beyond the concentration range described by the relationships, but the relationships were nevertheless considered sufficient for calculating the measured concentrations.

The average measured concentrations as a percent of nominal and the associated variability for each distillate are summarized in Table 2-3. The variability in the measured concentration is similar across the distillates. Average measured concentrations ranged from 45 (F2) to 91% (F3) of nominal, indicating a loss of constituents from the distillates during the preparation of test soils or subsamples. Loss of volatiles from F2 and F3a during soil preparation is expected based on their

composition, as previously discussed. Adherence of distillate to the mixing apparatus can also contribute to the loss, especially with F3b, which was highly viscous and tended to solidify on surfaces as it cooled during mixing. It is not known if this fully explains the low measured concentration of F3b.

Table 2-3: Summary of the variability in the measured concentration, as the percent of the nominal concentration, for distillates F2, F3, F3a, and F3b. Data from all concentrations were pooled for analysis.

Distillate	No. of repetitions	Mean % of nominal concentration (95% confidence limits)	Standard deviation	Standard error of mean	Coefficient of Variation (%)
F2	25	45.4 (40.2-50.6)	12.6	2.52	26
F3	6	90.7 (81.3-100.1)	8.86	3.66	9.9
F3a	7	69.0 (49.6-88.5)	21	7.94	30.4
F3b	4	62.2 (49.8-74.6)	7.81	3.9	12.6

Considering the lower measured concentration of the other distillates, the high measured concentration of F3 is surprising. The chemical make-up of F3, which is a mixture of both F3a containing volatile constituents and highly viscous F3b, might partly explain the higher measured concentration. The concentration, and thus loss, of volatile compounds would be reduced in F3 relative to F3a since the volatility of a compound is proportional to its concentration in the mixture (Potter and Simmons 1998). On the other hand, the presence of the lower molecular weight (F3a) constituents in F3 decreases the viscosity of this distillate, rendering it liquid at room temperature and mitigating the problems in mixing observed with F3b.

The variability within concentrations of F2 was also determined. The results were not concentration dependent (ANOVA, p = 0.222) and the variability (standard deviation 8.6 to 14.8) was similar to that observed for all F2 data and the other distillates.

Additional results and discussion on the relationships between nominal and measured concentrations not presented in this published article are provided in Appendix E.

2.4.2.2. Comparison to previous studies

Despite the use of a similar soil, distillates and spiking methodology, the measured concentration for F2 and F3 in the present study (Table 2-3) differed from the 29 to 31% nominal (F2) and 31% nominal (F3) observed by ESG over a concentration range comparable to that used in the present study (ESG 2003). The CCME recognized the uncertainty in the ESG measured concentrations for F3 only and noted that other studies suggest a higher recovery (typically 65 to 100%) (CCME 2008). The reason for the differences in measured concentrations for F2 and F3 between the present study and ESG are unknown. They might be due, in part, to the correction for analytical recovery used in the present study. The greater measured concentration of F2 might also be partly due to the timely extraction of the soil following sampling used here, which should limit the loss of F2. The larger data set from the present study, along with the higher recoveries noted by others (CCME 2008), suggest that the measured concentrations determined here are more accurate estimates of the actual exposure concentrations for the soil preparation methodology used.

2.4.3. Toxicity of individual oil distillates

Effect concentrations for distillates F2, F3, F3a, and F3b are presented in Table 2-4 as measured concentrations. Addition of distillates to soil had little effect on the initial or final pH and conductivity of the soil. All bioassays met the test validity criteria of Environment Canada (EC 2004; EC 2005a; EC 2007).

The decreasing order of toxicity of the distillates for each species was generally F2 > F3a > F3b. However, there were some deviations from this. For one endpoint, root length, F3b was more toxic than F3a. The reason for this is unknown, but might be related to the physical effect of F3b on soils. Soils contaminated with F3b repelled water, and the more severe effect of F3b on root growth might be due to physical changes in the soils' water holding capacity and structure and not the intrinsic toxicity of the distillate.

For invertebrates, the toxicity of F3 was intermediate to that of F3a and F3b, as expected, since F3 is a blend of F3a and F3b. However, with plants, F3 was less toxic than both F3a and F3b. The

reason for this is unknown, but the results highlight the fact that the response of organisms to petroleum distillates is complex and often species specific.

Because of the nature of each distillate, exposure was likely not at steady-state and differed between distillates. This was confirmed in a subsequent study (Chapter 3), which estimated the half-life for the loss from soil of aliphatics and aromatics of F2 to be less than the duration of all tests, of F3a to be similar to or greater than the duration of most tests, and of F3b to be considerably greater than the duration of all tests. Therefore, it could be assumed that in tests with F2, toxicity is due to a high, acute, initial exposure, while the toxicity of F3b is due to a more constant exposure and that of F3a is due to an exposure scenario intermediate to the two.

2.4.3.1. Comparison of toxicity with other studies

Only the study by ESG is known to have investigated the ecotoxicity of individual CCME distillates to terrestrial organisms (ESG 2003). Since that study was conducted using distillates, soil, and testing methodologies (ESG 2003) similar to those used here, the results of the two studies can be compared on a nominal basis, circumventing the differences in the measured concentrations noted earlier. On a nominal basis, the 50% effect concentrations for F2 and F3 were very similar between the two studies for invertebrates. Though the geometric mean of the ESG earthworm 14-d LC50s for F3 was similar to the 28-d LC50s determined in the present study, the variability was much greater, with values ranging from 5,400 to 22,400 mg/kg dry weight nominal (ESG 2003). Northern wheatgrass median inhibiting concentrations (IC50s) for F3 and shoot length were also comparable between the two studies, but this distillate appeared less toxic in the present study for root length. Plant biomass values could not be compared; the IC50 for biomass fell below the lowest concentration tested in the previous study (ESG 2003), and thus could not be reliably determined. Overall, the present study supports the values determined by ESG on a nominal concentration basis.

Table 2-4: Effective toxicity values for one plant (*Elymus lanceolatus*) and two invertebrate (*Orthonychiurus folsomi* and *Eisenia andrei*) species exposed to soil spiked with crude oil distillates F2, F3, F3a or F3b. The geometric mean of multiple tests and 95% confidence limits (in parenthesis if ≥ 4 test repetitions) or range of toxicity values [square brackets if <4 test repetitions] is provided. Single values represent values from a single test.

	50% Effect Concentration (mg/kg dry wt.)				20% Effect Concentration (mg/kg dry wt.)			
Parameter	F2	F3	F3a	F3b	F2	F3	F3a	F3b
Elymus lanceolatus								
Shoot length		13800 [12660-15080]	7480 [5240-10700]	12880 [10760-15380]		3190	770	2160
Shoot dry biomass		2890	1090	1910		980	280	770
Root length		35730 [31700-40330]	24720 [20690-29540]	15030 [12740-17750]		8380 [4270-16440]	8200 [3840-17500]	4610 [3490-6090]
Root dry biomass		4300	1760	2820		1075	320	1020
Emergence								
NOAEC		29110	20700	16920-25280				
LOAEC		38870-48630	24140-34460	33630				

Table 2-4 continued

	50% Effect Concentration (mg/kg dry wt.)				20% Effect Concentration (mg/kg dry wt.)			
Parameter	F2	F3	F3a	F3b	F2	F3	F3a	F3b
Orthonychiurus folsomi								
7-d lethality		8280	2500	19910		3230	1600	ND
35-d lethality		2330	~1090-1430	~6900		ND	ND	ND
Reproduction: No. of progeny		1100 [880-1390]	960	1540		550 [320-940]	650	700
Eisenia andrei								
14-d lethality	390 [310-480]				350 [340-360]			
28-d lethality		8630 [6640-11530]	2870 (2370-3480)	No mortality at 28070		6780 [5120-8910]	2290 (1680-3100)	No mortality at 28070

Table 2-4 continued

Parameter	50% Effect Concentration (mg/kg dry wt.)				20% Effect Concentration (mg/kg dry wt.)			
	F2	F3	F3a	F3b	F2	F3	F3a	F3b
Eisenia andrei								
Reproduction: No. of progeny		740 [690-790]	330 [320-340]	1570 [1080-2300]		350	230 [210-240]	870 [530-1440]
Reproduction: dry biomass of progeny		830 [680-1030]	660 [560-780]	1790 [1300-2470]		230 [100-540]	310 [280-340]	1010 [760-1350]

ND not determined; could not determine the 20% effect level from the data

NOAEC no observable adverse effect concentration

LOAEC lowest observable adverse effect concentration

Several others have investigated the toxicity of crude oils and petroleum products to soil organisms (Adam and Duncan 1999; Dorn et al. 1998; ESG 2003; Hanna and Weaver 2002; Li et al. 1997; Salanitro et al. 1997; Saterbak et al. 1999; Shin et al. 2005; Van Gestel et al. 2001; Visser et al. 2003; Xu and Johnson 1995). Comparisons between studies are difficult to make, since toxicity is dependent on the petroleum source, soil type, and species tested (Salanitro et al. 1997). The degree to which the PHCs in soil have aged and weathered might also affect toxicity (Alexander 1995). As well, co-contaminants such as metals and salts must be considered when comparing data from contaminated field soils. In two cases (Saterbak et al. 1999; Van Gestel et al. 2001), soils with high metal and/or salt concentrations were excluded from the following comparison.

The toxicity of crude oils and petroleum distillates to earthworms reported by others was generally comparable to that observed for earthworm mortality and reproduction with F3a and F3 (Dorn et al. 1998; Salanitro et al. 1997; Visser et al. 2003), except for a light oil that was considerably more toxic in one study (Salanitro et al. 1997). Several investigators have noted little or no earthworm mortality at PHC concentrations up to approximately 3,000 to 5,000 mg/kg (Saterbak et al. 1999; Shin et al. 2005; Van Gestel et al. 2001), but considerable mortality at concentrations greater than 10,000 to 12,000 mg/kg (Saterbak et al. 1999; Shin et al. 2005), similar to the results obtained with F3. The acute and chronic toxicity of crude oil and petroleum products to collembola (Van Gestel et al. 2001) were also comparable to that observed for F3.

Unlike that for invertebrates, the toxicity of crude oils and petroleum distillates to plants is highly variable and dependent on the species tested and endpoint considered (Adam and Duncan 1999; ESG 2003; Visser et al. 2003; Xu and Johnson 1995). For example, considering only PHC CWS distillate F3, IC50 values ranged from 3,140 to 53,000 mg/kg dry weight nominal for plant growth for three different species and various endpoints (the present study; ESG 2003). Because of this variability, comparison of responses between tests using different plant species and endpoints is not useful.

2.4.3.2. Effect of test duration on toxicity

An increase in toxicity with increasing exposure duration was noted for both earthworm and collembolan lethality (Table 2-4). The lethal toxicity of F3, F3a, and F3b to collembola was considerably greater at 35 d relative to the 7 d exposure. For earthworms, there was no additional mortality observed after 14 d of exposure to F2, while an increase in mortality occurred throughout

the 28-d exposure duration for F3 and F3a. To further explore this, the durations of two test repetitions with F3a were extended to 42 d. Mortality increased slightly up to 35 d, after which no further deaths were recorded. Earthworm lethality studies were not extended past 28 d with either F3 or F3b.

The threshold effect concentration is defined as the concentration at which there is no increase in observed effects with indefinite exposure (Persoone et al. 1990). From the limited data in the present study, it appears that for 50% lethality the threshold concentration for *E. andrei* was reached within 14 d for F2 and 35 d for F3a. The mean 28-d effective concentration (Table 2-4) is considered to be a close approximation of the 50% lethality threshold concentration for F3a since little additional mortality was noted after 28 d in the two extended tests. Only the 28-d LC50s are given in Table 2-4 as the larger data set for 28 d (6 repetitions versus 2 repetitions for the longer exposure data set) resulted in greater confidence in the mean LC50. The time required to reach the threshold concentration for 50% lethality for F3 and F3b is expected to be longer than that for F3a. The threshold concentrations for other organisms, endpoints and/or effect levels remain unknown.

Increases in toxicity with increased test duration have been observed in other studies with earthworm mortality (Hanna and Weaver 2002; Saterbak et al. 1999) and plant toxicity (Li et al. 1997; Xu and Johnson 1995). Results from short-term laboratory tests were not always reflective of field results, possibly due to the difference in exposure duration (acute versus chronic) (Visser et al. 2003). Mayer and Reichenberg (2006) argue that the lack of toxicity observed with some high K_{ow} compounds (>log K_{ow} of 5 to 6) is because the test durations are not sufficiently long for the accumulation of these compounds by the organisms. Since the log K_{ow} of oil constituents tend to increase as the boiling point range of the distillates increases, the time to reach a maximal tissue concentration (and thus a threshold concentration) should also increase as the boiling point range increases. Comparison of the toxicities of compounds should be made using threshold concentrations; otherwise, compounds may appear to be less toxic than they actually are. The change in LC50 from 7-d to 35-d with *O. folsomi* illustrates this well (Table 2-4), even though the 35-d toxicity values are not necessarily threshold concentrations. Since most toxicity tests with PHCs are of a short duration (Banks and Schultz 2005; Dorn et al. 1998; Hanna and Weaver 2002; Salanitro et al. 1997; Saterbak et al. 1999; Visser et al. 2003), there may be insufficient time for the accumulation of higher molecular weight compounds and thus for the full effect of crude oil and/or oil distillates containing these compounds to be manifested. Studies have noted the higher toxicity

of crude oils (Dorn et al. 1998; Salanitro et al. 1997) containing a large proportion of lower molecular weight compounds, which can reach toxic concentrations in the test organisms within the test period. Therefore, the toxicity of light oils and lower boiling point distillates might, in part, reflect differences in the accumulation rate of the petroleum hydrocarbons. Petroleum hydrocarbons that require longer test durations to manifest toxicity, such as distillates with higher boiling points, are either non-toxic or less toxic at shorter test durations. Therefore, it is difficult to say if the order of toxicity noted (F2 > F3a > F3b) is due to differences in intrinsic toxicity, or to the toxicity values not being threshold concentrations because of differences in the accumulation rate of each distillate. This needs further study, as remediation standards and risk assessments based on toxicity data from standard toxicity tests that are of insufficient duration to achieve threshold concentrations may underestimate the chronic toxicity of higher log K_{ow} compounds. While the data in the present study illustrate this for petroleum hydrocarbons, the same should be true for other high log K_{ow} organic contaminants.

2.4.3.3. *Sensitivity of species and endpoints*

A comparison of the sensitivity of organisms and endpoints could be made from the data for F3, F3a, and F3b (Table 2-4). A wide range in median toxicity values was obtained for each distillate, dependant on the species and endpoint tested. Toxicity was greatest for the sublethal invertebrate (reproduction) endpoints and plant dry biomass for these species, and the pattern of toxicity was similar for both the 50 and 20% effect levels.

The sublethal, invertebrate reproductive endpoints were significantly more sensitive than lethality. At the LC20 for each distillate, reproduction was inhibited from >50 to up to >90% with collembola, dependent on the test, and 100% with earthworms. Therefore, at contaminant levels displaying little or no mortality, significant effects on invertebrate populations can occur. Similarly, *E. lanceolatus* root and shoot biomass were very sensitive to PHC contamination. In most cases, a greater than 50% effect on biomass is expected at concentrations causing only a 20% decrease in shoot or root length in a 25-d test (Table 2-4). Others have also noted the sensitivity of biomass as a plant endpoint though no individual endpoint alone is sufficient for describing effects of PHCs to plants (Kulakow et al. 2000). Seedling emergence was consistently the least sensitive plant endpoint to PHCs, similar to the results of others (Dorn et al. 1998; ESG 2003; Li et al. 1997).

The large range in sensitivities of species and endpoints to PHCs is important for the assessment of risk of PHCs to soil organisms. Assessments that do not include sensitive, relevant species, or sensitive endpoints such as invertebrate reproduction or plant biomass, might result in soils being falsely judged as non-toxic. For example, seed germination and earthworm mortality assays are commonly conducted in investigations of the toxicity of PHCs (Banks and Schultz 2005; Dorn et al. 1998; Salanitro et al. 1997) or during site assessments (Saterbak et al. 1999). While no adverse effects may be noted with these endpoints, dramatic adverse effects might have been observed with other endpoints such as invertebrate reproduction or plant biomass. This phenomenon is likely not restricted to PHCs and therefore similar considerations should be undertaken during the assessment of toxicity and risk of other substances.

2.4.4. Comparison of toxicity of F3a to F3b

The toxicity of F3a and F3b was determined for a small number of organisms known to be sensitive to F3. For all endpoints other than root length, F3a was more toxic than F3b, though the magnitude of the differences in toxicity between the two distillates was small in many cases, especially for the sublethal endpoints (Table 2-4). There were insufficient data to determine if the differences between the two distillates were statistically significant. Therefore, for comparative purposes, the toxicity of F3a and F3b were judged to be different for each endpoint if the toxicity values for these distillates differed by an amount greater than the variability observed from repeated tests. For median toxicity, the range of values observed for repeated tests and for all endpoints was generally within a factor of two. This is similar to the variability observed in our lab and by others (J. Princz, Environment Canada, Ottawa, ON, Canada, personal communication) with reference toxicant tests for select endpoints with each species (EC 2004; EC 2005a; EC 2007). The 20% effect values were within a factor of 5.4 of each other due to the greater uncertainty in estimating responses at lower effect levels (Table 2-4). Thus, the toxicity of F3b for an endpoint was judged to be significantly different if the 50% effect value was two times greater and the 20% effect value was 5.4 times greater than that of F3a.

Using this definition, F3a is more toxic than F3b only for invertebrate lethality and earthworm reproduction at the 50% level, and for invertebrate lethality at the 20% effect level. Invertebrate lethality, as previously discussed, is a relatively insensitive endpoint and may not be protective of invertebrate populations. It is also unlikely that toxicity values for both F3a and F3b were at threshold concentrations for each endpoint. If threshold concentrations were used, it is likely that

the magnitude of difference between the toxicity of F3a and F3b would be less. Therefore, based on these results, it is not recommended to split F3 into F3a and F3b for regulatory purposes at this time.

2.5. CONCLUSIONS

Three distillates (F2, F3a, and F3b) were obtained using a combination of two distillation methods. A fourth distillate, F3, was created by combining appropriate proportions of F3a and F3b. The distillates have distinct physical and chemical properties that will affect their fate and behaviour, which might affect their toxicity.

Though the results of this study were comparable, on a nominal basis, to the toxicity values determined by ESG (ESG 2003), the relationships used to convert nominal concentrations of F2 and F3 into measured concentrations differed. The larger data set in the present study and the higher recoveries noted in other studies (CCME 2008) suggest that the relationships developed in the present study are more appropriate.

The order of toxicity was generally F2 > F3 (F3a > F3b). Several conclusions were reached in consideration of the toxicity data. From the data available, the toxicities of F3a and F3b are not sufficiently different to regulate F3 as these two separate subfractions; the magnitude of the difference in their toxicities was generally within the variability seen in repeated tests. In addition, test durations might be too short for determining the intrinsic toxicity of the higher ECN range distillates. The observed lower toxicity of these distillates might be an artefact of the test duration and the accumulation kinetics for the distillates. Consequently, not all toxicity values are threshold concentrations, making it difficult to compare the intrinsic toxicities of each distillate. Finally, when estimating the risk associated with contamination, test species and endpoints should reflect a range in sensitivities to decrease uncertainty associated with the risk estimates. Use of insensitive species and/or endpoints could result in toxic soils being classified as non-toxic, since significant effects on invertebrate reproduction and plant biomass occurred at concentrations showing little effect on invertebrate survival and plant germination and length measurements, respectively. These results have implications for regulatory remediation standards and risk assessment. Use of non-threshold concentration data and/or reliance on data from insensitive endpoints or species for petroleum hydrocarbon contamination, as well as with other organic contaminants, might lead to an

underestimation of risk and the development of remediation standards that are not truly protective of the terrestrial environment.

2.6. ACKNOWLEDGEMENTS

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2.7. APPENDICES FOR CHAPTER 2

2.7.1. Appendix A: ETL fractionation and analytical methodologies

2.7.1.1. ETL fractionation of distillates into aliphatic and aromatic chemical class fractions

Distillates of F2, F3a, and F3b were fractionated by EnviroTest Laboratories (Edmonton, AB), in triplicate, into four chemical class fractions designated as aliphatics, aromatics, nitrogen-containing heterocyclics, and polars following a procedure similar to that described previously (Later et al. 1981). The constituents of each chemical class fraction are described in Table A 1.

Each distillate was chromatographed on a neutral alumina column calibrated to the most appropriate deactivation level for the separation of distillates into the four chemical class fractions. The alumina column was prepared by adding 10 g of deactivated neutral alumina (70-290 mesh) in dichloromethane to a chromatography column, which was then pre-eluted with 20 ml of pentane prior to use.

The neutral alumina column was calibrated following a method modified from Later et al. (1985). Columns were prepared as above using alumina deactivated to 0, 1, 1.25, and 1.5% water. A standard containing compounds from each of the four chemical class fractions (Table A 2) was added to each column, and the compounds were eluted into the four chemical class fractions as described above. Gas chromatography coupled with mass spectrometry was used to determine into which fraction each compound eluted and at what concentration. The deactivation level that achieved the best chemical class separation was chosen for the fractionation of the oil distillates.

To fractionate the distillates, an aliquot of an oil distillate was placed directly on a prepared column at the appropriate level of deactivation, and each chemical class fraction was eluted, in sequence, with the following solvents: aliphatics with 20 ml of pentane; aromatics with 50 ml of benzene; N-containing heterocyclics with 100 ml of chloroform, and finally the polar material with 200 ml of a 19:1 methanol:water mixture. The aliphatic, aromatic, and N-containing heterocyclic fractions were concentrated to 1.0 ml using a Turbovap® (Zymark, Germany) evaporation system under nitrogen. The polar fraction was first extracted three times for one minute into 50 ml chloroform in a separatory funnel prior to concentrating to 1.0 ml. The quantity of each chemical

class fraction within the oil was determined gravimetrically by evaporating a 200-µl aliquot of concentrate to dryness.

Table A 1: Chemical class fraction of oil distillates, as fractionated by EnviroTest Laboratories, and the constituents of each fraction.

Chemical Class Fraction	Constituents
ETL Aliphatics	Aliphatic hydrocarbons and monoaromatics[1]
ETL Aromatics	PAHs, oxygen-, and sulphur-containing heterocyclics
ETL N-containing heterocyclics	Nitrogen-containing heterocyclics
ETL Polars	Hydroxy-PAHs

[1]the aliphatic fraction of Fraction 2 contains monoaromatic compounds as well as aliphatic compounds

Table A 2: Composition of standard used to determine the most appropriate level of alumina deactivation for the separation of mixtures into chemical class fractions

Chemical Class Fraction	Representative Compound in Standard
Aliphatics	n-pentacosane
Aromatics	Phenanthrene Benzo(g,h,i)perylene
N-containing heterocyclics	2-methylindole 2-aminofluoranthene 7,8-benzoquinoline or benzo(h)quinoline
Polars	2-naphthol

2.7.1.2. *ETL chemical analysis of soil*

Soil samples (50-200 g) were collected on the day of soil preparation, placed in amber glass jars and frozen immediately in a –20°C freezer. F2 soil samples were shipped on dry ice the same day sampled to EnviroTest Laboratories via overnight courier for extraction on the day they were received. This was done to reduce the loss of volatile components from the soil. All other samples were stored at -20°C for no more than 16 days before being shipped on dry ice.

As noted in Chapter 2 (Cermak et al. 2010), since each distillate contained very little N-containing heterocyclics or polar material (<3%), only the aliphatic and aromatic fractions were extracted and quantified. Extraction of the soil samples did not follow the CCME protocol (CCME 2001b). Instead, an aliquot of soil was mixed with anhydrous sodium sulphate and subjected to Soxhlet extraction overnight with 300 ml of dichloromethane:hexane (1:1). The resulting extract was concentrated, placed on a prepared gel permeation chromatography column (350 mm x 21.2 mm Phenomenex Envirosep ABC column, Phenomenex, Torrence, CA), and eluted with dichloromethane at a rate of 5 ml/min. The first 62.5 ml of eluent was discarded and the subsequent 60 ml collected for analysis. The eluent was concentrated using a Turbovap® evaporation system under nitrogen and exchanged into pentane. The soil extract was placed on a

calibrated neutral alumina column for fractionation into aliphatic and aromatic chemical class fractions, as described previously. Each fraction was concentrated to 1.0 ml using a Turbovap® evaporation system under nitrogen and analyzed by gas chromatography with a flame ionization detector (GC-FID) following the CCME protocol (CCME 2001b) with the following exception: an additional standard (*n*C23) was used to allow separate reporting of F3 as subfractions F3a and F3b.

The hydrocarbon analysis was conducted on an Agilent 6890 GC-FID equipped with a splitless injector. The GC separation was achieved using a 15 m x 0.53 mm id (0.25 µm film thickness) Restek MXT-1 column (Restek Chromatography Products, Bellefonte, PA). The carrier gas was helium at approximately 14 ml/min. The temperature regime was 40°C for two min, ramp 30°C/min to 350°C and held two min. The total run time was 14.33 min. Injection and detector temperatures were maintained at 350°C. For each sample, 1.0 µl extract was injected for analysis.

Aliphatic and aromatic fractions extracted from a control soil were analyzed by open scan gas chromatography coupled with mass spectrometry (GC-MS open-scan) to identify the background contamination. This analysis was conducted with an Agilent 6890 Series gas chromatograph coupled to an Agilent HP5973 quadrapole mass selective detector. The GC was equipped with a split/splitless injector, run in splitless mode, and a 30 m x 0.25 mm id (0.25 µm film) HP-5MS column. The carrier gas was helium with a flow rate of 1.1 ml/min. Injection and detector temperatures were 280°C and 290°C, respectively. The oven temperature was held at 40°C for four min, after which the temperature increased by 10°C/min until a final temperature of 290°C was attained. This final temperature was maintained for 16 min. The total run time was 45 min. For each sample, a 1.0-µl subsample was injected for analysis. 2,2-difluorobiphenyl (typically 25 mg/kg) was used as an internal standard. Data acquisition and analyses were conducted using Chemstation (Agilent).

Matrix spikes were prepared by EnviroTest Laboratories using clean Chernozem soil and each oil distillate, as provided by our laboratory. The soils were spiked with an aliquot of a distillate and then extracted and analyzed by GC-FID in the manner described above. This was done in duplicate with F2, F3, F3a, and F3b. The analytical recovery for the methodology was determined from the matrix spikes.

All concentrations of ETL aliphatics and ETL aromatics were corrected for the analytical recovery. Results for each distillate were also corrected for the background concentration of each distillate in the control soil. Only hydrocarbons occurring within the boiling point range of the distillate tested, and those immediately before and after this range, were considered to be petrogenic and used in the determination of concentration. One exception to this was F2, for which no hydrocarbons below the ECN of C10 were quantified. The analytical method was not suitable for the determination of such light hydrocarbons. These hydrocarbons constitute a very small proportion of F2 (see Table D 2, Appendix D).

2.7.2. Appendix B: Collection and Characterization of the Reference Soil

2.7.2.1. Materials and Methods

KBL Land Use Consulting Ltd. (Calgary, AB, Canada) collected the Ah horizon (0-0.3 m) of a Delacour Orthic Black Chernozem soil (MacMillan 1987) from a site east of Calgary, Alberta, and adjacent to the site (<5 m south) from which the soils used by ESG International Inc. were obtained (ESG 2003). Prior to soil collection, the grass and root mat was removed. Soil was then collected and sieved (4-mm) to remove larger stones and indigenous fauna. The screened material was shipped to Erb Cold Storage (Baden, ON) in 20-L plastic buckets for storage at approximately 3°C.

Soil subsamples were submitted by KBL Land Use Consulting Ltd. to Norwest Labs (Calgary, AB, Canada) to determine contaminant levels. Petroleum hydrocarbons were analyzed according to the CCME standard method (CCME 2001b). Metals were analyzed using in-house methods based on US EPA methods 245.5 and 3050B, while herbicides and pesticides were determined using in-house methods based on US EPA method 8151A and 8081A.

Physical-chemical properties of the soil were determined by the Soil and Nutrient Laboratory, Laboratory Services, University of Guelph (Guelph, ON, Canada). The pH of the soil was determined in-house using a soil slurry method (Soil Analysis Handbook 1992). A 1:2 (w/w) soil:deionized water slurry was made by adding deionized water to the soil in a beaker and stirring until a homogenous slurry was formed. The slurry was allowed to equilibrate for at least 20 min, after which it was stirred once more prior to determining the pH. The water holding capacity of the soil was determined, in triplicate, following the method of Greene et al. (1989).

A subsample of soil was submitted to EnviroTest Laboratories (ETL, Edmonton, AB, Canada) for fractionation into aliphatic and aromatic fractions and the identification of substances within these fractions by GC-MS open-scan, as described in Appendix A.

2.7.2.2. *Results and Discussion*

The results of the reference soil characterization are summarized in Table B 1.

Table B 1: Physical-chemical properties of the reference soil used

Parameter	Value
Available Phosphorus (mg/kg)	19
Available Potassium (mg/kg)	633
Available Magnesium (mg/kg)	489
Available Calcium (mg/kg)	4053
Sodium (mg/kg)	31
Chloride (mg/kg)	74
C:N Ratio	10.7
Organic Matter (%)	10.0
Total Carbon (%)	5.59
Total Organic Carbon (%)	5.54
Inorganic Carbon (%)	0.05
Total Nitrogen (%)	0.52
Ammonium (NH4-N) (mg/kg)	6.51
NO_2-N (mg/kg)	0
NO_3-N (mg/kg)	94.9
Cation Exchange Capacity (cmol+/kg)	34.5
Conductivity (mS/cm)	0.428
Soil Texture	Clay loam
Sand (%)	29.9
Silt (%)	42.4
Clay (%)	27.7

The reference soil was a clay-loam soil with high organic matter content (10%). This soil is classified as a fine-textured soil by ASTM D2487 (ASTM 2000), the classification system used by the CCME to differentiate fine- from coarse-textured soils in the PHC CWS (CCME 2000). This soil was similar to that used in previous petroleum toxicity testing (ESG 2003).

The average pH of the soil, calculated from 27 control test soils, was 6.1. The average water holding capacity (n=3) was 77.5 g/100 g dry weight soil (or 77.5%).

Pesticide and herbicide residues were below the detection limits. All metals were below the agricultural guideline values for contaminated sites in Ontario (OMEE 1997). No hydrocarbons within the ranges of petroleum distillate F1 or F2 were noted in the soil. A low concentration of hydrocarbons within the ranges of F3 (27 mg/kg) and F4 (101 mg/kg) was determined.

GC-MS open-scan analysis of the soil identified the hydrocarbons as long-chain alkanes falling within the boiling point ranges of F3a and F3b (Figure B 1 and Table B 2). These were found in both the aliphatic and aromatic fractions, indicating some crossover of aliphatics into the aromatic fraction during the alumina column fractionation, though the amount in the aromatic fraction was less than that in the aliphatic fraction. The hydrocarbons were likely biogenic in nature as no aromatic compounds were found, as would be expected if the contamination was petrogenic or pyrrogenic. Octadecanoic acid, butyl ester; hexadecanoic acid, butyl ester; and hexanedioic acid, bis(2-ethylhexyl)ester were also identified in the soil. These are sometimes found in method blanks at EnviroTest Laboratories (personal communication, Preston Kulmatycki, EnviroTest Laboratories, Edmonton, AB) and likely do not represent soil contamination.

This soil was determined to be suitable for plant growth and invertebrate survival and reproduction (EC 2004; EC 2005a; EC 2007).

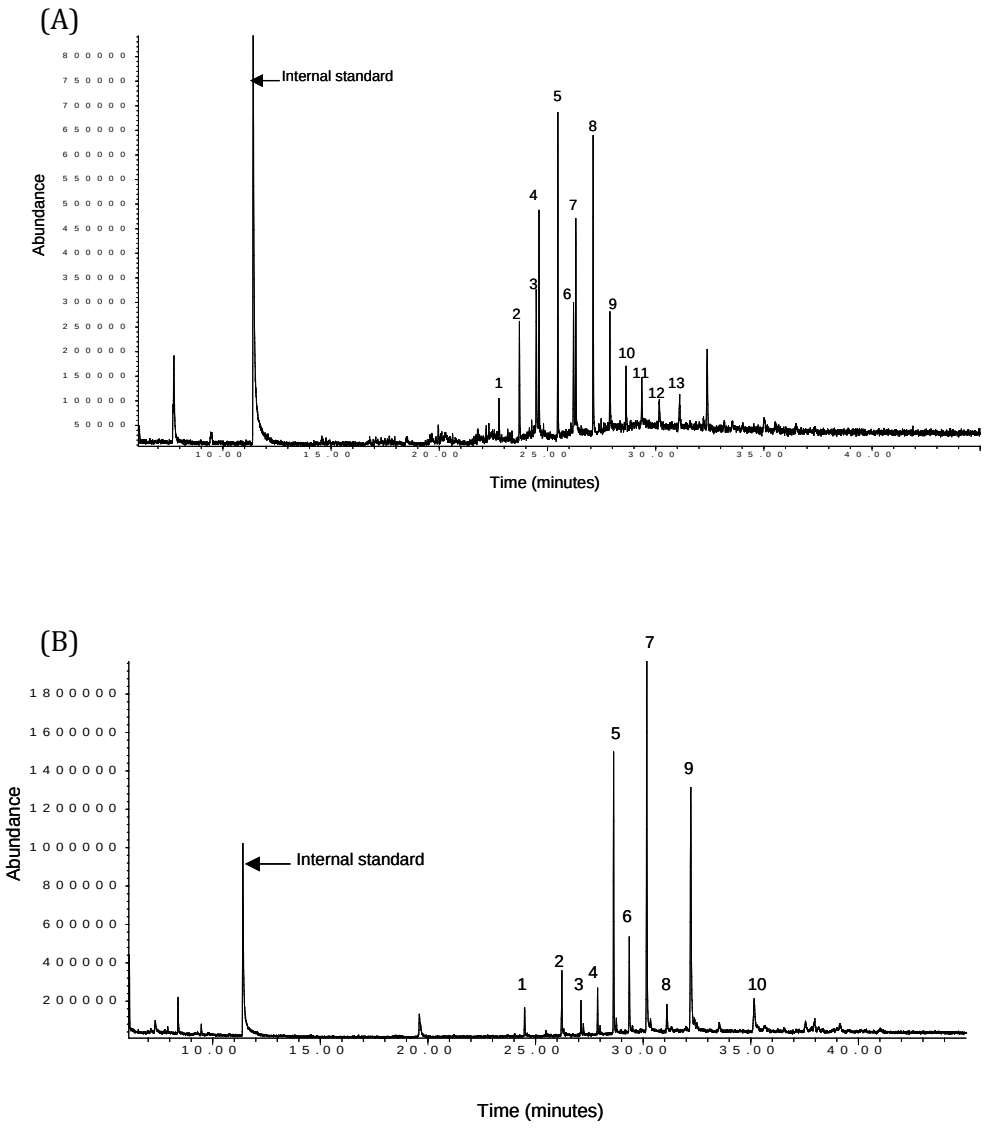


Figure B 1: Gas chromatography-mass spectrometry open-scan chromatograms of (A) aliphatics and (B) aromatics in clean control soil. The identities of the peaks correspond to the numbers provided in Table B 2.

Table B 2: Identity of peaks in the aliphatic and aromatic fractions of control soils as numbered in Figure B 1

Aliphatic Fraction		Aromatic Fraction	
Peak No.[1]	Compound	Peak No.[1]	Compound
1	$C_{20}H_{42}$	1	Hexadecanoic acid, butyl ester[2]
2	$C_{21}H_{44}$	2	Hexanedoic acid, bis(2-ethylhexyl) ester[2]
3	Octadecanoic acid, butyl ester[2]	3	$C_{25}H_{52}$
4	$C_{22}H_{46}$	4	$C_{26}H_{54}$
5	$C_{23}H_{48}$	5	$C_{27}H_{56}$
6	Octadecanoic acid, butyl ester[2]	6	$C_{28}H_{58}$
7	$C_{24}H_{50}$	7	$C_{29}H_{60}$
8	$C_{25}H_{52}$	8	Could not identify
9	$C_{26}H_{54}$	9	$C_{30}H_{64}$
10	$C_{27}H_{56}$	10	Could not identify
11	$C_{28}H_{58}$		
12	$C_{29}H_{60}$		
13	$C_{30}H_{62}$		

[1]see Figure B 1

[2]observed sometimes in laboratory blanks; therefore, likely not in the sample

2.7.3. Appendix C: Toxicity testing methodologies

2.7.3.1. Soil treatment preparation

Clean soil and oil distillates, as described in Chapter 2 (Cermak et al. 2010) and Appendix B, were used for all tests. Prior to use, all distillates were thoroughly mixed by inverting the storage container 70 times. After the appropriate amount of distillate was removed from the container, the air in the container was replaced with a nitrogen blanket by gently blowing a stream of high-purity nitrogen into the air space above the distillate for two minutes.

All treatment levels were determined on a soil dry-weight basis. For F3, F3a, and F3b, a stock soil was prepared, which was then proportionally diluted with clean reference soil for the preparation of each treatment. To make the stock soil, the distillate was added to the surface of the reference soil and the soil was mixed until visually homogenous using a hand-held electric mixer. The stock soil was covered to reduce water and petroleum hydrocarbon loss. To prepare each soil treatment level, an appropriate amount of the stock soil was diluted with clean reference soil and mixed thoroughly to produce a quantity of contaminated soil sufficient for all replicates. De-ionized water was added to each treatment in an amount sufficient to bring the moisture content of the soil to 33% (equivalent to 63.5% of the water holding capacity). The moisture content of the soil was calculated on a wet weight basis using the following equation:

$$\% \text{ moisture} = [(\text{wet weight} - \text{dry weight})/\text{wet weight}] \times 100 \qquad \text{Equation C 1}$$

The soil was then apportioned to the test units, which were sealed until the organisms were added.

F3b is solid at room temperature. Therefore, in order to make the stock soil, this distillate was heated to approximately 80°C in a water bath until melted. Once liquid, the distillate was mixed by inverting the container, and an appropriate aliquot of distillate was added to the soil and thoroughly mixed as described above.

A stock soil was not prepared with F2. This distillate contains some volatile constituents that could be lost from a stock soil during the course of preparing all test concentrations. For F2, each treatment level was individually made by spiking a sufficient amount of clean reference soil for all test replicates with an appropriate volume of the distillate. The volume of F2 corresponding to the weight of F2 required was determined from the temperature of the distillate and a graph of the

density of F2 (see Table D 1, Appendix D) versus temperature. To reduce the volatilization of petroleum hydrocarbons from the soil, the distillate was added to small indentations made into the soil; the indentations were covered immediately with soil following the addition of the distillate. The soil was hydrated to 33% with de-ionized water and mixed thoroughly for two minutes with a hand-held electric mixer. The soil was then quickly distributed among the test units and the test units sealed until the organisms were added.

Each experiment included a non-contaminated, negative control soil treatment. To prepare this treatment, clean soil was hydrated to 33% with de-ionized water and mixed thoroughly for two minutes with a hand-held electric mixer before apportioning the soil to the test units.

Test soils were prepared either the day before test initiation (d-1; invertebrate tests) or on the day of test initiation (d0; plant tests), as recommended by Environment Canada (EC 2004; EC 2005a; EC 2007). The initial pH and conductivity of all treatment levels were recorded. These were determined on a 1:2 soil:deionized water (w/w) slurry as described in Appendix B. The initial moisture content of each treatment level was also confirmed for the invertebrate tests only. All test organisms were introduced on day 0.

2.7.3.2. Toxicity test methodologies

Test conditions, including the number of replicates, number of organisms per replicate and descriptions of test units and environmental conditions, are provided in Table 2-1, Chapter 2 (Cermak et al. 2010). The toxicity tests followed the methods used by ESG (ESG 2003), which generally follows those prescribed by Environment Canada (EC 2004; EC 2005a; EC 2007) with minor deviations. The Environment Canada standard methods were not available when this project was initiated.

2.7.3.2.1. Plant definitive toxicity tests

A 25-day definitive test was conducted to determine the toxicity of each distillate to *E. lanceolatus*. On day 0, a subsample of seeds was taken out of refrigeration and acclimated to room temperature. At the time of planting, seeds were subjectively chosen based on uniformity in size, shape, and colour; those that were discoloured, an unusual size or shape, or that differed in any other manner from the general population of seeds, were discarded. Five seeds per 1-L test

unit (see Table 2-1, Chapter 2 (Cermak et al. 2010)) were planted at a depth of approximately 2-4 mm; one in the centre, and four around this central seed at a distance of approximately 2 cm. Immediately after planting the seeds, deionized water was sprayed onto the surface of the soil and clear plastic lids were placed on the test units to reduce water loss.

Test units were randomly placed within an environmentally-control growth chamber. Plants were watered with well water daily. The test units did not have drainage holes in order to prevent the leaching of the test material from the containers; therefore, care was taken to ensure that no more than 1-cm of the soil at the bottom of the test unit was saturated following watering. The clear plastic lids were kept on the test units until the time that one plant in any of the test units reached the level of the lid; after that time all of the lids were removed from all test units (EC 2005a).

At the end of the test, subsamples of soil from each test unit of a treatment level were combined and the soil pH was determined as described in Appendix B. The plants were removed from the test units and the soil cleaned from the roots under a gentle spray of water. The following parameters were determined per test unit: the number of plants emerged from the soil, the average length of the shoots and roots, and the total dry biomass of the shoots and roots.

2.7.3.2.2. Collembolan acute lethality and reproductive toxicity tests

Both a 7-d acute lethality and a 35-d reproduction toxicity test were conducted with *O. folsomi.* For both tests, on day 0, a subsample of adult collembola were removed from the culture and placed on a large (15-cm diameter) glass petri dish. Ten collembola were gently transferred from the petri dish to the test unit using a small paintbrush moistened with deionized water. As adult males are smaller than adult females, a mix of medium and large organisms was chosen to ensure that both male and female organisms were represented. It is difficult to visually determine the sex of this species (EC 2007).

Similar to previous studies (ESG 2003), a 7-d acute lethality test was conducted to determine the lethal toxicity of each fraction to *O. folsomi*. During this test, organisms were not fed. At the end of the test, mortality was assessed using a flotation method to determine the number of surviving adults. Water was added to each test unit and the contents of the test unit stirred. Air trapped under the carapace of collembola cause the organisms to float to the surface where they can be

assessed for survival. Collembola that remained immobile for 5 min following transfer to a moist petri dish were considered dead.

A 35-d reproduction test was also conducted with *O. folsomi* to determine the effect of each distillate on reproductive potential. Test soil moisture levels were visually assessed twice weekly and soil was misted with deionized water as needed. Collembola were fed three grains of dry active yeast once weekly. At the end of the test, adult survival and the number of progeny produced were determined using a flotation method. Water was added to the test unit and the contents stirred. The water, and any floating collembola, was poured into a large (15-cm) glass petri dish and the number of juvenile collembola was counted three times and an average was taken. This was repeated a second time by adding more water to the test unit, stirring, and then transferring the water and all remaining soil to a clean petri dish. Again, the number of juvenile collembola floating in the petri dish was counted three times and the average taken. The two averages were summed together to obtain the total number of juvenile collembola for each test unit. The number of surviving adult collembola was also recorded.

2.7.3.2.3. Earthworm lethality and reproductive toxicity tests

The methods used for both tests followed those used by ESG (ESG 2003), which is very similar to the standard method prescribed by Environment Canada (EC 2004). The Environment Canada standard method was not available when this project was initiated.

Toxicity tests for lethality and reproduction were conducted with the earthworm, *E. andrei*. For the lethal toxicity test, cumulative mortality was assessed on days 7, 14, 21, and 28. To do this, the contents of the test unit were emptied onto a tray and the number of live worms counted; dead earthworms cannot always be seen. An earthworm was considered to be dead if it did not respond to the touch of a probe. Mortality was the number of live worms subtracted from the initial number (five). Dead earthworms, if observed, were removed from the soil. The soil was moistened with a light spray of deionized water if needed and then placed back into the test unit. Surviving earthworms were then gently placed on the soil surface. On day 14, approximately 5-ml cooked oatmeal (1:3 v/v oatmeal:deionized water) was buried approximately 1-cm below the soil surface of each test unit. For two tests with F3a, the duration was extended to 42 d; in these cases, mortality was also assessed on days 35 (or 36) and 42, and oatmeal was added to the test unit on day 28.

Reproductive toxicity tests were conducted to determine the effect of each fraction on reproductive potential. Two clitellated adults were introduced to each test unit and allowed to reproduce for four or five weeks, after which they were removed. To do this, the contents of the test unit were emptied onto a tray and the adult earthworms removed from the soil. The soil was placed back into the test unit, ensuring that no cocoons or progeny remained on the tray. The test units were left for a further four weeks to allow cocoons to hatch and progeny to grow, after which the number of progeny and their total dry weight were determined for each test unit. Earthworms were fed approximately 5-ml cooked oatmeal every two weeks, starting on day 14. Deionized water was misted onto the soil surface on feeding days, as needed.

2.7.4. Appendix D: Additional results on the characterization of distillates

Table D 1 describes the physico-chemical properties of each distillate as provided by Imperial Oil.

Table D 1: Physico-chemical properties of distillates of crude oil provided by Imperial Oil

Property	Fraction 2	Fraction 3	Fraction 3a	Fraction 3b
Total sulphur content, wt. %	0.14	0.40	0.38	0.44
Total nitrogen content, wt. %	0.00036	0.059	0.034	0.098
Total oxygen content, wt. %[1]	5.6	0[2]	0.79	0.96
Percent of crude oil, by volume	23.5	26.7	16.7	10
Percent of crude oil, by weight	23.4	28.4	17.5	10.9
Flash point, °C	n/a	184	156	236
Reid vapour pressure, psig	<0.1	n/a	n/a	n/a
Density @ 0°C, g/ml[1]	0.8354	0.8918	0.8761	0.9143
Density @ 15°C, g/ml	0.8243	0.8814	0.8655	0.9041
Density @ 25°C, g/ml[1]	0.8160	0.8744	0.8584	0.8973

[1]calculated value

[2]value should not be zero since oxygen found in both Fractions 3a and 3b. Error in the determinations of the total S, N, C, and H content would lead to errors in the calculated oxygen content.

n/a not applicable

Table D 2 describes the amount of overlap between adjacent distillates (i.e., how "clean" the distillate cuts are). This, in combination with Figure 2-1 and Figure 2-2 from Chapter 2 (Cermak et al. 2010), demonstrate that there was minimal overlap between or among adjacent distillates, confirming that the combination of the two distillation methods produced fairly clean distillate cuts.

Table D 2: Percent of each distillate by weight found within each carbon range[1]

Distillate	ECN <C10	ECN >C10-C16	ECN >C16-C23	ECN >C23-C34	ECN >C34
Fraction 2	4.1%	87%	8.6%	0%	0%
Fraction 3a	0%	1.4%	87.5%	11.1%	0%
Fraction 3b	0%	0%	2.1%	90.1%	7.8%
Fraction 3[2]	0%	0.7%	96.3%		3%

[1] as determined by the Emergencies Science and Technology Division, Environment Canada (Ottawa, ON, Canada).

[2]Fraction 3 was determined to be 61.6% F3a and 38.4% F3b by weight by Imperial Oil Ltd. (Sarnia, ON)

2.7.5. Appendix E: Additional results and discussion on "Soil treatment: Nominal versus measured concentrations"

The relationship between the measured concentration and the nominal exposure concentration of the oil distillates was described by applying regression procedures to the analytical results from the test soils. Select treatment levels from one or more toxicity tests for each distillate were analyzed to determine the concentration of aliphatic and aromatic compounds in the spiked soils. Because the soils were spiked with the distillates, there was potential for systematic errors to occur that could result in measured concentrations being consistently higher or lower than what was expected. For this reason, the regression lines were neither forced through the origin, nor were the control (non-spiked) treatments used, in the regression analyses. The resulting regression equations for the lines fit to these data were used to adjust all toxicity values given as nominal concentrations to measured concentrations.

2.7.5.1. Fraction 2

The average analytical recovery (n = 2) of the method, as determined through matrix spikes, was 30.6% (range of 30.2-31.0%), indicating a significant loss of the distillate during the extraction, fractionation, and gas chromatography analysis of the soils. Because of this loss, all analytical results were corrected for recovery.

Eighty-seven percent of the constituents of the F2 distillate lie within the ECN >C10-C16 range (Table D 2, Appendix D) while the remainder fall within the F1 and F3a ranges. Therefore, when calculating the concentration of F2 in the soil, only PHCs within the F2 (ECN >C10-C16) and F3a (ECN >C16-C23) ranges were included, as these were considered to be petrogenic in origin and derived from the distillate spiked into the soil. While F2 contains hydrocarbons within the F1 (ECN <C10) range, these were not analytically determined and they were assumed to contribute minimally to the F2 concentration. Hydrocarbons found within ranges ECN >C23 were generally low and considered to be native to the soil used and of non-petrogenic origin.

Figure E 1 shows the relationship between the total measured concentration of F2 (sum of the aliphatic and aromatic compounds) and the nominal concentration of F2. Equations were derived relating total F2, as well as the concentration of aliphatic and aromatic compounds of F2, to the nominal concentration of F2 spiked into the soil (Table E 1).

Table E 1: The relationship between the nominal concentration of each distillate and the measured concentration of aliphatic compounds, aromatic compounds, and the total (sum of aliphatics and aromatics) of each distillate.

Equation relating measured to nominal concentration	r^2 value
Fraction 2	
Total F2 = 0.589(nominal F2 concentration) – 85.4	0.813
F2 aliphatics = 0.470(nominal F2 concentration) – 56.9	0.779
F2 aromatics = 0.119(nominal F2 concentration) – 28.5	0.732
Fraction 3	
Total F3 = 0.976(nominal F3 concentration) – 174.0	0.999
F3 aliphatics = 0.764(nominal F3 concentration) – 188.8	0.998
F3 aromatics = 0.211(nominal F3 concentration) + 14.4	0.999
Fraction 3a	
Total F3a = 0.688(nominal F3a concentration) + 55.0	0.944
F3a aliphatics = 0.560(nominal F3a concentration) + 51.1	0.900
F3a aromatics = 0.128(nominal F3a concentration) + 3.93	0.967
Fraction 3b	
Total F3b = 0.557(nominal F3b concentration) + 217.4	0.987
F3b aliphatics = 0.425(nominal F3b concentration) + 129.8	0.999
F3b aromatics = 0.132(nominal F3b concentration) + 87.6	0.862

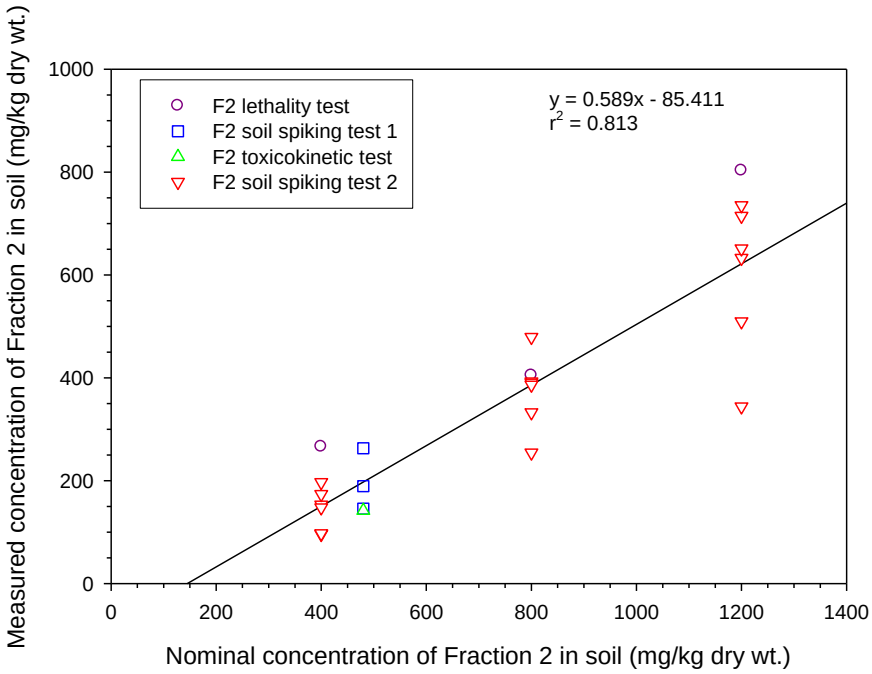


Figure E 1: Relationship between the analytically measured concentration of Fraction 2 (sum of the aliphatics and aromatics) and the nominal concentration spiked into the soil.

The regression obtained suggests that at low concentrations (below 150 mg/kg dry weight) all of F2 was lost. The validity of this has not been tested and extrapolations to concentrations below the lowest concentration tested should not be made. However, the soil concentrations tested ranged from those having no-effect to those with significant adverse effects in earthworms; thus, the relationship derived can be used to convert toxic effect levels from nominal to measured concentrations.

Table E 2 provides the concentration of each distillate as a percentage of the nominal concentration. The measured concentration of F2 in the soil ranged from 24 to 67% of the nominal concentration, with an average of 45.4 (40.2-50.6)%.

In two instances, soils were spiked with F2, separate from any toxicity tests, in order to attain further data on the variability of the measured concentration of F2. In these studies, three or six replicates of each concentration were analyzed. The measured concentrations from these two tests were within the range of those obtained from the toxicity tests, and thus all data were combined for analysis. The variability associated with the measured concentrations of F2 in the spiked soils, reported as a percent of the nominal concentration, is summarized in Table E 3.

Table E 2: The nominal and measured concentration of each oil distillate from different tests.

Experiment	Nominal concentration (mg/kg dry wt.)	Measured concentration (mg/kg dry wt.)	% of nominal
Fraction 2			
Lethality test	400	265.76	66.4
	800	404.48	50.6
	1200	802.78	66.9
Soil spiking test 1	480	263.05	54.8
	480	189.26	39.4
	480	145.51	30.3
Soil spiking test 2	400	95.98	24.0
	400	152.93	38.2
	400	97.16	24.3
	400	173.52	43.4
	400	196.60	49.2
	400	147.44	36.9
	800	392.30	49.0
	800	390.17	48.8
	800	332.42	41.6
	800	479.04	59.9
	800	386.06	48.3
	800	254.13	31.8
	1200	632.84	52.7
	1200	651.03	54.2
	1200	714.53	59.5
	1200	735.03	61.2
	1200	509.40	42.4
	1200	343.91	28.7
Toxicokinetic test	480	154.65	32.2
Average (95% confidence limit)			45.4 (40.2-50.6)

Table E 2 continued

Experiment	Nominal concentration (mg/kg dry wt.)	Measured concentration (mg/kg dry wt.)	% of nominal
Fraction 3			
Lethality test	2000	1537.57	76.9
	8000	7537.55	94.2
	16000	15523.58	97.0
Reproduction test	260	219.49	84.4
	1000	1016.34	101.6
	3800	3423.71	90.1
Average (95% confidence limit)			90.7 (81.3-100.1)
Fraction 3a			
Lethality test	1000	698.81	69.9
	3000	2068.48	69.0
	6000	4181.49	69.7
Reproduction test	200	99.3	49.6
	1000	539.48	54.0
	2500	1474.61	58.2
Toxicokinetic test	1700	1918.66	112.9
Average (95% confidence limit)			69.0 (49.6-88.5)
Fraction 3b			
Reproduction test	500	361.64	72.3
	4000	2300.94	57.5
	16000	8770.90	54.8
Toxicokinetic test	10000	6420.42	64.2
Average (95% confidence limit)			62.2 (49.8-74.6)

Figure E 1 and Table E 2 illustrate that there was considerable variability in the measured concentrations of F2 as a fraction of the nominal, even for tests where multiple replicate subsamples of the same treatment level were analyzed. Table E 3, however, demonstrates that the variability in the dose was similar across all concentrations. Therefore, variability in the dose was not concentration dependent within the range of exposure concentrations tested. The variability in the F2 data was generally within the ranges observed for F3 (Tindal 2005)[1]. The average measured concentration of F2 was also not concentration dependent. While the concentration, expressed as a percent of the nominal concentration, increased with increasing nominal concentration, this was not significant (ANOVA, $p = 0.224$).

The variability in the measured concentration, as a percent of the nominal concentration, is likely due to heterogeneity within the spiked test soils. Because of the high toxicity of F2, low contaminant concentrations were required. It is difficult to obtain homogeneously spiked soils at low contaminant concentrations due to the small amount of contaminant that is mixed into a large amount of soil. The small amount of soil used to analytically determine the soil concentration (approximately 5-10 g, or 0.3-0.5% of the total amount of test soil, by weight, prepared for a treatment level) increased the possibility that the sample did not represent the true concentration within an individual treatment level. While heterogeneity within the test soil is acknowledged, the biological toxicity data suggest that across replicates (test units) within each soil treatment, the concentration was similar (i.e., the toxicity observed across replicates is similar). This, along with the similar variability in the F2 data as compared to that of F3, indicates that the derived relationship between the measured and nominal concentrations of F2 describes the average concentration of F2 in the soil.

The study conducted by ESG International, using a similar distillate, soil, and spiking methodology, reported a greater loss of F2 during treatment preparation than noted in the current study (measured concentrations ranged from 29-33% of nominal for the concentration range 500-1,000 mg/kg nominal) (ESG 2003).

[1] Using data from Tindal (2005), the variability in the measured concentrations given as a percent of the nominal concentrations was determined. The standard deviation ranged from 7.6-16.3, the standard error from 2.5-5.4, and the coefficient of variation from 7.2-16.3% (n=6 for one data set, n= 9 for three data sets).

2.7.5.2. *Fraction 3*

The average analytical recovery (n = 2) of the method, as determined through matrix spikes, was 67.0% (range of 65.6-68.8%), indicating a loss of the distillate during the extraction, fractionation, and analysis of the sample. All results were corrected for recovery.

The constituents of the F3 distillate overlap into the F2 (ECN >C10-C16) and F4 (ECN >C34) boiling point ranges (Table D 2, Appendix D). Therefore, when determining the measured concentration of F3 in the soil, PHCs within all boiling point ranges (F2, F3a, F3b, and F4) were considered to be petrogenic in origin and used to determine the total concentration of petroleum hydrocarbons in the soil.

On average, the measured concentrations of F3 in soil were 90.7% (81.3-100.1%) of the nominal concentrations (Table E 2). Figure E 2 demonstrates that there is a very good linear relationship between the measured and nominal concentrations (r^2 = 0.999). The soil samples tested ranged from no-effect concentrations to those with significant adverse effects in sensitive species (earthworm and collembola) and sensitive endpoints (shoot length and biomass in plants). From this, an equation was developed that can be used to estimate the actual soil concentrations of F3 based on the nominal concentrations (Table E 1). It was assumed that the relationship could be extrapolated to higher concentration to adjust toxicity values to measured concentrations for those endpoints affected at higher concentrations.

Table E 3: Summary of the variability in the measured concentration of Fraction 2, as a percent of the nominal concentration, for soils spiked with Fraction 2.

Nominal Concentration (mg/kg dry wt.)	n	Mean % of nominal concentration	Standard deviation	Standard error of mean	Coefficient of Variation (%)
400	7	40.34	14.77	5.58	37
480	4	38.52	11.74	5.87	30
800	7	47.12	8.65	3.27	18
1200	7	52.26	12.95	4.89	25
All data combined	25	45.4 (40.2-50.6)	12.60	2.52	26

Since there were no replicates for any of the concentrations, all data for F3 were pooled and the variability in this pooled data determined. This was done for F3a and F3b as well. Table E 4 summarizes the variability in the measured concentration, as the percent of the nominal concentration, for F3, F3a, and F3b.

These results (Table E 2 and Table E 4 and Figure E 2) indicate that there was little loss of F3 from the soils and little error in spiking the soils with the distillate. Variability (Table E 4), as described by the standard deviation, was likely due to heterogeneity in the soil concentrations and is similar to that observed in another study (Tindal 2005) (see footnote 1, page 73). In that study, a Chernozem soil was spiked with different concentrations of F3 and triplicate subsamples analyzed for measured F3 by the CCME method (CCME 2001b) and the same Environment Canada in-house method used in the study by ESG (ESG 2003). It was determined that, on average, the measured concentration of F3 for the three replicate samples was approximately equal to the nominal concentration (Tindal 2005).

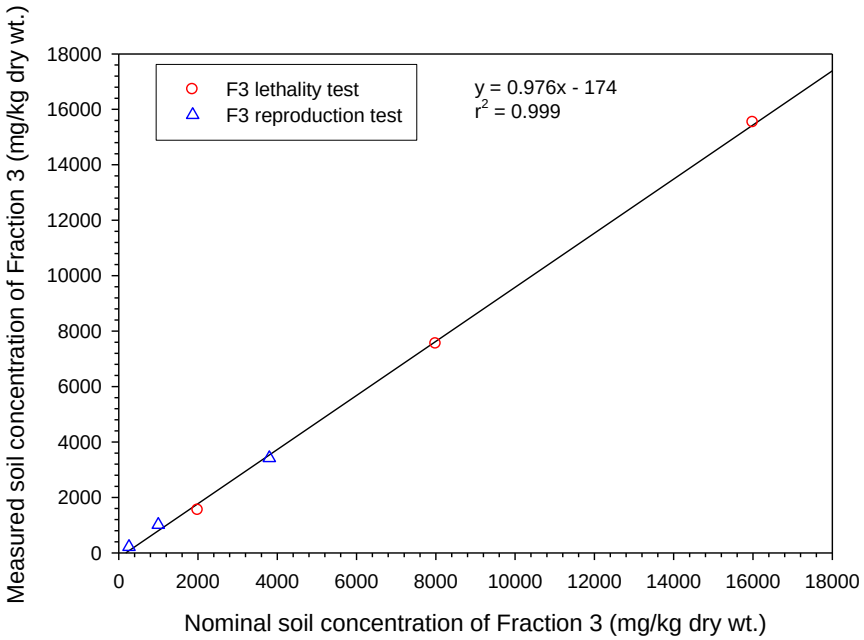


Figure E 2: Relationship between the analytically measured concentration of Fraction 3 (sum of the aliphatics and aromatics) and the nominal concentration spiked into the soil.

Table E 4: Summary of the variability in the measured concentration, as the percent of the nominal concentration, for Fractions 3, 3a, and 3b.

Fraction	Mean % of nominal concentration	Standard deviation	Standard error of mean	Coefficient of Variation (%)
F3	90.7	8.86	3.66	9.9
F3a	69.0	21.00	7.94	30.4
F3b	62.2	7.81	3.90	12.6

In the current study, the relationship between the measured and nominal concentrations of Fraction 3 was linear and resulted in measured concentrations greater than 90% of the nominal concentration. This agrees with the results observed by Tindal (2005) who obtained measured concentrations close to 100% of nominal, but differs from the relationship observed by ESG (2003). ESG tested only two samples within the data range investigated in this study and by Tindal (≤16,000 mg/kg nominal); within this range, the measured concentration was 31% of the nominal

concentration, which was much lower than that observed in this study or by Tindal. Differences in the analytical methodology might explain the difference in the results between this study and ESG; however, it cannot explain the different results obtained by Tindal (2005), as that study used the exact same methodology as ESG as well as the CCME analytical method (CCME 2001b). The weight of evidence suggests that measured concentrations of Fraction 3 are close to the nominal concentrations and that there was an error in the analysis reported by ESG. Therefore, when comparing the results of toxicity tests from this study to those of ESG (2003), the toxicity results from ESG, as nominal concentrations, was assumed to be equivalent to the results as measured concentrations.

This apparent discrepancy in the determination of the measured concentration of Fraction 3 by this study and the one conducted by Tindal (2005), and that conducted by ESG (2003), has implications for the PHC CWS. The guideline values for F3 were derived primarily from data on the toxicity of F3 determined by ESG, with measured concentrations adjusted to 31% of nominal (CCME 2000; ESG 2003). If the measured concentration is much closer to 100% of nominal, then the PHC CWS for F3 is approximately 59% lower than it should be, and is thus overly conservative.

2.7.5.3. *Fraction 3a*

The average analytical recovery (n = 2) of the method, as determined through matrix spikes, was 88.55% (range of 87.6-89.5%), indicating a slight loss of the distillate during the extraction, fractionation, and analysis of the sample. All results were corrected for recovery.

The constituents of F3a overlap into the F2 (ECN >C10-C16) and F3b (ECN >C23-C34) boiling point ranges (Table D 1, Appendix D). Therefore, during analysis, PHCs within the boiling point ranges for F2, F3a, and F3b were considered to be petrogenic in origin and used to determine the total concentration of petroleum hydrocarbons in the soil.

On average, the measured concentrations of F3a in soil were 69.0% (49.6-88.5%) of the nominal concentration (Table E 2). These results indicate that, in general, there was some loss and/or error during the treatment preparation. Figure E 3 demonstrates that there was a good linear relationship between the measured and nominal concentrations. While most measured concentrations ranged from 50-70% of nominal, one concentration was greater than the nominal concentration, probably due to heterogeneity within the soil. The soil samples tested ranged from no-effect concentrations to those with significant adverse effects in sensitive species (earthworm

and collembola) and sensitive endpoints (biomass in plants). From this, an equation was developed that can be used to estimate the actual soil concentrations of F3a based on the nominal concentrations (Table E 1). It was assumed that the relationship could be extrapolated to concentrations greater than 6,000 mg/kg nominal for those endpoints with toxicity values above this concentration.

As with F3, all F3a data were pooled and the variability in these pooled data was determined. Table E 4 summarizes this for the measured concentration, as the percent nominal concentration. The variability in the measured concentrations was the greatest for F3a when compared to the other distillates due to the one data point that was 112.9% of the nominal concentration. If this data point is excluded, the variability is similar to that of F3 and F 3b.

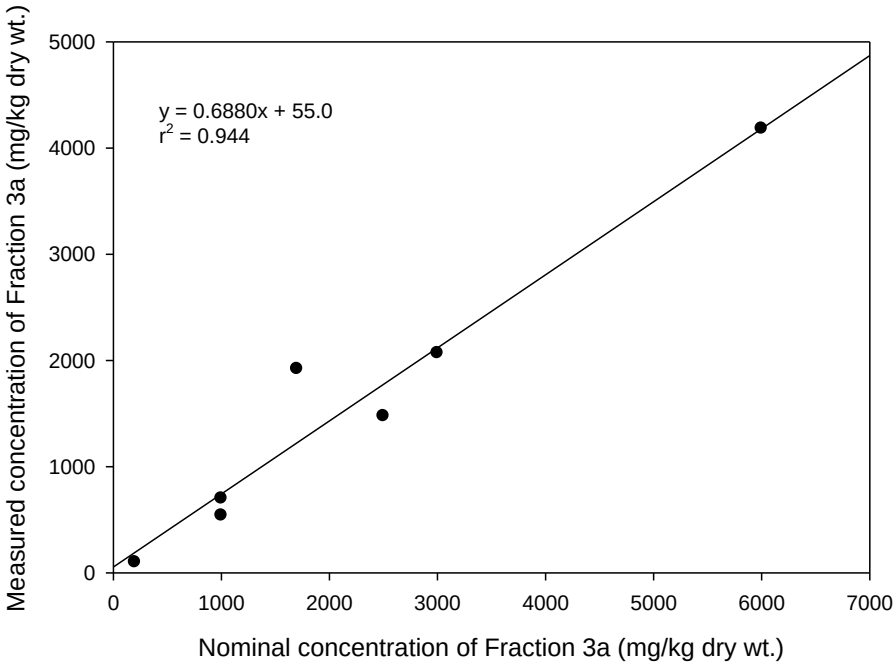


Figure E 3: Relationship between the analytically measured concentration of Fraction 3a (sum of the aliphatics and aromatics) and the nominal concentration spiked into the soil.

2.7.5.4. *Fraction 3b*

The average analytical recovery (n=2) of the method, as determined through matrix spikes, was 100% (range of 96.0-104.6%), indicating there was very little, if any, loss of the sample during sample extraction, fractionation, and analysis. No correction for recovery was thus required.

The constituents of F3b overlap into the F3a (ECN >C16-C23) and F4 (ECN >C34) boiling point ranges (Table D 2, Appendix D). Therefore, during analysis, PHCs within the boiling point ranges for F3a, F3b, and F4 were considered to be petrogenic in origin and used to determine the total concentration of petroleum hydrocarbons in the soil.

On average, the measured concentration of F3b in soil was 62.2 (49.8-74.6)% of the nominal concentration (Table E 2). These results indicate that there was some loss and/or error during the treatment preparation. F3b is a solid at room temperature, and is highly viscous even when heated, as was done for treatment preparation. Thus, during treatment preparation, some of the distillate adhered to the mixing apparatus (i.e., the mechanical mixer and stainless steel bowl). This would result in a decrease in the concentration of distillate in the soil, though this likely does not explain all of the loss observed. This low percentage of the nominal concentration might also reflect the small number of samples used in the derivation, as well as the heterogeneity within the spiked soil as a result of difficulties in mixing a highly viscous material into soil.

Figure E 4 demonstrates that there was a good linear relationship between the measured and nominal concentrations (r^2 = 0.987). From this, an equation was developed that can be used to estimate the actual soil concentrations of F3b based on the nominal concentrations (Table E 1).

The soil samples tested ranged from no-effect concentrations to those with significant adverse effects in sensitive species and endpoints (invertebrate reproduction, plant length and biomass), and thus the relationship could be used to adjust toxicity values determined as nominal concentrations to measured concentrations. It was assumed that the relationship could be extrapolated to higher concentration to adjust toxicity values to measured concentrations for those endpoints affected at higher concentrations

The relationship between measured and nominal concentrations developed for F3b suggests that there was a significant amount of F3b (217 mg/kg) in the control soil, which was not the case. Therefore, this equation cannot be used to extrapolate to nominal concentrations below those used in the derivation. No extrapolations were required for data in this study.

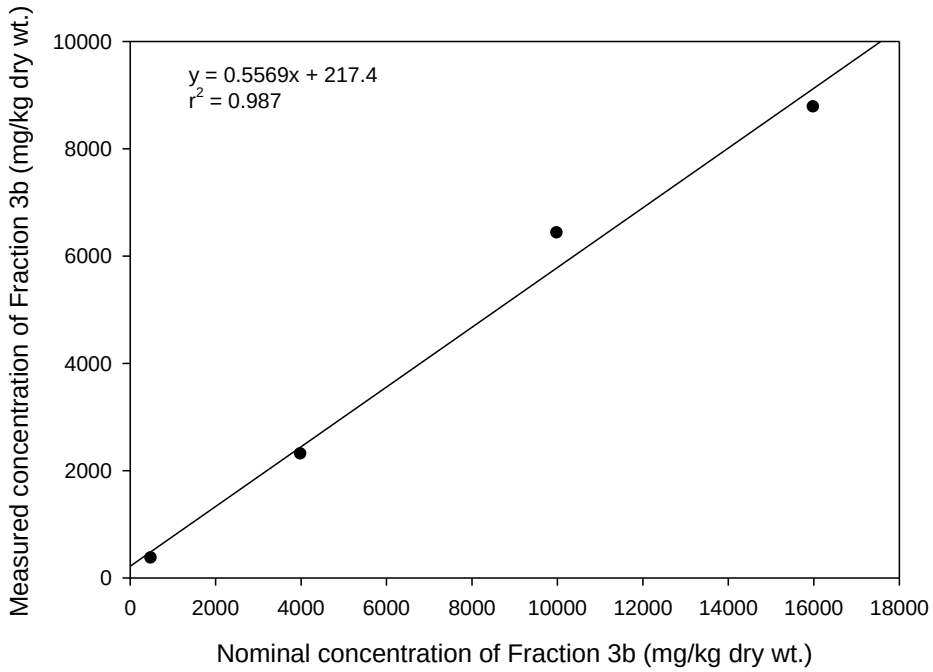


Figure E 4: Relationship between the analytically measured concentration of Fraction 3b (sum of the aliphatics and aromatics) and the nominal concentration spiked into the soil.

As with F3 and F3a, all of the F3b data were pooled and the variability of the pooled data determined. Table E 4 summarizes this for the measured concentration, as the percent nominal concentration. The variability was similar to that observed with F3.

Chapter 3 **INVESTIGATION OF THE TOXICOKINETICS OF PETROLEUM HYDROCARBON DISTILLATES WITH THE EARTHWORM *EISENIA ANDREI***

3.1. INTRODUCTION

Petroleum crude is a complex and variable mixture of thousands of individual compounds, the majority of which are aliphatic and aromatic hydrocarbons (Potter and Simmons 1998). The potential for environmental contamination with petroleum hydrocarbons (PHCs) is high. Within Canada, approximately 286,000 tonnes of oil and petroleum products are used daily (Wang and Fingas 2006) and the Canadian Council of Ministers of the Environment (CCME) estimates that there are over 250,000 potential or actual terrestrial sites contaminated with PHCs (CCME 2000).

The constituents of crude oil and petroleum products have wide-ranging physico-chemical properties and toxicity, making it difficult to develop suitable soil criteria for PHC-contaminated soil on the basis of the total petroleum hydrocarbon concentration. To address this problem, many jurisdictions have developed criteria based on groupings of petroleum constituents with similar environmental fate, behaviour, and toxicity (CCME 2000; Gustafson et al. 1997; King et al. 1996; Verbruggen 2004). These groupings are based on boiling point ranges, given as equivalent carbon number (ECN) ranges. ECN, commonly used by the petroleum industry, normalizes the carbon number of a compound, based on its boiling point, to that of the *n*-alkanes such that compounds with the same boiling point will have the same equivalent carbon number (Gustafson et al. 1997).

In Canada, the Canada-wide Standards for petroleum hydrocarbons in soil (PHC CWS) (CCME 2001a; CCME 2008) for the protection of both human health and the environment were developed to address the management of lands contaminated with petroleum hydrocarbons. These standards regulate petroleum hydrocarbons based on the concentration of petroleum hydrocarbons within four ECN ranges: F1 (C6-C10); F2 (>C10-C16); F3 (>C16-C34); and F4 (>C34) (CCME 2000; CCME 2008). The results of toxicity studies with these distillates (Cermak et al. 2010; ESG 2003), as well as two subfractions of F3, F3a (ECN >C16-C22) and F3b (ECN >C22-C34) (Cermak et al. 2010), suggest that though toxicity generally decreased with increasing ECN range (Cermak et al. 2010; ESG 2003), threshold effect concentrations (defined as the concentration at which there is no increase in observed effects with indefinite exposure (Persoone et al. 1990)) were likely not reached during the duration of the test for all organisms and/or distillates, especially with the highly hydrophobic, higher ECN distillates despite the use of longer-than-standard lethality test durations (Cermak et al. 2010). Mayer and Reichenberg (2006) state that, due to the slow accumulation kinetics of highly hydrophobic compounds (i.e., log K_{ow} > 6), acute test durations are often insufficient for toxicity to be manifested. Due to their very different physical-chemical

properties (Cermak et al. 2010), different toxicokinetics are expected for each distillate. Tests of sufficient duration to achieve threshold effect concentrations, however, implicitly take into consideration differences in toxicokinetics allowing for valid comparisons between toxicity values, and are thus more appropriate to use (McCarty et al. 2011; Sprague 1969). Furthermore, loss of lower ECN constituents of some distillates is expected over the course of the test duration (Chapter 2 (Cermak et al. 2010)) leading to dissimilar exposure scenarios for each distillate.

The PHC CWS regulate soil contamination based on the concentration of petroleum hydrocarbons within the four ECN ranges, assuming an 4:1 ratio of aliphatics to aromatics (CCME 2008). While this ratio is approximately true of some crude oils (Wang et al. 2003) and petroleum products (CCME 2008), it is not true of others. For instance, the constituent composition of fuel oil no. 2/diesel is approximately 10% aromatics, while that for fuel oil no. 5 and heavy fuel oil range from approximately 41-47% aromatics (Wang et al. 2003). Some jurisdictions regulate aliphatic and aromatic PHCs separately (Edwards et al. 1997; Gustafson et al. 1997; Massachusetts Department of Environmental Protection 2003; Potter and Simmons 1998; Weisman 1998) while other organizations recommend considering aliphatics and aromatics separately when estimating toxicity (i.e., CONCAWE (http://concawe.be/content/default.asp?PageID=778, (Redman et al. 2006)) and the Netherlands (Verbruggen et al. 2008)). Studies indicate differences in the toxicity of aliphatic and aromatic PHCs (Feuston et al. 1994; Khan et al. 1986; Payne et al. 1995; van Overbeek and Blondeau 1954; Vandervaart Cook 2002).

A study was initiated to determine the toxicokinetics of three PHC distillates (F2, F3a, and F3b) by the earthworm, *Eisenia andrei*, as well as the loss of PHCs from the test unit, in order to gain an understanding of how toxicokinetics may affect toxicity and to assist in the interpretation of results from previous toxicity tests (Chapter 2 (Cermak et al. 2010)) with these petroleum distillates. PHC accumulation was determined as accumulated aliphatics and aromatics separately in this study to determine if it is reasonable to regulate PHCs as total PHC concentration within a given ECN range, as is currently done for the Canada-wide Standards for Petroleum Hydrocarbons in soil, or whether further division to aliphatic and aromatic hydrocarbons is advisable.

3.2. MATERIALS AND METHODS

3.2.1. Experimental design

Clean soil (Orthic Black Chernozem: 29.9% sand, 42.4% silt, 27.7% clay, pH 6.1, 10.0% OM, 5.54% OC) and three crude oil distillates (F2, ECN >C10-C16; F3a, ECN >C16-C23; and F3b, ECN >C23-C34) were used as previously described (Chapter 2 (Cermak et al. 2010) and Appendices B and D). Earthworms (*Eisenia andrei*) were cultured in-house (Chapter 2 (Cermak et al. 2010)) and used to determine the accumulation and elimination of aliphatic and aromatic compounds following exposure to soil amended with the individual distillates. A summary of the test conditions is provided in Table 3-1.

Clean soil was spiked at one concentration (Table 3-1) for each individual oil distillate and hydrated to 33% moisture (wet weight basis) with deionized water, as described in Chapter 2, Appendix C. Each test unit consisted of 300 g soil wet weight in a 500-mL glass mason jar with an aluminum foil lid. The lids were perforated on day 0 for tests with F3a and F3b, and on day 8 for F2, similar to that done for earlier toxicity tests (Chapter 2 (Cermak et al. 2010)). Tests were conducted at non-lethal concentrations. The test concentrations for F3a and F3b were within the range affecting reproduction (Table 2-4, Chapter 2 (Cermak et al. 2010)). As reproduction is affected by F2 at concentrations similar to those causing mortality (ESG 2003), it was tested at a concentration approximately half of the LC50 (Table 2-4, Chapter 2 (Cermak et al. 2010)).

Due to the amount of test soil required, it was prepared in batches. One replicate from each batch was destructively sampled during each sampling episode. Test soils were prepared and placed into the test units one day prior to the addition of five sexually mature, fully-clitellated adult *E. andrei*. All earthworms were 350 to 600 mg wet weight and were not depurated prior to test initiation. This was done to ensure that exposure conditions during this study were similar to those of toxicity tests conducted previously (Chapter 2 (Cermak et al. 2010)), such that these toxicity results could be interpreted in light of the accumulation and elimination kinetics for each distillate.

Each test consisted of an accumulation phase and an elimination phase. The duration of the accumulation phase was 16 d for F2 and 64 d for F3a and F3b, with six or seven sampling times during the course of the accumulation phase (Table 3-1). At each sampling time, three or four independent replicates were destructively sampled, providing 4 to 8 g wet weight tissue for

analysis. On day 8 (for F2) or day 16 (for F3a and F3b), earthworms from 12-16 test units were removed from the contaminated soil and placed into clean soil (33% moisture, wet weight basis) for the elimination phase of the study; this became day 0 of the elimination phase. There were four sampling times during the elimination phase: one, two, four, and eight days following transfer of the earthworms into clean soil. At each sampling time, three or four independent replicates were destructively sampled, providing 4 to 8 g wet weight of tissue for analysis.

During the accumulation phase, both soil and earthworm tissues were sampled. During the elimination phase, only earthworm tissue was sampled. Soil samples were collected in 250-ml amber glass jars with minimal headspace and frozen (-20°C) until extracted. For tissue samples, earthworms were removed from the soil, rinsed in ultrapure (Type 1) water (Barnstead E-Pure, Thermo Fisher Scientific, Waltham, MA), placed on moistened glass-fibre filter paper in a glass petri dish and allowed to depurate their gut contents for 24 h in the dark at room temperature. *E. andrei* were found to depurate 97% of their gut contents within this time period (unpublished data). Following depuration, the earthworms were rinsed with ultrapure water, blotted dry, weighed, and flash frozen in liquid nitrogen. Frozen earthworms were placed into 40-ml amber glass vials with Teflon-lined caps and frozen at -20°C pending extraction.

Earthworm tissues from replicates collected per sampling episode were pooled for analysis because of the cost. Therefore, the resultant value for each sampling episode is considered to represent the average tissue concentration for that sampling time. Doing this ensured that a sufficient number of sampling times could be incorporated into the test design to describe the accumulation and elimination kinetics for each distillate. Soil samples from each sampling time were also pooled to obtain an average soil concentration for each sampling time.

Table 3-1: Experimental conditions for studies on the accumulation of elimination of oil distillates from soil by *E. andrei*

Distillate (nominal concentration, dry weight)	No. of replicates sampled per sampling period	No. of organisms/ replicate	Sampling days for accumulation	Day of transfer of earthworms to clean soil for elimination	Lighting and temperature regime
F2 480 mg/kg	3-4	5	Days 0, 1, 2, 4, 8, and 16	Day 8	Continuous fluorescent, 50-100 µmol/(m^2·s) 22°C
F3a 1,700 mg/kg	3-4	5	Days 0, 2, 4, 8, 16, 32, and 64	Day 16	Continuous fluorescent, 50-100 µmol/(m^2·s) 22°C
F3b 10,000 mg/kg	3-4	5	Days 0, 2, 4, 8, 16, 32, and 64	Day 16	Continuous fluorescent, 50-100 µmol/(m^2·s) 22°C

Earthworms were not fed for the first 16 d of the accumulation phase or for the elimination phase. For those tests where the accumulation phase of the study was greater than 16 d, approximately 5 ml of cooked oatmeal (1:3 oatmeal:deionized water, v/v) was added to each of the remaining test units in the accumulation phase of the study on days 16 and 32. This is similar to the toxicity tests, where earthworms were fed every 14 d starting on day 14. As well, deionized water was added on days 16 and 32 to each test unit equivalent to the measured amount lost (by weight).

All glassware and metal instruments were initially washed with soap and water, solvent rinsed with dichloromethane, washed a second time and rinsed with deionized water. Glassware and glass fibre filters were baked at 475°C for 4 h prior to use to remove any organic contaminants; aluminum foil used to line trays onto which the contents of the test units were emptied during sampling was baked at 450°C for 4 h since higher temperatures resulted in brittle foil.

3.2.2. Chemical analysis

Since aliphatic and aromatic compounds comprise the majority of each distillate (Table 2-2, Chapter 2 (Cermak et al. 2010)), only the concentration of these compounds were determined for both soil and tissue samples. Constituents of the aliphatic fraction include normal-, branched-, and cyclo-alkanes; those of the aromatic fraction include polycyclic aromatic hydrocarbons (PAHs), sulphur-containing heterocyclics, and polycyclic furans. Monoaromatics, present only in F2, also elute with the aliphatic fraction. Soil samples (~5-10 g) were extracted and fractionated into aliphatics and aromatics by EnviroTest Laboratories (ETL; Edmonton, AB, Canada) as described in Chapter 2, Appendix A. Frozen tissue samples were ground with sodium sulphate and then extracted, fractionated, and analyzed in the same manner as soils. A 1.0-ml aliquot of the tissue extract was removed following extraction for the gravimetric determination of the tissue lipid content.

The aliphatic and aromatic fractions of all samples were analyzed by gas chromatography with flame ionization detection (GC-FID) (as described in Chapter 2, Appendix A), to determine the total concentration of petroleum hydrocarbons within each distillate range. Select samples were analyzed by selective ion monitoring (SIM) gas-chromatography mass spectrometry (GC-MS) to determine the concentration of specific aromatic hydrocarbons. SIM GC-MS was conducted using an Agilent 6890 Series gas chromatograph oven coupled to an Agilent HP5973 quadrapole mass

selective detector. The GC was equipped with a split/splitless injector, run in splitless mode, and a 30 m x 0.25 mm id x 0.25 µm film DB-5MS column (J&W Scientific). The carrier gas was helium with a flow rate of 1.2 ml/minute. The injection and detector temperatures were 290°C. The temperature program was: 80°C initial temperature, ramp by 15°C/min to 315°C and hold for 3.83 min. A 1.0-µl subsample of extract was injected for analysis. For SIM GC-MS, data acquisition and analysis was conducted using Chemstation (Agilent).

GC-FID results for all soil and tissue samples were corrected for analytical recovery, as determined through matrix spikes. Soil matrix spikes used whole distillates, while tissue matrix spikes consisted of clean tissue spiked with appropriate amounts of aliphatic and aromatic fractions of a distillate. GC-MS SIM results were also corrected using the same analytical recoveries despite different detection methods. It was assumed that the recovery was similar between the GC-FID and GC-MS methods.

All tissue results are reported on a lipid basis (mg/kg lipid) following correction for the average background concentration of aliphatics and aromatics of all control earthworm tissues. Tissue concentrations were considered to be significantly greater than background if they were greater than two standard deviations above the average control tissue concentration for each ECN range (Jager et al. 2003a). Tissue concentrations less than this were considered to be zero. Likewise, soil concentrations were also corrected for the background concentrations of aliphatic and aromatic compounds in the clean soil used for each test.

3.2.3. Data analysis for accumulation and elimination

All models were fit using nonlinear regression procedures (nonlin program in SYSTAT® 11 (Systat Software Inc. 2004b)).

3.2.3.1. Loss of petroleum hydrocarbons from soil

Changes in the concentration of PHCs in soil over time were modelled using a first-order exponential decay model:

$$C_{soil}(t) = C_{soil}(0)e^{-ks(t)}$$

Equation 3-1

where $C_{soil}(t)$ is the concentration of aliphatics or aromatics (mg/kg dry weight) at time t (day), $C_{soil}(0)$ is the concentration of aliphatics or aromatics (mg/kg dry weight) at the start of the test (i.e., day 0), and k_s (day^{-1}) is the rate constant for loss from soil.

The half-life for loss from soil ($t_{1/2\ soil}$; day) was calculated as:

$$t_{1/2\ soil} = \ln 2/k_s$$

Equation 3-2

3.2.3.2. *Earthworm elimination*

Earthworm elimination data were fitted to a one-compartment, first-order kinetic elimination model:

$$C_{worm}(t) = C_{worm}(0)e^{-k2\ elim(t)}$$

Equation 3-3

where $C_{worm}(t)$ is the tissue concentration of the earthworm (mg/kg lipid) at time t (day) during the elimination phase, $C_{worm}(0)$ is the tissue concentration (mg/kg lipid) of the earthworm at the start of the elimination phase, and $k_{2\ elim}$ is the elimination rate constant (day^{-1}).

The half-life for elimination from earthworms ($t_{1/2\ elim}$; day) was calculated as:

$$t_{1/2\ elim} = \ln 2/k_{2\ elim}$$

Equation 3-4

3.2.3.3. *Earthworm accumulation*

Accumulation data were fitted to a modified first-order kinetic model that accounted for a decrease in the amount of the distillate bioavailable for accumulation (Jager et al. 2000; Landrum 1989):

$$C_{worm}(t) = [(k_1 C_{soil}(0))/(k_{2\ elim} - k_{dis})] \times [e^{-kdis(t)} - e^{-k2\ elim(t)}]$$

Equation 3-5

where $C_{soil}(0)$ is the soil concentration (mg/kg dry weight) on day 0, k_1 is the accumulation rate constant (kg dry weight/(kg lipid·day)), k_{dis} is a rate constant (day^{-1}) describing the change in the bioavailable concentration of the contaminant, $k_{2\ elim}$ is the elimination rate constant (day^{-1}), and t is the time (day) during the accumulation phase. The elimination rate constant as determined from the elimination phase data ($k_{2\ elim}$) was used in this model rather than calculating an elimination rate constant for the accumulation phase ($k_{2\ uptake}$) since the model could not estimate k_{dis}, k_1, and $k_{2\ uptake}$ simultaneously. $C_{soil}(0)$ was analytically determined from a composite sample of soil collected on day 0.

When there was no change in the available concentration of distillate, k_{dis} was zero and the equation reverted to the standard one-compartment, first-order kinetic model:

$$C_{worm}(t) = [(k_1 C_{soil}(0))/k_{2\ uptake}] \times [1 - e^{-k2\ uptake(t)}]$$

Equation 3-6

In this case, $k_{2\ uptake}$ (d^{-1}), is the elimination constant estimated for the accumulation phase.

3.2.4. Biota-soil accumulation factors

Soil accumulation factors (BSAFs) were determined using Equation 3-7 for total aliphatics and aromatics as determined by GC-FID using both the highest measured earthworm concentration and the maximum modelled earthworm concentration (defined as the peak value or as 95% of the maximum). In addition, time-dependent BSAFs were also determined using Equation 3-7 for the individual aromatic hydrocarbons quantified by SIM GC-MS on days having approximately the greatest tissue concentrations, according to the experimental data, for each individual distillate (day 2 for F2, day 16 for F3a, and day 32 for F3b).

$$BSAF_{(t)} = C_{worm(t)}\ (mg/kg\ lipid)/C_{soil\text{-}OC(0)}\ (mg/kg\ OC)$$

Equation 3-7

where $BSAF_{(t)}$ is the biota-soil accumulation factor at time (t), $C_{worm(t)}$ is the lipid-normalized PHC concentration in the earthworm tissue at time (t), and $C_{soil\text{-}OC(0)}$ is the organic carbon (OC)-normalized concentration in the soil on day 0.

3.3. RESULTS

3.3.1. Loss of distillates from soil

A loss of aliphatics and aromatics with time was noted for each distillate, with rate constants (k_s) and half-lives presented in Table 3-2. The rate of loss from the soil was greatest for F2 ($t_{½}$ ~ 10 d) and least for F3b ($t_{½} \geq 87$ d), confirming the expectation of greatest loss of F2, followed by F3a, over the duration of the toxicity tests and the much lower loss of F3b (Cermak et al. 2010). While little difference was observed in the half-lives for aliphatics and aromatics for F2, the half-lives for aliphatics were less than those for aromatics for both F3a and F3b. The rate constants describing the loss of F2 from soil, however, should be used with caution, as there was considerable variability in the data and one data point (day 16) was excluded from the analysis as it was unexpectedly greater than the concentrations for any previous sample time. The reason for this is unknown but the tissue concentrations (see below) suggest that the day 16 data point was erroneous as F2 tissue concentrations were decreasing by that time.

Table 3-2: Rate constants for loss from soil and accumulation and elimination by *E. andrei* of petroleum distillates

Parameter	Fraction 2	Fraction 3a		Fraction 3b
	d0-d16	d0-d16	d0-d64	d0-d64 data
Aliphatics				
k_1 (kg dry wt.·[kg lipid·d]$^{-1}$)	25.85	2.06	0.24	0.149
k_{dis} (d^{-1})	0.039	n/a	n/a	n/a
$k_{2\ uptake}$ (d^{-1})	nd	0.955	0.025	0.035
$k_{2\ elim}$ (d^{-1})	0.496	0.328	0.328	0.302
$t_{1/2\ elim}$ (d)	1.4	n/a	2.1	2.3
k_s (d^{-1})	0.071	n/a	0.02	0.008
$t_{1/2\ soil}$ (d)	9.8	n/a	34.7	86.6
Aromatics				
k_1 (kg dry wt.·[kg lipid·d]$^{-1}$)	400.65	n/a	18.22	0.907
k_{dis} (d^{-1})	0.147	n/a	0.013	n/a
$k_{2\ uptake}$ (d^{-1})	nd	n/a	nd	0.072
$k_{2\ elim}$ (d^{-1})	0.71	n/a	0.241	0.095
$t_{1/2\ elim}$ (d)	1.0	n/a	2.9	7.3
k_s (d^{-1})	0.072	n/a	0.014	0.006
$t_{1/2\ soil}$ (d)	9.6	n/a	49.5	115.5

nd not determined; used $k_{2\ elim}$ in the model

n/a not applicable

3.3.2. Accumulation and elimination of F2, F3a, and F3b

Accumulated aliphatic and aromatic compounds were predominantly within the boiling point range of the distillate tested, as illustrated for F3a in Figure 3-1, supporting that they were petrogenic in nature (parent compounds, metabolites and/or degradation products). Significant accumulation of petrogenic aliphatic and aromatic compounds was noted even for the highest boiling point range distillate, F3b, despite the high hydrophobicity and thus presumed low availability of these compounds for uptake from pore water.

Figure 3-2 illustrates the aliphatic fraction of F3a in soil. From Figure 3-1 and Figure 3-2, it is observed that there is a difference in the F3a aliphatic compounds accumulated by earthworms as compared to what is in the soil. The chromatograph of F3a aliphatic compounds in the soil has distinct peaks corresponding to *n*-alkanes above a large unresolved "hump" comprising the unresolved complex mixture (UCM). Aliphatics accumulated by earthworms also appeared as an unresolved hump at day 16 and 64 but there were no discernible *n*-alkane peaks. GC-MS chromatographs were unfortunately lost for F2 and F3b; however, GC-MS chromatographs from another study (Chapter 4) on the accumulation of mixtures of F3a and F3b show that both *n*-alkanes and the UCM in the F3b range are accumulated by earthworms.

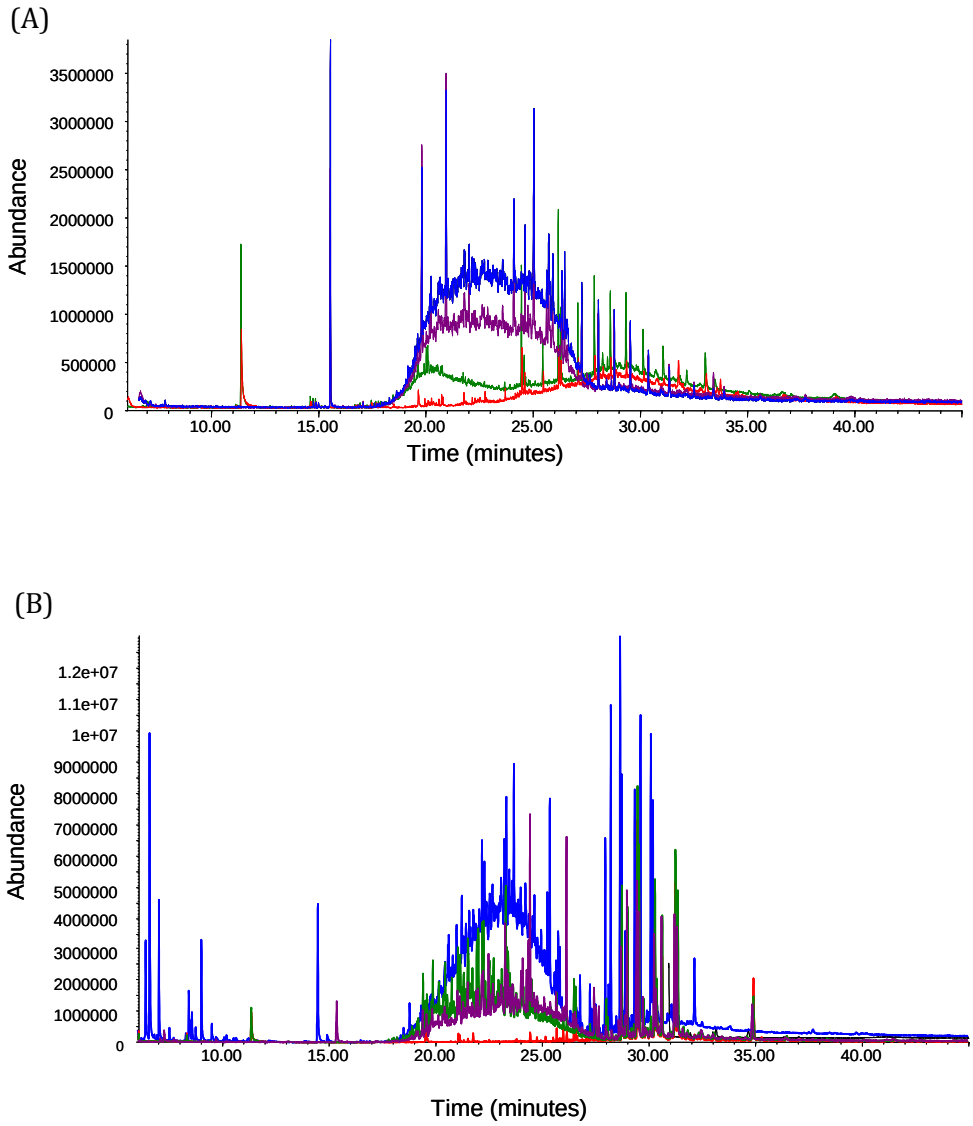


Figure 3-1: Gas chromatography-mass spectrometry open-scan chromatograms of tissues from the earthworm *E. andrei* exposed to soil contaminated with oil distillate Fraction 3a for zero (red), 16 (green), 32 (purple), and 64 (blue) days. (A) aliphatic compounds in tissues; (B) aromatic compounds in tissues. Fraction 3a distillate elutes between approximately 18 and 28 min.

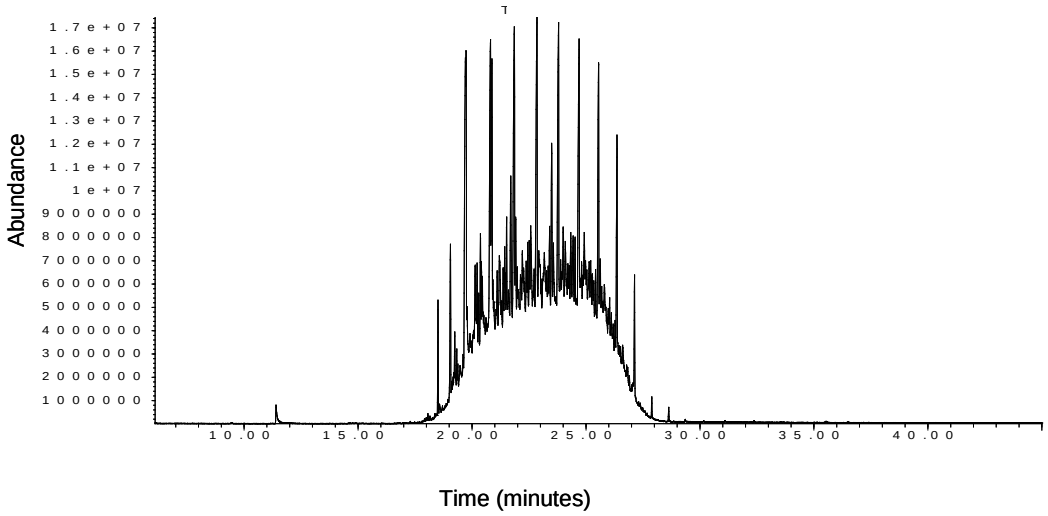


Figure 3-2: Gas-chromatography mass-spectroscopy chromatogram of the initial soil (day 0) contamination for Fraction 3a aliphatics.

Figure 3-3 to Figure 3-5 illustrate the accumulation and elimination of aliphatic and aromatic compounds from F2, F3a, and F3b by *E. andrei* as determined by GC-FID. Accumulation and elimination rate constants are provided in Table 3-2. A variety of accumulation curves were observed. F2 aliphatics and aromatics (Figure 3-3, A and B, respectively), as well as F3a aromatics (Figure 3-4 B), had accumulation curves that peaked at a maximum tissue concentration followed by a decline with longer test duration and were modelled using the modified first-order accumulation model (Equation 3-5). Data points for F3a aliphatics closely followed standard first-order accumulation up to day 16, with a further slower increase in tissue concentration thereafter; accumulation of F3a aliphatics for both the day 0 to day 16 and the day 0 to day 64 data were modelled separately using Equation 3-6 (Figure 3-4 A, Table 3-2). The standard first-order accumulation curves (Equation 3-6) best described the accumulation of F3b aliphatics and aromatics (Figure 3-5 A and B, respectively); however, a decrease in the concentration of F3b aromatics at day 64 is suggestive of a peak-accumulation pattern, but no peak could be modelled with Equation 3-5 ($k_{dis} \sim 0$). It is not known if this represented variability in the data or if a further decline in tissue concentration would have been noted had the accumulation phase continued past day 64.

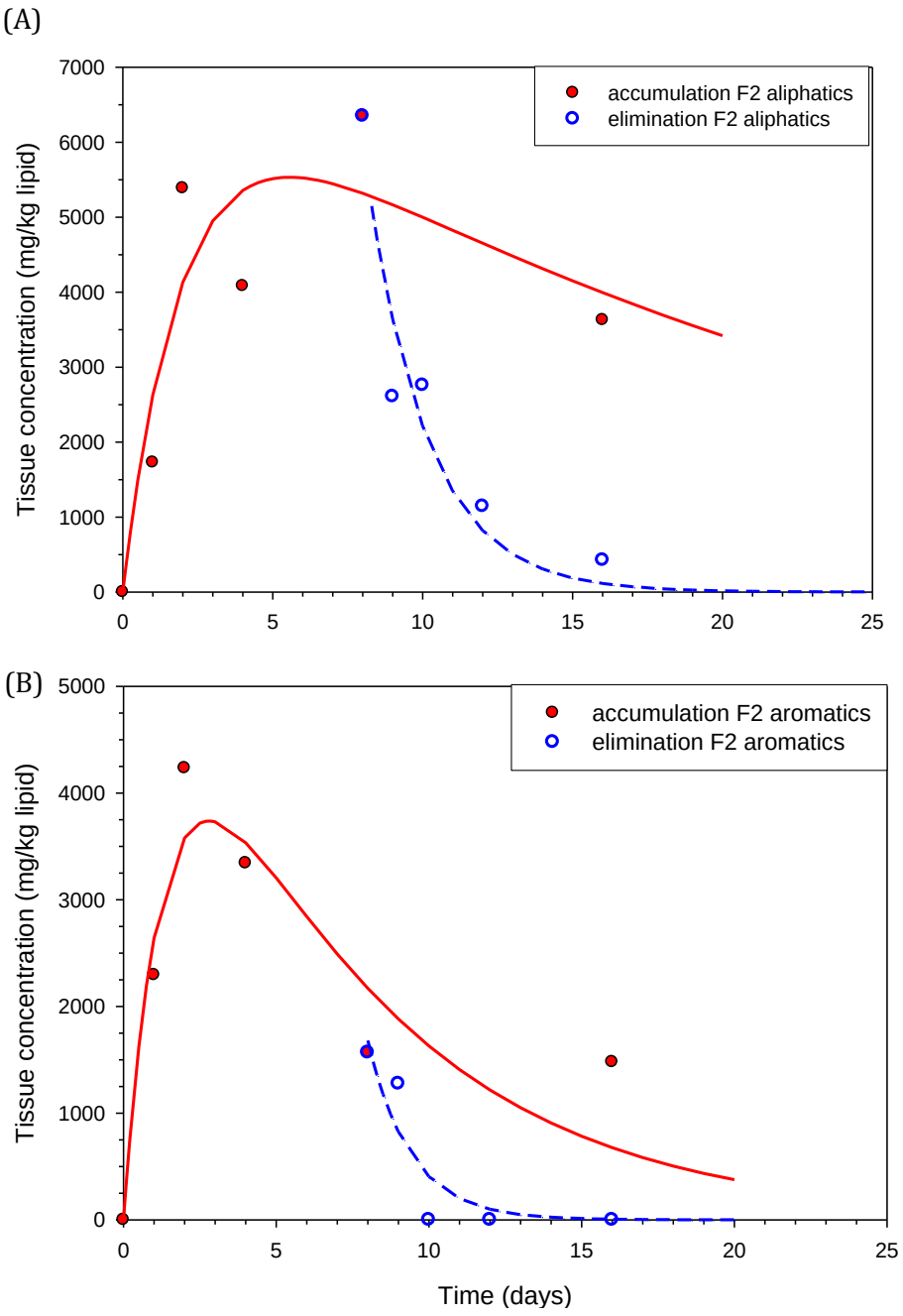


Figure 3-3: Accumulation and elimination of Fraction 2 (A) aliphatic and (B) aromatic compounds from soil by the earthworm *E. andrei*

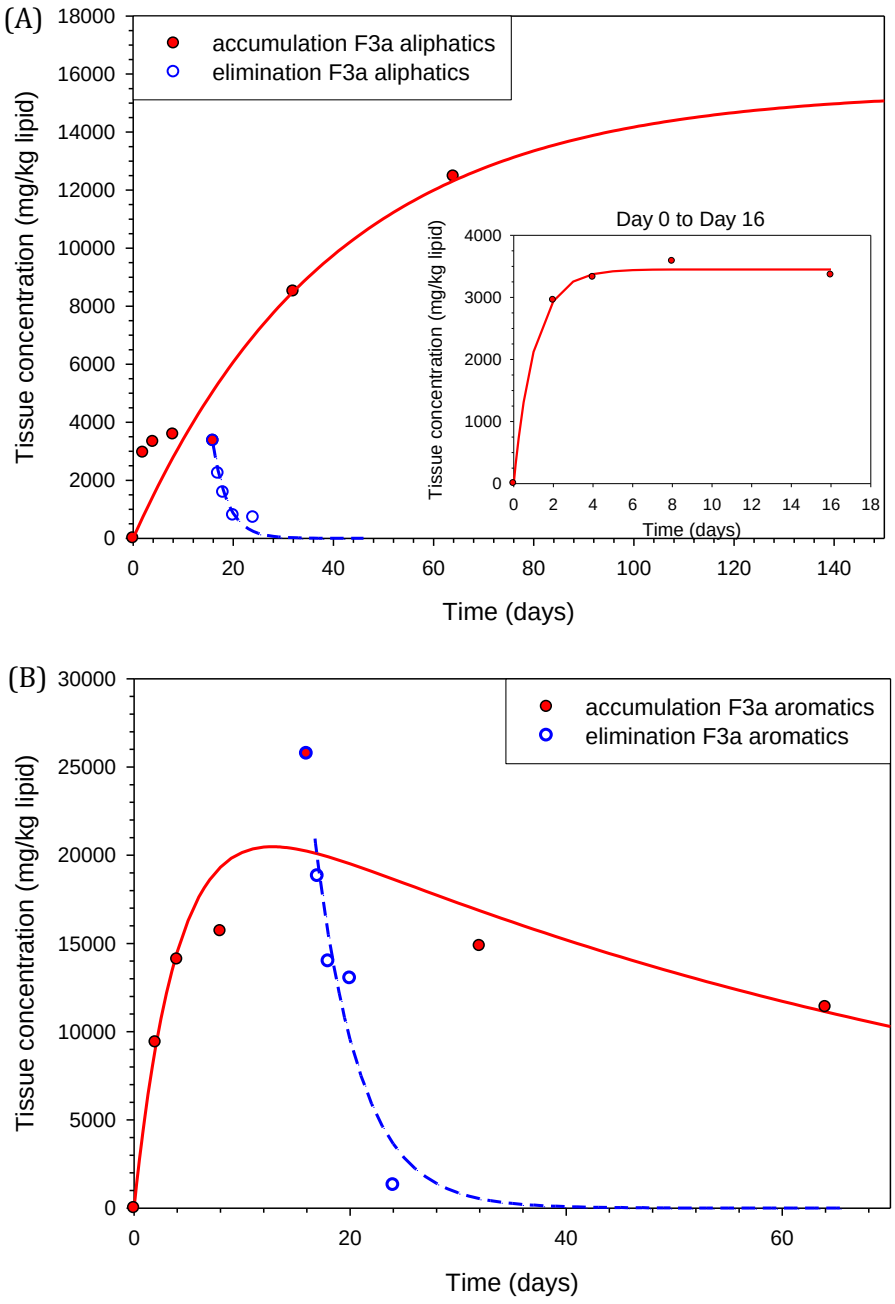


Figure 3-4: Accumulation and elimination of Fraction 3a (A) aliphatic and (B) aromatic compounds from soil by the earthworm *E. andrei.* Note the different *x*-axis for each graph.

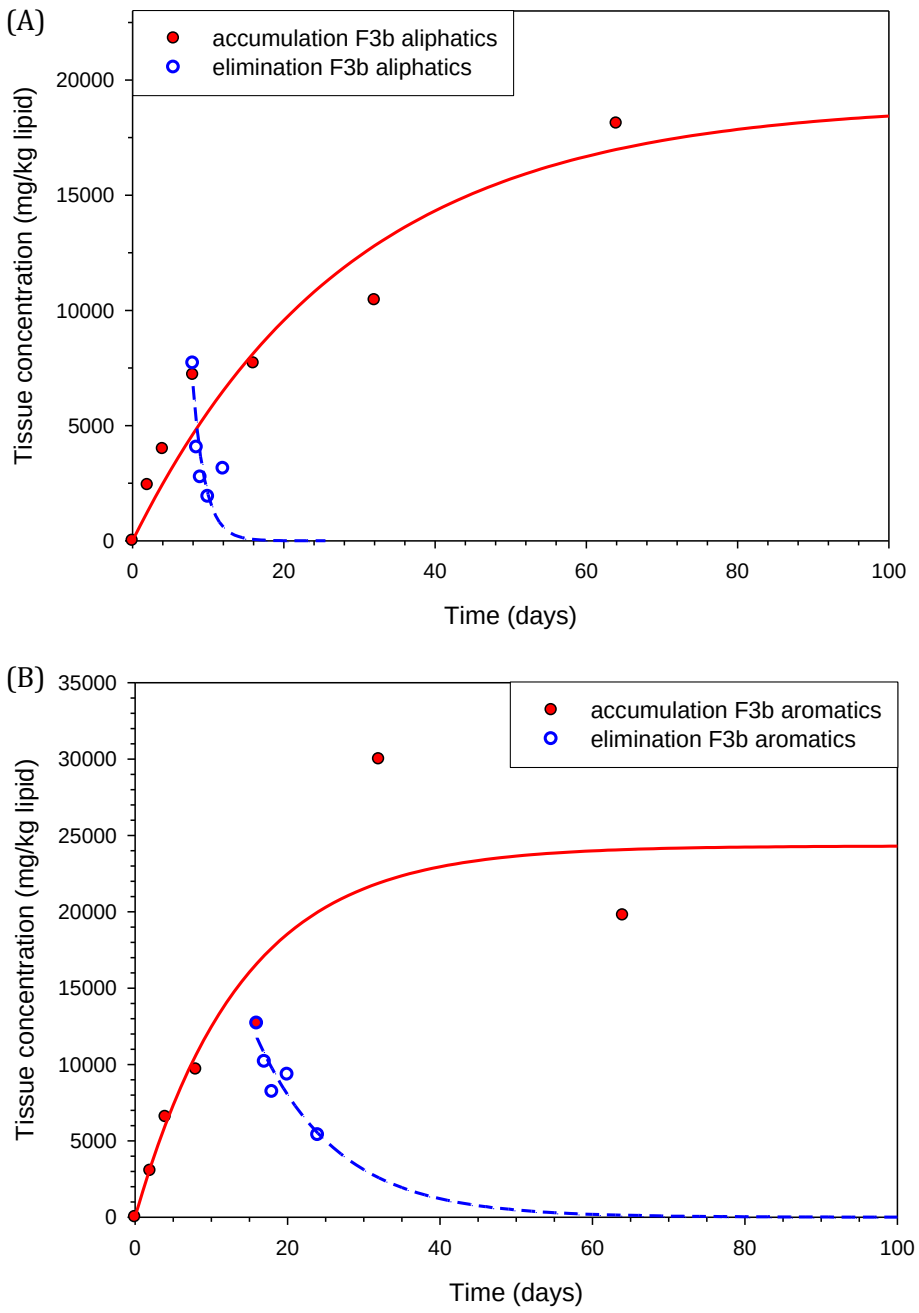


Figure 3-5: Accumulation and elimination of Fraction 3b (A) aliphatic and (B) aromatic compounds from soil by the earthworm *E. andrei.* Note the different *x*-axis for each graph.

Similar to the findings of others (Jager et al. 2000; Matscheko et al. 2002), the elimination of aliphatic and aromatic compounds by earthworms was monophasic and followed one-compartment, first-order elimination kinetics. Over the eight-day elimination phase, only F2 aromatics were completely eliminated (Figure 3-3 B). For distillates and chemical classes where both $k_{2\ uptake}$ and $k_{2\ elim}$ could be determined, $k_{2\ elim}$ was usually greatest (Table 3-2).

The following can be said of the accumulation and elimination rate constants (Table 3-2): 1) they were always in the order F2 > F3a > F3b; 2) they were consistently greater for accumulation than for elimination; and 3) they were consistently greater for aromatics than aliphatics. The higher accumulation rates for aromatics translate to a disproportionate accumulation, over the duration of this study, of aromatics over aliphatics by earthworms as compared to the proportions found in the soils. This is illustrated in Figure 3-6 for all distillates.

3.3.3. Biota-soil accumulation factors

The concentrations of select aromatic compounds were determined by selective ion monitoring GC-MS analysis for day 0 soils and for tissue samples following 2, 16, and 32 d of exposure for F2, F3a, and F3b, respectively. These exposure times had the highest measured tissue concentrations of aromatic compounds for each respective distillate (Figure 3-3 to Figure 3-5). Alkylated-aromatics represented the majority of the compounds in the soils and tissues; this was expected because the majority of aromatic compounds in the distillates are alkylated (Chapter 2 (Cermak et al. 2010)). The time-dependent BSAF for individual F3a aromatics ranged from 2.8 to 15.6 and that for individual F3b aromatics ranged from 2.0 to 13.4, indicating enrichment of these specific aromatic compounds in earthworms (Table 3-3). Only acridine, a N-heterocyclic detected in the aromatic fraction of F3a, had a BSAF = 0 as no acridine was detected in earthworm tissue. For F2, the concentrations of most compounds within the soil were below the detection limit (0.02 μg/g). For those compounds with concentrations in soil below the detection limit but with measureable tissue concentrations, the detection limit was used as the soil concentration in order to estimate the BSAF. This resulted in time-dependent BSAFs for F2 ranging from 5.85 to >1,500 for individual compounds identified by SIM GC-MS. While these values and the fact that detectable concentrations of these compounds were found in earthworm tissues indicate that bioaccumulation is occurring with F2, confidence in the F2 BSAF values is low due to the low compound concentrations in soil.

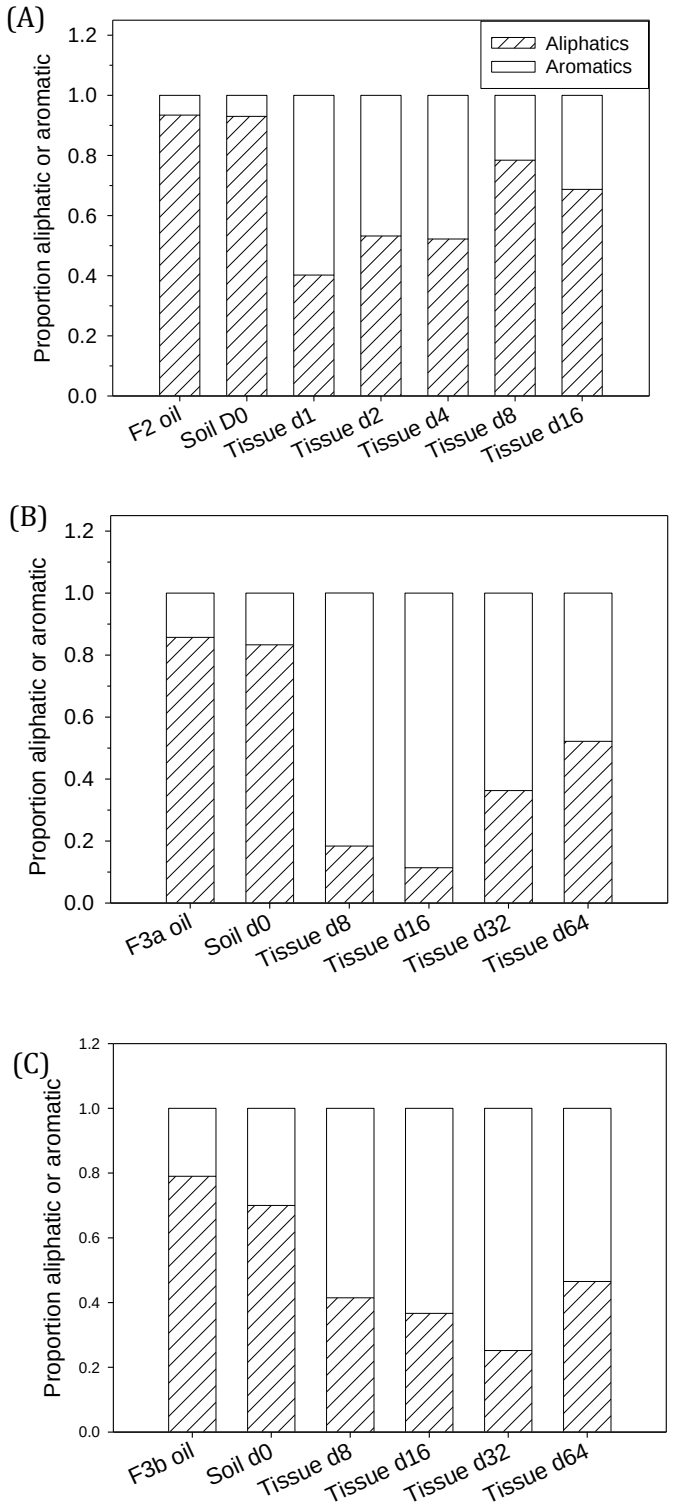


Figure 3-6: Proportion of aliphatics and aromatics in oil, soil and *E. andrei* tissue over the accumulation phase of the toxicokinetic test. (A) Fraction 2, (B) Fraction 3a, and (C) Fraction 3b.

Table 3-3: Time-dependent Biota-soil accumulation factors (BSAFs, kg OC/kg lipid) for aromatic compounds accumulated by the earthworm, *Eisenia andrei,* from petroleum distillates. Aromatics were measured by selective ion monitoring gas-chromatography/mass spectroscopy for Fractions 2, 3a, and 3b determined on days 2, 16, and 32, respectively.

Compound	F2	F3a	F3b
Naphthalene	24.5		4.1[1]
2-methylnaphthalene			5.6[1]
Methyl naphthalene	397.3		5.6[1]
Biphenyl			2.0[1]
Methyl biphenyl	47.7		
C2 biphenyl			
C2 naphthalene	1569.6		4.0[1]
C3 naphthalene	131.0	10.50	3.8[1]
C4 naphthalene	26.3	15.65	
Acenaphthylene			
Acenaphthene			
Fluorene		8.58	
Methyl acenaphthene	0.0		0.0
Methyl fluorene	18.4	10.31	0.0
C2 fluorene		13.49	0.0
Phenanthrene		7.05	0.0
Anthracene			
Acridine		0.00	0.0
Fluoranthene			0.0
Pyrene		3.96	0.0
C1 phenanthrene/anthracenes		9.53	4.5
C2 phenanthrene/anthracenes		6.87	7.6
C3 phenanthrene/anthracenes		4.20	13.4
C4 phenanthrene/anthracenes		4.11	5.9
Methyl Fluoranthene/pyrene		3.17	4.7

[1]these compounds are not expected in F3b

Table 3-3 continued

Compound	F2	F3a	F3b
Dibenzothiophene		5.10	0.0
Methyldibenzothiophene		9.09	0.0
C2 dibenzothiophene		6.98	4.8
C3 dibenzothiophene		5.02	4.3
C4 dibenzothiophene		2.76	5.2
Benz(a)anthracene			
Chrysene			
Methyl Benz(a)anthracene/chrysene		7.0	
C2 Benz(a)anthracene/chrysene			7.6
Benzo(b)fluoranthene			
Benzo(k)fluoranthene			
Benzo(a)pyrene			
Methyl Benzo(bk)Fluoranthene/Benzo(a)pyrene			5.1
C2 Benzo(bk)Fluoranthene/Benzo(a)pyrene			3.5
Indeno(123-cd)pyrene			
Dibenz(ah)anthracene			
Benzo(ghi)perylene			

[1]these compounds are not expected in F3b

BSAFs were also calculated based on the GC-FID results for total aliphatics and aromatics, using the measured soil concentrations on day 0 and both the highest measured tissue concentration in earthworms and the maximum tissue concentrations predicted by the model. Results using both the measured and estimated tissue concentrations were similar (Table 3-4). BSAFs were greater than one for F2 aliphatics and aromatics and for F3a aromatics. BSAFS were less than one for F3a aliphatics and both F3b aliphatics and aromatics.

Table 3-4: Biota-soil accumulation factors (BSAFs) for total aliphatic and aromatic fractions of petroleum distillates F2, F3a, and F3b accumulated by *Eisenia andrei* . BSAFs were determined using the maximum measured tissue concentration or the maximum modelled tissue concentration.

	BSAF (kg OC/kg lipid)			
	Aliphatics		Aromatics	
	measured	modelled	measured	modelled
Fraction 2	2.67	2.32	23.47	20.72
Fraction 3a	0.43	0.53	4.46	3.55
Fraction 3b	0.22	0.24	0.86	0.70

3.4. DISCUSSION

3.4.1. Accumulation and elimination of F2, F3a, and F3b

Petrogenic compounds from all distillate ranges were accumulated by earthworms, even those in the highest boiling point range (ECN >C23-C34) (Figure 3-3 to Figure 3-5) despite their high hydrophobicity and thus presumed low bioavailability of these compounds in pore water. Muijs and Jonker (2010) also noted accumulation of high molecular weight compounds, including *n*-alkanes up to C34, by the aquatic worm *Lumbriculus variegates* and they concluded that uptake mechanisms other than via pore water must occur such as ingestion. Ingestion of contaminated soils has been shown to be an important accumulation pathway in earthworms for compounds with $\log K_{ow} > 5$ (Jager et al. 2003b). Ingestion is likely an important exposure pathway for most aliphatics and many aromatics, especially for the higher ECN distillates, based on the $\log K_{ow}$ coefficients for select aliphatic and aromatic compounds provided by Gustafson et al. (1997).

Accumulation rates were greatest for the lowest boiling point distillate (F2) and progressively decreased as the boiling point range of the distillate increased (Table 3-2). This was noted for TPH accumulation by aquatic worms as well (Muijs and Jonker 2010). This is likely due to a decrease in the bioavailability of oil constituents due to changes in sorption and water solubility as the boiling point range for hydrocarbons increases (Battelle 2007; Gustafson et al. 1997). Steric hindrance to the diffusion of molecules into membranes may also contribute to the observed lower accumulation (Barron 1990) and various molecular size cutoff criteria have been suggested; however, many large molecules above the criteria can still accumulate to high levels (Arnot et al. 2009). With regard to bioavailability, as the boiling point range for hydrocarbons increases, so does the average log K_{ow} (Battelle 2007) while the water solubility decreases (Gustafson et al. 1997) leading to greater sorption to soil organic carbon and lower pore water concentrations. Similarly, others (Jager et al. 2000; Landrum 1989) observed that accumulation rates were inversely proportional to the log K_{ow} when expressed on a soil concentration basis. Slow desorption of contaminants from soil into pore water can also decrease accumulation rates (Belfroid et al. 1996). Accumulation rates were also consistently higher for aromatics over aliphatics. This, again, can be related to the log K_{ow} and water solubility as aliphatics within a distillate range have higher log K_{ow}'s and lower water solubility than the aromatics or because of steric effects. Studies on the dermal absorption of JP-8 jet fuel demonstrated that aromatic compounds generally had higher permeability rates through skin than aliphatic compounds, and that the accumulation of aliphatic compounds decreased with increasing chain-length (McDougal and Robinson 2002).

Peak accumulation curves were observed for three of the fractions: F2 aliphatics, F2 aromatics, and F3a aromatics. Similar curves have been observed by others in bioaccumulation studies of PAHs in terrestrial (Contreras-Ramos et al. 2009; Jager et al. 2000; Ma et al. 1995; Matscheko et al. 2002) and aquatic (Kraaij et al. 2001; Landrum 1989; Landrum et al. 1991; Penry and Weston 1998) species. Muijs and Jonker (2010) investigated the accumulation of total petroleum hydrocarbons (TPH) from sediments by the aquatic worm *L. variegates* in carbon ranges similar to those used in the current study. Like that observed for both F2 aliphatics and aromatics, they observed a peak pattern for TPH accumulation in the C11-C16 range in one sediment. No peak accumulation curves were noted within the C16-C22 (F3a) or C22-C28 (~F3b) ranges. The lack of a peak in the F2 and F3a ranges of other sediments may be due to the weathered nature of the petroleum contamination in the sediments and/or because the authors did not distinguish between the accumulation of aliphatic and aromatic hydrocarbons.

Peak accumulation curves have been attributed to various phenomena. One possibility is a decrease in lipid content (Jager et al. 2000). However, in this study no decrease in lipid levels in earthworms was observed.

Induced metabolism may also result in peak accumulation curves (Penry and Weston 1998; Widianarko and Van Straalen 1996); however, this is likely not the case in this study as metabolism is not thought to be an important elimination route for PAHs in earthworms (Jager et al. 2000; Ma et al. 1998; Matscheko et al. 2002). While earthworms do possess a cytochrome P450 monooxygenase enzyme system (Achazi et al. 1998; Eason et al. 1998; Saint-Denis et al. 1999), enzyme activities are considerably lower than those found in many other organisms (Achazi et al. 1998; Eason et al. 1998) with only 1% or less of pyrene metabolized by *E. andrei* in one study (Jager et al. 2000).

The peak accumulation curve is likely due to a change in the exposure concentration with time. This may be due to the loss of contaminant from the soil over time via biodegradation and volatilization (Muijs and Jonker 2010; Widianarko and Van Straalen 1996), or a decrease over time in the bioavailable pool of contaminant in the pore water via sorption to non-bioavailable sites in the soil (Landrum 1989; Ma et al. 1995) or through the induction of fast microbial degradation of the contaminant coupled with its slow desorption from the soil (Jager et al. 2000). In all cases, a decrease in concentration in the pore water with time occurs.

Loss of contaminant from soil via degradation and volatilization may have caused or contributed to the peak accumulation patterns observed. A rate of loss from soil (k_s) similar to k_{dis} implies that the loss of contaminant from the soil is mainly responsible for the peak pattern. However, if k_s is less than k_{dis}, then loss of contaminant from the soil is not sufficient to explain the size of the peak and suggests other processes affecting the bioavailability of the contaminant must be occurring. The rate constant k_s was greater than or similar to k_{dis} for both F2 aliphatics and F3a aromatics (Table 3-2), suggesting that for these fractions the loss of contaminant through degradation and/or volatilization is the main driver for the peak accumulation pattern observed. The larger value of k_s compared to k_{dis} for F2 aliphatics may be due to the uncertainty surrounding the k_s value or to an underestimation of k_{dis} (see below). For F2 aromatics, k_s was less than k_{dis}

(Table 3-2), indicating that other processes are implicated in determining the magnitude of the peak accumulation pattern.

It should be noted that k_{dis} was determined by setting the value of k_2 in Equation 3-5 to that determined during the elimination phase ($k_{2\ elim}$). For fractions for which both $k_{2\ uptake}$ and $k_{2\ elim}$ could be determined, $k_{2\ elim}$ was often greater than $k_{2\ uptake}$ (Table 3-2). Similar to Jager et al. (2005), this was attributed to a higher feeding activity of earthworms exposed to clean soil during the elimination phase as it was visually noted that earthworms egested a greater quantity of material during depuration in this phase, at least for F2, as compared to during the accumulation phase. If $k_{2\ elim}$ overestimates $k_{2\ uptake}$, then k_{dis} is underestimated and may be greater than k_s, indicating that processes affecting the bioavailability of the contaminant to the earthworms are also influencing the peak accumulation curve.

A decrease in bioavailability with time may occur due to the movement of contaminant to non-bioavailable sites within the soil (Landrum 1989; Ma et al. 2012; Ma et al. 1995). Landrum (1989) observed reductions in bioavailability with phenanthrene, a compound associated with the F3a aromatic fraction which had a peak accumulation curve, within the timeframe of the exposures of the current study, but not with benzo[a]pyrene or benzo[a]anthracene, aromatics associated with F3b which did not have a peak accumulation curve. Peak accumulation curves were also noted in another study with PAHs with $\log K_{ow} < 5$ (e.g., fluorene, phenanthrene, and anthracene, all associated with F3a) while k_{dis} was zero for those with $\log K_{ow} > 5$ (Landrum et al. 1991). White et al. (1999) noted a significant decline in the accumulation of phenanthrene by earthworms following 14 d of aging compared to 3 d of aging, and found that some phenanthrene was associated with a fraction strongly resistant to desorption even after only 3 d. Measurements of bioavailability or of rapid and slowly desorbing fractions in soils or sediments (Kelsey et al. 1997; Kraaij et al. 2001; Ma et al. 2012) indicate that movement to non-bioavailable sites in soils can occur fairly quickly. Accumulation rates and bioaccumulation of phenanthrene and pyrene by earthworms was significantly decreased after 15 d aging of contaminant in soil (Ma et al. 2012). Therefore, it is possible that this may play a role in the shaping of the bioaccumulation curves of these distillates.

Biodegradation coupled with slow desorption of contaminants from the soil (Jager et al. 2000) also likely contributes to the peak accumulation curves. At the start of the bioaccumulation test, pore water concentrations are high. However, as biodegradation begins the concentration of

contaminant in the pore water decreases. If the rate of biodegradation exceeds the rate of desorption of contaminant from the soil, the pore water concentration of contaminant can approach zero (Jager et al. 2000). Desorption is known to be rate-limiting for the biodegradation of 2-3 ring PAHs, such as those found in F2 and F3a, with most of these PAHs degrading as quickly as they are desorbed (Huesemann et al. 2003); therefore, the concentration of these aromatic compounds available to earthworms for accumulation via the pore water may be very low once biodegradation begins. Four to six ring PAHs, such as those found within F3b, were also readily desorbed from soils and bioavailable despite their high $\log K_{ow}$ values but at a rate higher than their biodegradation, at least in the initial stages of biotreatment (Huesemann et al. 2003), such that these contaminants will be present in the pore water for accumulation even after biodegradation commences. Therefore, biodegradation of aromatics in the pore water may also contribute to the peaks observed with F2 and F3a aromatics. The decrease in tissue concentration of aromatics in F3b-exposed worms at day 64 may be due to variability or may indicate the initiation of biodegradation of these higher molecular weight aromatics and, consequently, a decrease in the pore water concentration of F3b.

F3a aliphatics followed a biphasic accumulation pattern, with tissue concentrations quickly attaining an apparent plateau (2-16 d, Figure 3-4 A), followed by another slower increase in concentration. This is similar to what was observed with the uptake of PAHs by poly(dimethylsiloxane) coated fibers from a contaminated soil (Ter Laak et al. 2006) which was attributed to the presence of both a fast and slow desorbing fraction of contaminant in the soil. *Normal*-alkanes are expected to be readily biodegraded (Englert et al. 1993; Huesemann et al. 2003) and biodegradation rates for *n*-alkanes in the range of C12-C24 can be higher than desorption rates from soil (Huesemann et al. 2003). Despite this no peak accumulation curve was observed for F3a (ECN >C16-C23) as was seen with F3a aromatics. In contrast to Muijs and Jonker (2010) who observed *n*-alkane peaks in most samples over the entire boiling point range, GC-MS chromatographs of the aliphatic fraction in the soil and accumulated by earthworms (Figure 3-1 and Figure 3-2) demonstrate that though *n*-alkanes are present within the soil, they are not accumulated by the earthworms and that the accumulated aliphatic fraction consists of an unresolved complex mixture. It is speculated that the readily degradable *n*-alkanes are biodegraded within the pore water leaving only the less degradable aliphatics, such as highly branched alkanes and cycloalkanes (El-Gendy and Farah 2011; Englert et al. 1993; Penet et al. 2004) available for uptake.

In contrast to F3a, *n*-alkanes in the F3b range were accumulated by earthworms (see Chapter 4), suggesting that the biodegradation rate of these larger molecular weight alkanes was not sufficient to limit accumulation. A preferential degradation of lower molecular weight *n*-alkanes over higher molecular weight *n*-alkanes was noted by others (El-Gendy and Farah 2011).

It should be noted that the concentrations of F3b used in this study likely resulted in the formation of a non-aqueous phase liquid (NAPL) as there was 18% F3b in organic carbon (w/w); values above 15% oil in organic carbon are expected to result in a NAPL (Jonker et al. 2003). This should, theoretically, result in saturated pore water concentrations. However, the presence of a NAPL can also act as another sorption phase and may affect the partitioning of hydrocarbons (Jonker et al. 2003). The accumulation of F3b and/or its constituents at lower, non-NAPL-forming concentrations may not be the same as that observed in the current study because of differences in bioavailability when no NAPL is present.

3.4.2. Biota-Soil Accumulation Factors

Biota-soil accumulation factors were calculated for both specific individual aromatic compounds, as determined by SIM GC-MS (Table 3-3), and total aromatic and aliphatic compounds as determined by GC-FID. There were some differences in results between the two methods. BSAFs were generally lower when determined based on the total aromatic concentration (Table 3-4) as compared to those determined for individual compounds determined by SIM GC-MS (up to 1570, 15.6, and 13.4 for F2, F3a, and F3b, respectively), though some individual compounds were within the range for total aromatics (Table 3-3). The individual aromatics quantified were mainly parent PAHs and some of their alkylated homologues which make up only a small portion of the total aromatic fraction (Chapter 2 (Cermak et al. 2010)). The remainder are likely much more complex petroleum compounds that are less bioavailable, as reflected in the BSAFs for the total aromatic concentration. The results of both methods indicate that aromatic hydrocarbons in both the F2 and F3a ranges can bioaccumulate in earthworms. The SIM GC-MS results demonstrate that bioaccumulation occurs and can be high for some specific compounds, even up into the F3b range (Table 3-3).

Compared to others, the range of BSAFs for individual PAHs in F3a and F3b encompass the range of maximum time-dependent BSAFs (2.4-8.2 kg OC/kg lipid) determined by Jager et al. (2000) for four PAHs spiked into an artificial soil and aged for one week. The F3a BSAFs determined in the present study at day 16 for phenanthrene (7.05 kg OC/kg lipid) and pyrene (3.96 kg OC/kg lipid), two aromatic compounds found predominantly in F3a, corresponded well with the BSAFs at day 19 determined by Jager et al. (2000) (7.3 and 3.9 kg OC/kg lipid for phenanthrene and pyrene, respectively).

The BSAFs determined for individual and total aromatic hydrocarbons using freshly amended soils are considerably higher than those determined for PAHs in aged, field soils (Jager et al. 2003a; Ma et al. 1998; Matscheko et al. 2002).

BSAFs for total aliphatic compounds for each distillate demonstrate that the aliphatic compounds are accumulated to a lesser extent, with only F2 aliphatics bioaccumulating to levels above ambient (Table 3-4).

3.4.3. Accumulation of aliphatics and aromatics with respect to toxicity

In order for toxicity to occur, a substance must first be taken up by the organism and reach the site of toxic action in sufficient quantity and for a sufficient length of time to elicit a toxic response (McCarty et al. 2011). Therefore, all toxicity data for the oil distillates must be interpreted in light of their accumulation kinetics. The kinetics determined in the current study can be applied to previous investigations on the toxicity of the individual distillates (Cermak et al. 2010) to earthworms as similar methodologies were followed for both studies. In the toxicity study, it was determined that threshold effect concentrations were not necessarily reached over the exposure duration which, from the current study, can be attributed to differences in the accumulation kinetics between the different petroleum distillates. A presence of a peak in the accumulation pattern for some distillates also suggests that the internal exposure is transitory, in particular for aromatic fractions. Petroleum hydrocarbons are expected to cause toxicity via nonpolar narcosis, a reversible mode of action (King et al. 1996). Therefore, if the toxic response is reversible, then sublethal toxic effects will be transitory as well.

The time to reach the peak or 95% of the maximum body burden, as well as the time during which ≥ 80% of maximum is achieved, was determined using the fitted accumulation curves (Table 3-5); however, these should be used with caution because, as noted earlier, there are some questions regarding the accumulation of F3a aliphatics (apparent change in k_1 after day 16) and F3b aromatics (possible peak at day 32 that could not be modelled). This demonstrates that, for a given distillate and chemical class fraction (i.e., aliphatics or aromatics), the maximum body burden is reached at different exposure durations. Standard toxicity test methods may not embrace these different exposure durations.

Assuming that the accumulation patterns observed at non-lethal concentrations are similar to those that occur at higher, lethal concentrations, the results of the earthworm lethality tests (Table 2-4, Chapter 2 (Cermak et al. 2010)) can be compared to the accumulation patterns observed for each distillate and chemical class fraction. This assumption may not be true, as accumulation patterns are dependent on soil concentrations (Jager et al. 2000). However, the accumulation patterns for F3a and F3b should be suitable for the interpretation of the results of earthworm reproduction tests as the concentrations were within the range of those affecting reproduction.

Table 3-5: Time (days) to reach maximum body burden and time during which ≥80% of maximum is achieved

Distillate	Aliphatics		Aromatics	
	≥80%	Maximum	≥80%	Maximum
F2	2.3 – 13	5.5	1.25 – 5.5	2.8
F3a	≥64	120[1]	5 – 34	13
F3b	≥46	86[1]	≥22	42[1]

[1]determined as 95% of maximum for data fit by standard first-order accumulation curves

For F2, maximum body burdens for both aliphatics and aromatics occurred within the first six days (Table 3-5). This corresponds well to the results of the toxicity tests, where most mortality was observed within the first seven days of exposure, and the threshold effect concentration for survival was reached by 14 days (Chapter 2 (Cermak et al. 2010)). In addition, studies have demonstrated that earthworm reproduction is affected at F2 concentrations similar to those

affecting survival (ESG 2003). During a reproduction test, adult earthworms are exposed to contaminated soil for 28-35 days (Cermak et al. 2010; EC 2004; ESG 2003). If the earthworms survive the initial, acute internal exposure to F2, body burdens quickly decline (<80% of maximum after 6-13 days, Table 3-5), recovery occurs and the earthworms are able to survive and reproduce at levels similar to earthworms in clean soil resulting in similar 50% effect concentrations for reproduction and lethality (ESG 2003).

With F3a, the threshold effect concentration for survival was reached by day 35 (Chapter 2 (Cermak et al. 2010)), a duration similar to that for adult exposure in the reproduction test. Only the maximum body burden for aromatics was reached during this time (day 13). In addition, while for the majority of the exposure duration the tissue concentration of aromatics was within 80% of the maximum body burden, the aliphatic tissue concentrations did not attain even 80% of the maximum during this time (Table 3-5).

With F3b, the threshold effect concentration for lethality was not determined but is assumed to be greater than that of F3a (Chapter 2 (Cermak et al. 2010)). The maximum body burden for neither aromatics nor aliphatics was reached during the 28 d duration for lethality or during the 28-35 d test duration for reproduction (Table 3-5) and only aromatic compounds attained a tissue concentration within 80% of the maximum body burden but only near the end of the exposure duration (≥22 days). No mortality was observed during the 28 d survival assay (Table 2-4, Chapter 2 (Cermak et al. 2010)); while this could be due to a low intrinsic toxicity of F3b, the accumulation data indicate that it may just as well be due to an insufficient test duration to reach a toxic body burden as proposed by Mayer and Reichenberg (2006). Effects were noted on reproduction with F3b (Table 2-4, Chapter 2 (Cermak et al. 2010)); however, as maximum body burdens were not achieved during the adult exposure duration, it is likely that lower concentrations of F3b will prove to be toxic if the exposure duration is extended.

Earthworms exposed to the three distillates displayed very different internal exposure scenarios. Those exposed to F2 had short-term exposures to both the aromatic and aliphatic fractions, those exposed to F3a were exposed to 80% of the maximum concentration of aromatics over most of the test duration, and those exposed to F3b were exposed to less than 80% of the maximum amount aliphatics and aromatics for all, or nearly all, of the exposure duration. Though the results of the toxicity tests have been used to compare the toxicities of the distillates, these

differences suggest the results of the toxicity tests are not strictly comparable. This also suggests that toxicity testing protocols might need to be modified for use with petroleum hydrocarbons or other compounds. Where applicable (i.e., distillates or compounds with high K_{ow} values), exposure durations should be extended as the exposure duration for lethality and reproduction toxicity tests with earthworms is generally 7-28 d (ASTM 1999; EC 2004; OECD 2004). In other cases, where the loss of the contaminant could significantly affect the accumulation pattern (i.e., more volatile or easily degraded distillates or compounds), methods should be developed to minimize or adjust for the loss.

Studies have demonstrated a greater toxicity of aromatics to plants (van Overbeek and Blondeau 1954) and mammals (Feuston et al. 1994; Khan et al. 1986), and a relatively low toxicity of aliphatics to marine organisms (Hamoutene et al. 2004; Payne et al. 1995). The results of the current study and those in Chapter 2 (Cermak et al. 2010) indicate that the aromatic hydrocarbons, though present in lower concentrations in the oil and soil, are the main contributors to the toxicity of the distillates, in agreement with Verbruggen et al. (2008). Aromatics always reached maximum tissue concentrations before aliphatics and within the exposure duration of the toxicity test (Table 3-5) and were accumulated to the greatest degree by earthworms (Table 3-2). One study looked specifically at the toxicity of aromatics and aliphatics from Federated crude oil to earthworms (*Lumbricus terrestris*) using a 28-d exposure and concluded that the aromatic fraction was more toxic (Vandervaart Cook 2002); however, this likely reflects the different toxicokinetics of these two fractions as observed in the present study. Some studies observed higher toxicity of crude oils containing a large proportion of lower molecular weight compounds (Dorn et al. 1998; Salanitro et al. 1997). The results of the present study indicate that this was likely due, at least in part, to the different accumulation rates for lower versus higher molecular weight constituents leading to an apparent higher toxicity of the lighter oils compared to the heavier oils during a fixed duration toxicity test.

The PHC CWS are based on the total concentration of petroleum hydrocarbons within specific boiling-point ranges in the soil, and assume a 1:4 ratio of aromatic to aliphatic compounds. While this ratio is generally true for fresh crude oils, other petroleum products such as fuel oils have different ratios, with some having considerably more aromatics (Wang et al. 2003). Field sites, which have been weathered and/or remediated, might also have different proportions of aromatics to aliphatics. Therefore, the regulation of petroleum aromatics separate from petroleum aliphatics

in the soil is recommended instead of the total concentration of PHCs in a given ECN range. Further studies should be conducted to determine if the disproportional accumulation of aromatics occurs across phyla and different exposure routes.

The results of these tests cannot be directly translated to the field. However, it is expected that the general trends in the rate constants (e.g., k_1 and k_2 decreasing with increasing boiling point range) and the disproportionate accumulation of aromatics would be similar.

3.5. CONCLUSION

This study demonstrates that varying accumulation patterns for petroleum hydrocarbons result from the complex interaction of loss of contaminant from soil and changes in bioavailability over time such that internal exposure scenarios for earthworms vary greatly for each distillate and for aromatic versus aliphatic hydrocarbons. This indicates that toxicity results for earthworms from standard static, fixed duration toxicity tests are incomparable for petroleum hydrocarbons and those differences in the toxicities of aromatics and aliphatics as well as between light and heavy oils/distillates are likely due, in part, to the differences in their toxicokinetics. Similar problems are expected when comparing the toxicities of other compounds with highly dissimilar physico-chemical properties. The results also indicate that standard toxicity test durations are likely not sufficient for determining the ultimate threshold toxicity of other highly hydrophobic compounds. Using the information from the accumulation kinetics, it was determined that for the test system used, the threshold effect concentration was likely reached for F2 and F3a, at least with regard to aromatics. The toxicity of F3b is likely greater than that observed in Chapter 2 (Cermak et al. 2010) as the exposure duration was not sufficient to reach maximum tissue residue levels.

As petroleum hydrocarbons act via narcosis, all accumulated hydrocarbons, aliphatic or aromatic, will contribute to the observed toxicity; however, the observed disproportionate accumulation of aromatic over aliphatic compounds indicate that the toxicity of each distillate observed in Chapter 2 (Cermak et al. 2010) is mainly attributable to the aromatic fraction. It is recommended that petroleum hydrocarbons be regulated based on different boiling point ranges of aliphatic and aromatic hydrocarbons separately, as is done in some jurisdictions (Edwards et al. 1997; Gustafson et al. 1997; Massachusetts Department of Environmental Protection 2003; Potter and Simmons 1998; Weisman 1998) rather than as total petroleum hydrocarbons within a distillate range as is done with the Canada-wide Standards (CCME 2008).

Chapter 4 TOXICITY AND TOXICOKINETICS OF BINARY COMBINATIONS OF PETROLEUM HYDROCARBON DISTILLATES WITH THE EARTHWORM *EISENIA ANDREI*

4.1. INTRODUCTION

Petroleum crude is a complex and variable mixture of thousands of individual compounds, the majority of which are aliphatic and aromatic hydrocarbons (Potter and Simmons 1998). The potential for environmental contamination with petroleum hydrocarbons (PHCs) is high. Within Canada, approximately 286,000 tonnes of oil and petroleum products are used daily (Wang and Fingas 2006) and the Canadian Council of Ministers of the Environment (CCME) estimates that there are over 250,000 potential or actual terrestrial sites contaminated with PHCs (CCME 2008).

The constituents of crude oil and petroleum products have wide-ranging physico-chemical properties and toxicity, making it difficult to develop suitable soil criteria for PHC-contaminated soil on the basis of the total petroleum hydrocarbon concentration. To address this problem, many jurisdictions have developed criteria based on groupings of petroleum constituents with similar environmental fate, behaviour, and toxicity (CCME 2008; Gustafson et al. 1997; King et al. 1996; Verbruggen 2004). These groupings are based on boiling point ranges, given as equivalent carbon number (ECN) ranges. ECN, commonly used by the petroleum industry, normalizes the carbon number of a compound, based on its boiling point, to that of the *n*-alkanes such that compounds with the same boiling point will have the same equivalent carbon number (Gustafson et al. 1997).

In Canada, the Canada-wide Standards for petroleum hydrocarbons in soil (PHC CWS) (CCME 2001a; CCME 2008) for the protection of both human health and the environment were developed to address the management of lands contaminated with petroleum hydrocarbons. These standards regulate petroleum hydrocarbons based on the concentration of petroleum hydrocarbons within four ECN ranges (F1, ECN C6-C10; F2, ECN >C10-C16; F3, ECN >C16-C34; and F4, ECN >C34) and assumes that each has an aliphatic:aromatic ratio of 4:1 (CCME 2008).

Though contaminated sites are generally contaminated with more than one distillate fraction, the PHC CWS ecological values are based on the toxicity of individual distillates and do not consider interaction effects when more than one distillate is present. Hydrocarbons, of which petroleum is mainly composed, have a similar mode of action, non-polar narcosis, and thus their toxicities are expected to be concentration-additive (King et al. 1996). However, previous work with binary combinations of CCME distillates F1, F2, and F3 resulted in less-than-additive lethality with the earthworm *Eisenia andrei* (ESG 2003). This was unexpected and the less-than-additive toxicity was considered to be possibly due to differing toxicokinetics between distillates such that there was a

temporal separation in exposure to each distillate. This is corroborated by the toxicokinetics of individual distillates (see Chapter 3), especially when peak-shaped accumulation curves were present. Results show that there is the potential for organisms being internally exposed to only one distillate at a time even if they are present as a mixture.

The current study was initiated to investigate the lethal toxicity and toxicokinetics of binary combinations of CCME distillate F2 and two subfractions of CCME distillate F3 used in earlier studies, F3a (ECN >C16-C23) and F3b (ECN >C23-C34) (Chapters 2 (Cermak et al. 2010) and 3), with the earthworm *E. andrei* to first confirm the less-than-additive toxicity to earthworms and, secondly, to explore the cause of this phenomenon through toxicokinetic studies. As it is unlikely for soils to be contaminated with disparate boiling point ranges of petroleum hydrocarbons (i.e., PHCs in the F2 and F3b range only), binary combinations consisting of only distillates with consecutive boiling point ranges were used for both the toxicity and toxicokinetic studies. The duration of the lethal toxicity studies was also extended beyond what was used in previous binary tests (ESG 2003) as 14 days was found to be insufficient for distillates with boiling point ranges greater than that of F2 (see Chapter 2 (Cermak et al. 2010)). In addition, the toxicokinetics of both the aliphatic and aromatic hydrocarbon fractions were determined as differences in the accumulation kinetics of these two chemical classes were previously noted (Chapter 3). The results of this study are discussed with regard to regulations and the assessment of risk at contaminated sites.

4.2. MATERIALS AND METHODS

4.2.1. Crude oil distillates

Three crude oil distillates (Fraction 2 (F2), ECN >C10-C16; Fraction 3a (F3a), ECN >C16-C23; and Fraction 3b (F3b), ECN >C23-C34) were used as previously described (Chapter 2 (Cermak et al. 2010) and Chapter 2, Appendix D).

4.2.2. Soils and soil preparation

A clean Orthic Black Chernozem soil (29.9% sand, 42.4% silt, 27.7% clay, pH 6.1, 10.0% OM. 5.54% OC) was used as previously described (Chapter 2 (Cermak et al. 2010) and Chapter 2, Appendix B). All treatment concentrations were determined on a dry weight basis of soil. Test soils

were prepared as described in Chapter 2, Appendix C. Briefly, a stock soil was made for both F3a and F3b. When preparing binary combinations of F3a and F3b, an appropriate amount of each stock soil was diluted with clean reference soil and mixed thoroughly together. The soil was hydrated with de-ionized water to a moisture content of 33% on a wet weight basis.

As F2 is more volatile, a stock soil was not made. Thus, for each treatment of when binary combinations of F2 and F3a, an appropriate amount of F3a stock soil was diluted with clean reference soil and mixed thoroughly, followed by the addition of an appropriate volume of F2 as described in Chapter 2, Appendix C. De-ionized water was added in an amount sufficient to bring the moisture content of the soil to 33% and the soil was mixed thoroughly for an additional two minutes.

Treatments were prepared the day before test initiation (day -1), as recommended by Environment Canada (EC 2004). Earthworms were introduced on day 0, the day after the test soils were prepared.

4.2.3. Test species

Toxicity tests and accumulation and elimination studies with binary combinations of petroleum distillates were conducted using the earthworm, *Eisenia andrei*, as described in Chapter 2 (Cermak et al. 2010).

4.2.4. Test methods

The methods for conducting lethality tests with *E. andrei,* as described in Chapter 2 (Cermak et al. 2010) and Chapter 2, Appendix C, were followed. Experimental conditions for tests with binary combinations of distillates are provided in Table 4-1. Tests were either 28- or 42-d in duration.

Cumulative mortality was assessed on days 7, 14, 21, 28, 35, and 42, where applicable. If needed, deionized water was added to the test soil at these times. Earthworms were fed approximately 5-ml of cooked oatmeal (1:3 oatmeal:deionized water, v/v) on day 14 of all tests, and on day 28 of tests extended to 42 d.

Table 4-1: Summary of the experimental conditions for determining the lethal toxicity of binary combinations of Fractions 2, 3a, and/or 3b to *Eisenia andrei*

Test	Duration	No. of treatments including control	No. of replicates/ treatment	No. of organisms/ test unit	Test unit	Amount soil (@ 33% moisture)	Lighting and temperature regime
Acute lethality	28 or 42 days[1]	7-8	4	5	500-mL glass mason jar with perforated aluminum foil lid[2]	300-375 g	Continuous fluorescent, 50-100 µmol/(m^2·s) 22 ° C

[1]one F2F3a and one F3aF3b test was extended to 42 days

[2]lids were not perforated until day 7 for all tests with Fraction 2

Two binary combinations of oil distillates were used: F2 with F3a (F2F3a), and F3a with F3b (F3aF3b). Only combinations of distillates with consecutive boiling point ranges were used, as these are most likely to be found concurrently at contaminated sites. Two repetitions of the lethal toxicity test were conducted with each mixture.

A toxic unit approach (Altenburger et al. 2000) was used to assess the interaction of mixtures of distillates. With this approach, the concentration of each distillate is expressed in toxic units (TU), which is the ratio of the concentration of the distillate to the endpoint of interest (in this case, the LC50). The concentration of the mixture in toxic units would thus be the sum of the toxic units of each component present (see Equation 1-1, Chapter 1). Similarly, the toxicity of the mixture can be expressed in toxic units. Following this approach, if the distillates are concentration-additive in their toxicity, the LC50 of the mixture will occur at a concentration of 1 TU. If the toxicity of the mixture occurs at a concentration other than 1 TU, then the toxicities of the individual contaminants are not concentration-additive.

For mixtures of F2 and F3a, equitoxic combinations of the two distillates (i.e., each component of the mixture contributes an equal number of toxic units to the mixture), based on the nominal concentrations, were made. In treatment calculations, a nominal LC50 of 960 mg/kg dry weight and 3,400 mg/kg dry weight were used for Fraction 2 and Fraction 3a, respectively. These LC50 values were based on the results of a single lethal toxicity test conducted by our laboratory with each distillate prior to the present investigation. In Table 2-4, Chapter 2 (Cermak et al. 2010), the results of multiple tests for lethality are provided; these additional results were obtained during the current investigation with the individual distillates run concurrently with the binary toxicity tests (see below).

For combinations of F3a and F3b, the above approach was modified since F3b was not lethal; therefore, the concentration of F3b could not be expressed in toxic units. For this combination, the concentration of Fraction 3b was kept constant in all treatments (10,000 mg/kg dry weight nominal) while the concentration of F3a varied. The concentration of 10,000 mg F3b/kg dry weight was one that produced effects on reproduction but did not result in extreme hydrophobicity in the soils. The concentration of F3a was expressed in toxic units and the LC50 was expected to occur at 1 TU (i.e., the LC50 for F3a). As in the F2F3a tests, a nominal LC50 of 3,400 mg F3a/kg dry weight was used in treatment calculations.

Concurrent with each binary test, lethal toxicity tests with the individual distillates were also conducted. Experimental conditions for tests with individual distillates are provided in Table 2-1, Chapter 2 (Cermak et al. 2010) with the exception that, for F3b, only one concentration (10,000 mg/kg dry weight) was used. The toxic unit values in the mixture test were adjusted using the LC50s determined in the concurrently run individual distillate toxicity tests. Thus, any changes in the sensitivity of the organisms or variation in the test conditions that might affect the toxicity were accounted for. The results from the tests with the individual distillates were included in Table 2-4, Chapter 2 (Cermak et al. 2010).

From one F2F3a test and one F3aF3b test, soil samples from the lowest mixture concentration, the highest mixture concentration, and the concentration equal to 1 TU (nominal) were submitted to EnviroTest Laboratories (Edmonton, AB) for fractionation into aliphatics and aromatics and chemical analysis by GC-FID, as described in Chapter 2 (Cermak et al. 2010) and Chapter 2, Appendix A. All soil results were corrected for the analytical recovery (Chapter 2 (Cermak et al. 2010) and Chapter 2, Appendix A) and for the concentration of PHCs in the control soil. Relationships between the measured and nominal concentrations for each distillate in the soils were derived. All nominal soil concentrations of the mixtures were translated into measured concentrations and the concentrations, in toxic units, were adjusted accordingly.

4.2.5. Toxicokinetics of binary combinations of distillates by earthworms

The experiments investigating the toxicokinetics of binary combinations of petroleum distillates by earthworms were conducted as described in Chapter 3. The experimental conditions are listed in Table 4-2. Initial earthworm weights were generally 350 to 600 g wet weight and the same nominal concentrations used in the investigation of the accumulation and elimination of individual distillates (480, 1,700, and 10,000 mg/kg dry weight for Fractions 2, 3a, and 3b, respectively (Chapter 3)) were used in the present study. These concentrations were non-lethal and within the range affecting reproduction in the case of F3a and F3b (Chapter 2 (Cermak et al. 2010)) or at approximately one half of the LC50 in the case of F2. Briefly, each test consisted of an accumulation phase and an elimination phase. The duration of the accumulation phase was 32 d for the binary combination of F2 and F3a (F2F3a) and 64 d for the binary combination of F3a and F3b (F3aF3b). At each sampling time, three or four independent replicates were destructively sampled, providing approximately 4 to 8 g wet weight tissue for analysis. On day 8 (for F2F3a) or day 16 (for

Table 4-2: Experimental conditions for the tests on the toxicokinetics of binary combinations of oil distillates (Fractions 2, 3a, and 3b) and the earthworm *Eisenia andrei*

Test	Sampling times for accumulation	Day transfer earthworms to clean soil for elimination	No. of independent replicates sampled per sampling date	No. of organisms/ replicate	Test unit	Amount soil (@ 33% moisture)	Lighting and temperature regime
F2F3a	Days 0, 1, 2, 4, 8, 16, and 32	Day 8	4	5	500-mL glass mason jar with perforated aluminum foil lid[1]	300	Continuous fluorescent, 50-100 µmol/(m^2·s) 22°C
F3aF3b	Days 0, 2, 4, 8, 16, 32, and 64	Day 16	3-4	5	500-mL glass mason jar with perforated aluminum foil lid	300	Continuous fluorescent, 50-100 µmol/(m^2·s) 22°C

[1] lids were only perforated on day 8 for all remaining test units in order to be consistent with the toxicity test protocol (Appendix C). All elimination phase test units had perforated lids.

F3aF3b), earthworms from 12-16 test units were removed from the contaminated soil and placed into clean soil (33% moisture, wet weight basis) for the elimination phase of the study; this became day 0 of the elimination phase. There were four sampling times during the elimination phase (day 1, 2, 4, and 8; Table 4-2). At each sampling time, three or four independent replicates were destructively sampled, providing approximately 4 to 8 g wet weight of tissue for analysis. Soil samples were collected on either three (F3aF3b) or six (F2F3a) different sampling events during the accumulation phase only as described in Chapter 3. Similar to Chapter 3, test soils were prepared in batches and one replicate from each batch was destructively sampled during each sampling event. For each sampling event, tissue samples or soil samples were pooled for analysis (Chapter 3).

Earthworms were not fed for the first 16 d of the accumulation phase or during the elimination phase. Earthworms were fed approximately 5 ml of cooked oatmeal (1:3 oatmeal:deionized water, v:v) on days 16 and 32 of the accumulation phase, when applicable. As well, at this time deionized water was added to each test unit equivalent to the amount lost (by weight) over the test period.

Accumulation and elimination data were analyzed as described in Chapter 3. The accumulation and elimination of both aliphatic and aromatic compounds for each distillate range in the binary combination, as well as total aliphatics and total aromatics for each test, were determined.

Soil and tissue samples were fractionated and analyzed by GC-FID by Envirotest Laboratories as described in Chapters 2 (Cermak et al. 2010) and 3 and Chapter 2, Appendix A. All tissue results are reported on a lipid basis (mg/kg lipid) following correction for the average background concentration of aliphatics and aromatics of all control earthworm tissues. Tissue concentrations were considered to be significantly greater than background if they were greater than two standard deviations above the average control tissue concentration for each ECN range (Jager et al. 2003a). Tissue concentrations less than this were considered to be zero. Likewise, soil concentrations were also corrected for the background concentrations of aliphatic and aromatic compounds in the clean soil used for each test.

Biota-soil accumulation factors (BSAFs) for aliphatics and aromatics in each distillate range as well as the total accumulated aliphatics and aromatics were determined as described previously

(Equation 3-7, Chapter 3) for both measured and estimated maximum tissue concentrations, when applicable.

4.2.6. Statistical analysis

Statistical analyses of the toxicity data followed the recommendations of Environment Canada (EC 2005b). The results of the lethality studies were analyzed by logit (WEST and Gulley 1996), non-trimmed Spearman-Karber (WEST and Gulley 1996), or binomial procedures where appropriate. For toxicity tests with individual distillates, all concentrations were log-transformed for analysis (EC 2005b). For toxicity tests with binary combinations of distillates, which were based on toxic units, the concentrations in toxic units were log-transformed.

Statistical analyses of measured soil concentrations, as a percent of the nominal concentration, were also conducted. All analysis of variance (ANOVA) procedures and comparison of treatments were conducted using SYSTAT® (Systat Software Inc. 2004b). Data normality and homoscedasticity of residuals were determined by the Shapiro-Wilk normality test and Levene's test, respectively. Treatment parametric comparisons were conducted using Fisher's Least Significant Difference test and non-parametric comparisons were performed using the Kruskal-Wallis One-way Analysis of Variance test.

4.3. RESULTS

4.3.1. Relationship between nominal and measured concentration for binary combinations of distillates

The concentrations of aliphatic and aromatic compounds were analytically determined for soil samples from three treatment levels from one F2F3a and one F3aF3b toxicity test. In addition, concentrations of aliphatic and aromatic compounds were determined in soils amended with binary combinations of F2 with F3a and F3a with F3b in earthworm toxicokinetic experiments. These data were combined and used to determine linear relationships relating measured concentrations to nominal concentrations of each distillate in each binary combination (Table 4-3). Nominal concentrations for each distillate in the binary toxicity tests were adjusted to measured concentrations using these relationships. Linear relationships for measured versus nominal

Table 4-3: Relationship between the measured and nominal concentrations of distillates when in combination

	Average measured concentration as % of nominal at beginning of test	Standard deviation of % nominal	Standard error of % nominal	Coefficient of variation of % nominal (%)	Linear relationship for measured vs. nominal concentration (mg/kg dry wt.)
F2F3a test					
F2 in F2F3a combination	66.5	13.67	6.84	20.57	F2 measured = 0.872(nominal) – 104.0 r^2 = 0.971
F3a in F2F3a combination	67.1	7.05	3.53	10.51	F3a measured = 0.788(nominal) – 193.1 r^2 = 0.993
F3aF3b test					
F3a in F3aF3b combination	71.8	4.58	2.29	6.38	F3a measured = 0.679(nominal) + 112.2 r^2 >0.999
F3b in F3aF3b combination	71.1	3.24	1.62	4.56	Not applicable

concentrations found in Table E1 (Chapter 2, Appendix E) were used to adjust nominal concentrations for toxicity tests with individual distillates.

The average measured concentration, as a percent of the nominal concentration, of each distillate when spiked into soil in binary combination were not significantly different from one another (p >0.05).

4.3.2. Toxicity of binary combinations of oil distillates to the earthworm, Eisenia andrei

The results of toxicity tests conducted with binary combinations of Fractions 2, 3a, and 3b are presented in Table 4-4. For all combinations, the toxicity of the mixture occurred at concentrations greater than 1 TU, indicating that the toxicities of the individual distillates are less-than-concentration-additive when the distillates are in combination (i.e., the toxicity of the binary combinations are less toxic than expected based on the toxicities of the individual distillates). The approximate concentration of each distillate at the concentration of the LC50 for the mixture as well as the observed LC50 from concurrently run tests with the individual distillates is noted in Table 4-4 for comparative purposes.

4.3.2.1. Tests with Fractions 2 and 3a

As observed in Table 4-4, the median lethal toxicity of binary combinations of F2 and F3a occurred at concentrations above 1 TU, suggesting that the toxicity of the distillates was less-than-concentration-additive when present together in this test system. In both cases, at the median lethal concentrations for the binary mixtures, the concentration of F2 was greater than the LC50 for the distillate alone (F2 contributed 1.35-2.26 TU).

Table 4-4: Median lethal concentration of binary combinations of the oil distillates Fractions 2, 3a, and 3b.

Experiment	Duration	LC50 of mixture (Toxic Units with 95% CI)	LC50 from concurrent tests with individual distillates (mg/kg dry wt. with 95% CI)	Estimated concentration of each distillate at the LC50 of the mixture (mg/kg dry wt.)		
				F2	F3a	F3b
F2F3a test 1	28 days	3.21 (2.91-3.56)	F2: 320 (280-350) F3a: 2,490 (2,230-2,790)	720	2,390	n/a
F2F3a test 2	28 days[1]	1.92 (1.74-2.12)	F2: 380 (350-400) F3a: 3,120 (2,780-3,480)	510	1,780	n/a
F3aF3b test 1	28 days	>2.13[2]	F3a: 2,440 (2,150-2,770) F3b: not toxic	n/a	>5,190	7,110[3]
F3aF3b test 2	28 days	~2[2]	F3a: 3,670 (3,230-4,160) F3b: not toxic	n/a	7,040	7,110
	42 days	1.57 (1.48-1.67)	F3a: 3,360 (2,990-3,770) F3b: not toxic	n/a	5,270	7,110

[1]this test was extended to 42 days but no additional mortality occurred after 28 days

[2]LC50 is approximately equal to or greater than the highest concentration tested; therefore LC50 values could not be calculated

[3]concentrations of F3b in this test ranged from 7,109-7,678 mg/kg dry wt.

n/a –not applicable

While equitoxic concentrations of F2 and F3a were the aim when preparing the treatment levels, this was not achieved. In general, F2 contributed more to the total concentration, in toxic units, than F3a. Thus, at the LC50 in F2F3a test 1, the concentration of F2 was 2.26 TU while that of F3a was 0.95 TU. In F2F3a test 2, at the LC50 of the mixture the concentration of F2 was 1.35 TU and that of F3a was 0.57 TU.

It is noted that in each test with F2 and F3a, the majority of the deaths occurred within the first 14 d of exposure. Smaller increases in toxicity were observed after 14 d. One test was extended to 42 d and no additional deaths were recorded after 28 d. Therefore, the threshold effect concentration, defined as the concentration at which there is no increase in observed effects with indefinite exposure (Persoone et al. 1990), for this mixture appears to be reached by 28 d.

4.3.2.2. Tests with Fractions 3a and 3b

As observed with binary combinations of F2 and F3a, the median lethal toxicity of F3a in combination with F3b occurred at concentrations equal to or greater than 1.57 TU (Table 4-4). Because F3b was not lethally toxic, its concentration was held constant. Thus, the concentration of the mixture expressed in toxic units refers only to the concentration of F3a in the mixture, and a concentration of 1.57 TU reflects a concentration of F3a that was 1.57 times greater than the LC50 of F3a alone.

During the 28-d test duration, mortality was not observed until day 14, unlike what was observed with combinations of F2 and F3a. With all tests, after 28 d of exposure, mortality remained below 50% at the highest test concentration, even though 28-d LC50s could be determined for F3a alone and were close to the threshold toxicity values (see Chapter 2 (Cermak et al. 2010)).

The duration of F3aF3b test 2 was extended to 42 d. Similar to F3aF3b test 1, mortality was first recorded on day 14 and increased over the extended test period, such that a median lethal toxicity value of 1.57 TU could be calculated at day 42. No apparent threshold concentration for lethality was reached during this time. It is possible that the toxicity of the binary mixture may approach 1 TU at the threshold concentration for lethality; however, the data demonstrate that the time to reach the threshold concentration when F3a and F3b are in binary combination occurs at a longer exposure duration than that for F3a alone (35 d, see Chapter 2 (Cermak et al. 2010)).

While the concentration of F3b was held constant among treatments in each test, in F3aF3b test 1 an error was made during treatment preparation that resulted in the concentration of F3b ranging from 10,000 to 10,800 mg/kg dry weight nominal (7,109-7,678 mg/kg dry weight measured). Since similar, less-than-additive toxicity was observed in the other F3aF3b test, this variability in the concentration of F3b among the test units minimally affected the results.

4.3.3. Toxicokinetics for binary combinations of distillates by earthworms

4.3.3.1. Loss of distillates from soil

Rate constants describing the loss of aliphatic and aromatic compounds from the soil (k_s) are given in Table 4-5. Little to no loss of hydrocarbons from soil was noted over the first 16 days of the test with F2F3a and therefore no k_s could be determined. Rate constants were estimated for the F3aF3b test but there was considerable variability in the soil concentrations over time. These values should be used with caution. Similar to that observed with individual distillates (Chapter 3), half-lives in soil, when determined, were less for aliphatic compounds compared to aromatic compounds.

Table 4-5: Rate constants for individual distillates when they are present in binary combination with a second distillate

Parameter	Units	F2F3a test					F3aF3b test			
		F2	F3a		Total		F3a	F3b	Total	
Aliphatics			*d0-d16*	*d0-d32*	*d0-d16*	*d0-d32*			*d0-d16*	*d0-d64*
k_1	kg dry wt./ (kg lipid·d)	5.809	0.964	---	2.05	0.795	nd[1]	nd[1]	nd[1]	nd[1]
k_{dis}	day^{-1}	0.008	---	---	---	---	---	---	---	---
$k_{2\ uptake}$	day^{-1}	nd[3]	0.444	---	0.350	0.059	nd[1]	nd[1]	nd[1]	nd[1]
$k_{2\ elim}$	day^{-1}	0.252	0.069	---	0.192	0.192	nd[2]	nd[2]	nd[2]	nd[2]
$t_{1/2\ elim}$	day	2.8	10.0		3.6	3.6				
k_s	day^{-1}	nd	nd	---	nd	nd	0.013	0.012	nd	0.012
$t_{1/2\ soil}$	day	nd	nd	---	nd	nd	53.3	57.8	nd	57.8

Table 4-5 continued

Parameter	Units	F2F3a test					F3aF3b test			
		F2	F3a		Total		F3a	F3b	Total	
Aromatics			*d0-d16*	*d0-d32*	*d0-d16*	*d0-d32*			*d0-d16*	*d0-d64*
k_1	(kg dry wt./ (kg lipid·d)	82.136	---	10.761	---	16.601	3.422	0.250	2.176	0.551
k_{dis}	day^{-1}	0.044	---	0.05	---	0.018	---	---	0.318	---
$k_{2\ uptake}$	day^{-1}	nd[3]	---	nd[3]	---	nd[3]	0.122	0.016	nd[3]	0.042
$k_{2\ elim}$	day^{-1}	0.625	---	0.128	---	0.199	0.053	0.025	0.040	0.040
$t_{1/2\ elim}$	day	1.1		5.4		3.5	13.1	27.7	17.3	17.3
k_s	day^{-1}	nd	---	nd	---	nd	0.008	0.005	nd	0.006
$t_{1/2\ soil}$	day	nd	---	nd	---	nd	86.6	138.6	nd	115.5

[1]data could not be modelled

[2]tissue concentrations were zero for all days during elimination phase

[3]used k_2 derived from elimination phase in the model

--- not used as a parameter in the model

nd not determined

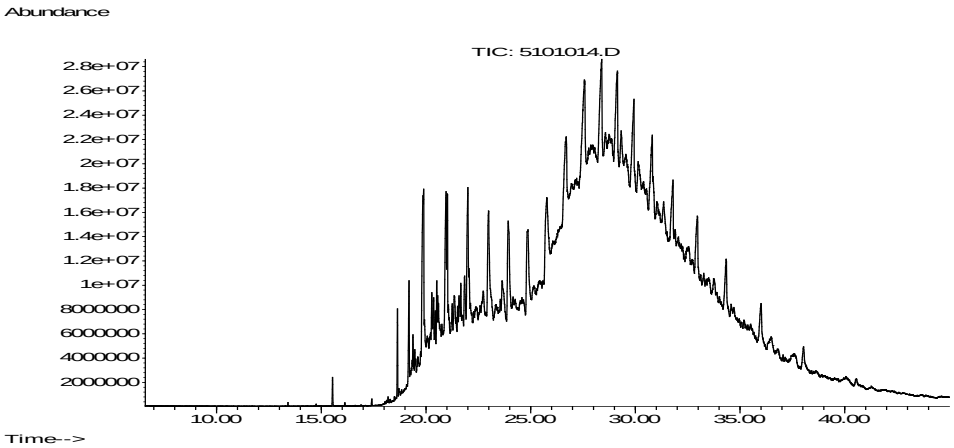
4.3.3.2. Accumulation and elimination of binary combinations F2F3a and F3aF3b

Earthworms accumulated both aliphatic and aromatic compounds from soil and predominantly within the boiling point range of the distillates used within a test supporting that they were mainly petrogenic in origin.

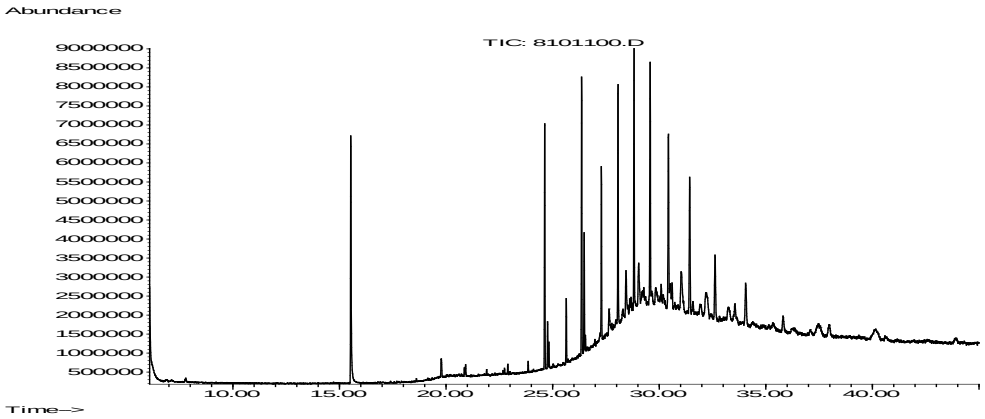
Figure 4-1 depicts GC-MS open scan chromatograms of the aliphatic fraction from the F3aF3b test in soil and earthworm tissue. *N*-alkanes (approximately C16-C35) and a hump representing the unresolved complex mixture (UCM) were both observed in the soil covering the full range of both F3a and F3b. Notwithstanding, though tissue samples had an UCM in both the F3a and F3b range, *n*-alkane peaks were observed predominantly within the F3b range (from approximately C21-C33).

GC-MS chromatograms were not available for samples from the F2F3a test. GC-FID chromatograms of soil (Figure 4-2) and tissue (Figure 4-3) samples illustrate the accumulation of aliphatic compounds by earthworms. Prominent peaks indicative of *n*-alkanes were observed in soil samples from days-2 through 16; these peaks were mostly absent in the F2 range and greatly diminished in the F3a range by day 32. Two large peaks (between approximately 6-6.5 min) observed at day 32 may be associated with laboratory contaminants (see Appendix B). *N*-alkane peaks above the UCM appeared to be absent in tissue samples (Figure 4-3). Two or three small peaks were observed in the higher molecular weight F3a range by day 32 that may be associated with *n*-alkanes though this cannot be confirmed; the presence of higher molecular weight *n*-alkanes would be consistent with what was observed in the F3aF3b test. Similar to what was observed with soils, the two larger peaks at retention times of 6-6.5 min may be associated with laboratory contaminants.

(A)



(B)



(C)

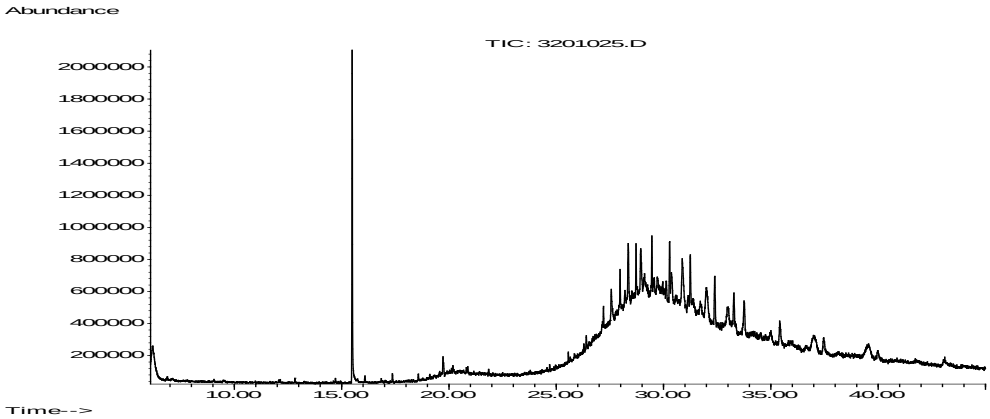


Figure 4-1: GC-MS open scan chromatograms of aliphatic compounds from the toxicokinetic study with *Eisenia andrei* and a binary combination of petroleum distillates F3a and F3b: (A) soil d0; (B) tissue following 2 d of accumulation; and (C) tissue following 16 d of accumulation

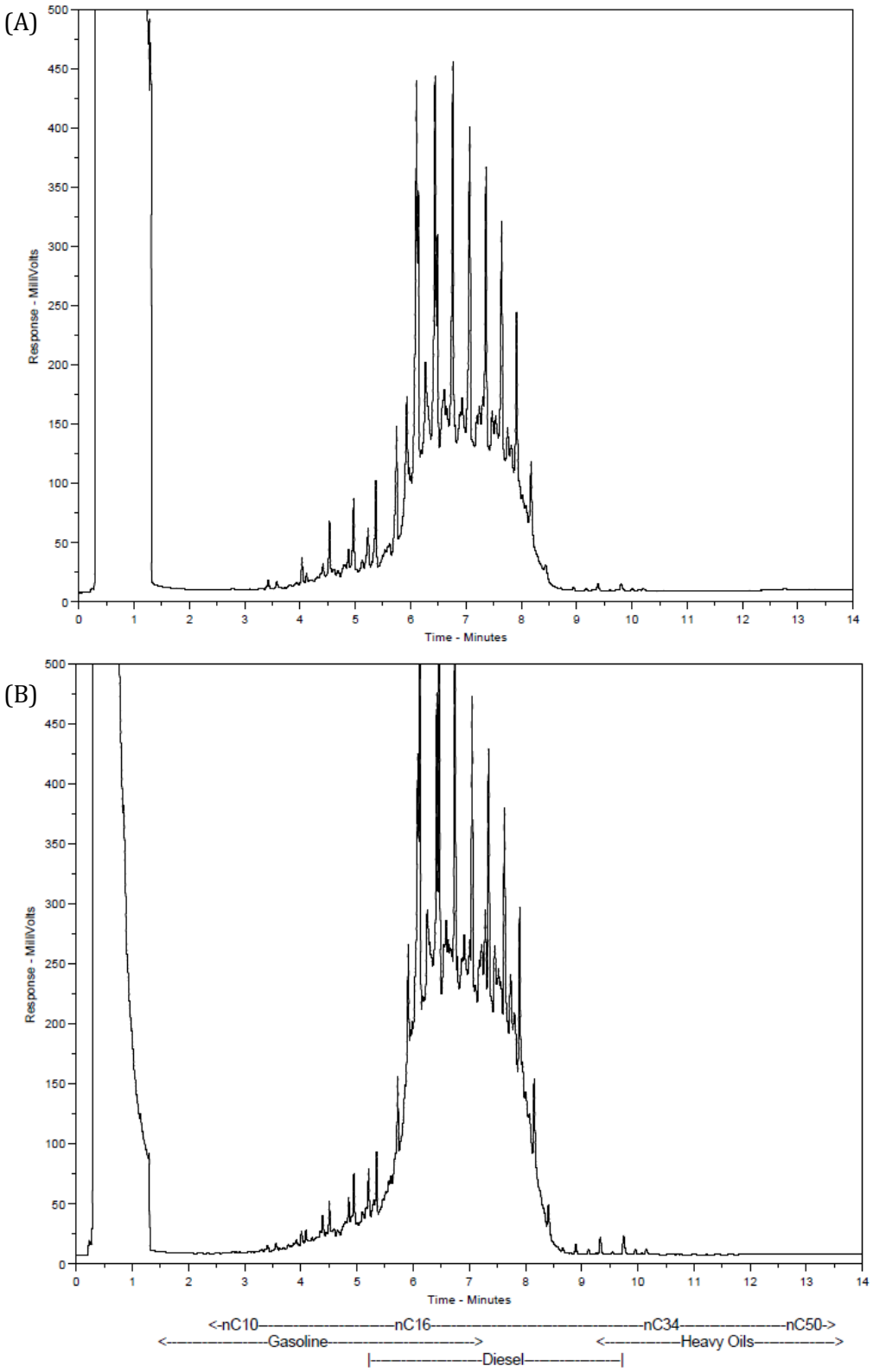


Figure 4-2: GC-FID chromatographs of aliphatic compounds in soil at (A) day 2, (B) day 16, and (C) day 32 of the toxicokinetic study with a binary combination of petroleum distillates F2 and F3a

(C)

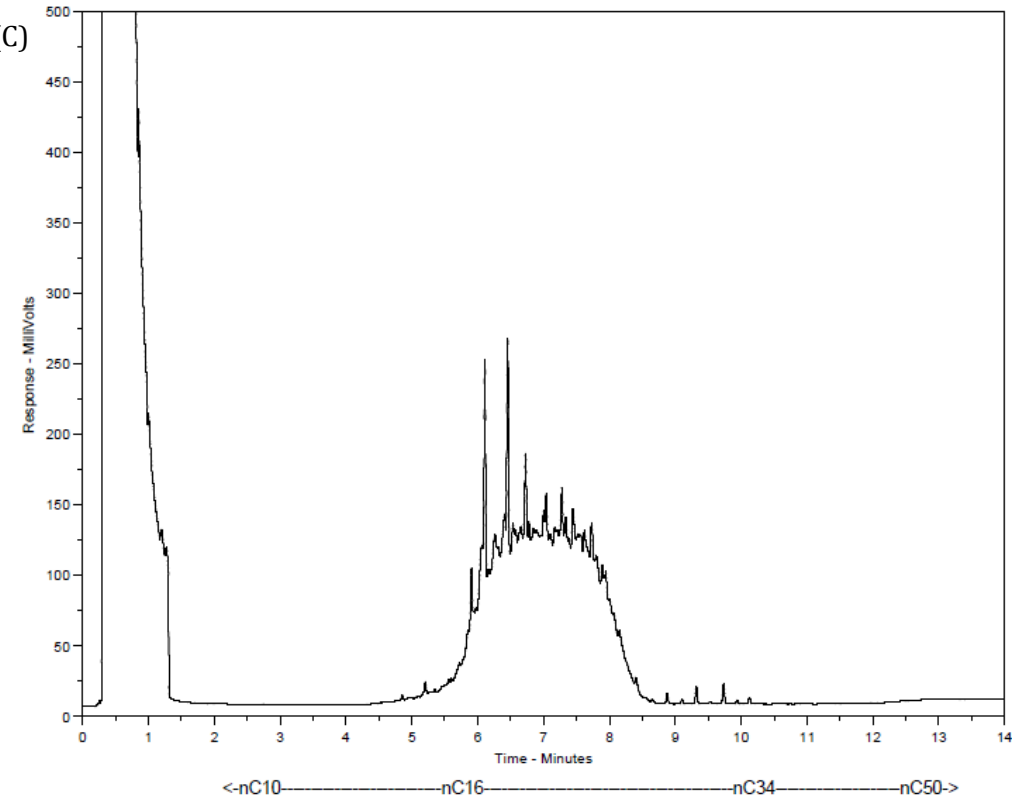


Figure 4-2 continued: GC-FID chromatographs of aliphatic compounds in soil at (A) day 2, (B) day 16, and (C) day 32 of the toxicokinetic study with a binary combination of petroleum distillates F2 and F3a

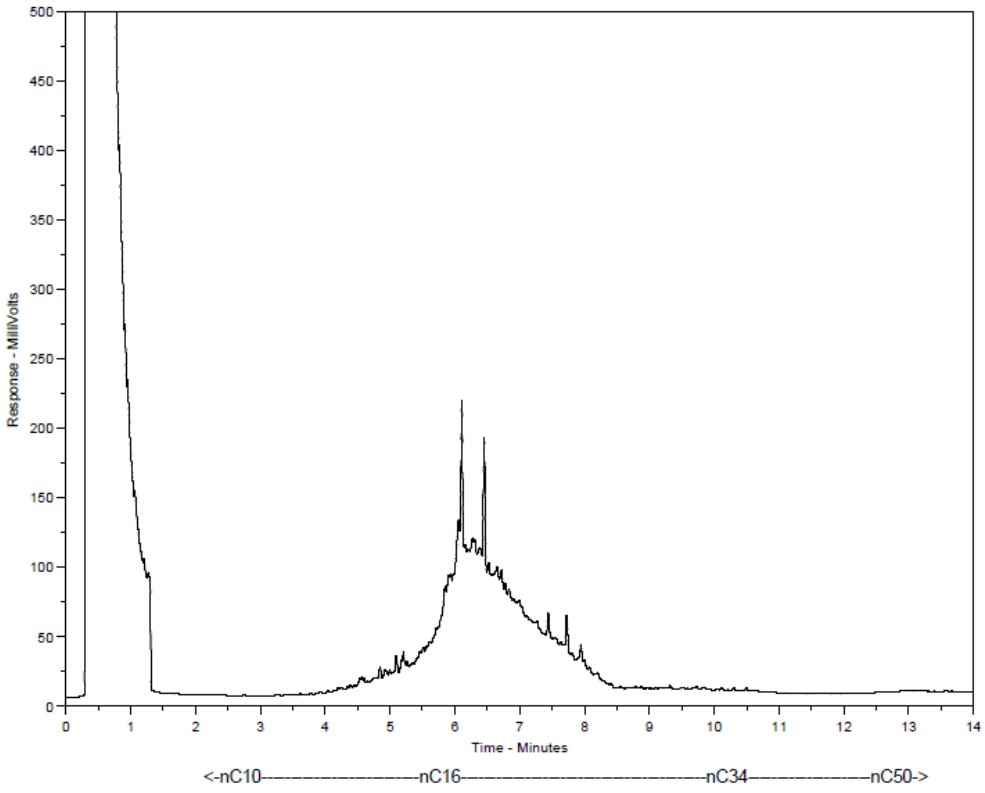


Figure 4-3: GC-FID chromatographs of aliphatic compounds in *E. andrei* tissue at day 32 of the toxicokinetic study with a binary combination of petroleum distillates F2 and F3a

Figure 4-4 to Figure 4-7 illustrate the accumulation and elimination of the aliphatic and aromatic fractions of individual distillates when in the presence of a second distillate. Accumulation and eliminations rate constants are given in Table 4-5 for each distillate tested as well as for total aliphatics and aromatics. Most of the data could be modelled, except for F3a and F3b aliphatic compounds in the F3aF3b test; the data from this test were not amenable to the accumulation models and are thus presented as the measured tissue concentrations in Figure 4-6 and Figure 4-7.

A variety of accumulation curves were noted. Similar to that observed with individual distillates (Chapter 3), a peak in the accumulation curve was noted for both F2 aliphatic and aromatic compounds, as well as for F3a aromatic compounds, in the F2F3a test. A peak could not be modelled from the data for F3a aromatics in the F3aF3b test ($k_{dis} \sim 0$), though it is noted that the tissue concentration appeared to be decreasing after day 32 similar to what was observed when F3a was tested alone. An additional data point after day 64 is required to determine if this trend of decreasing tissue concentrations continues or if the data point at day 32 simply represents variability in the data.

Also similar to what was observed with individual distillates (Chapter 3), the accumulation of F3a aliphatics in the F2F3a test closely followed standard first-order accumulation up to day 16 with a slower increase in tissue concentration thereafter. The accumulation of F3a aliphatics were only modelled for day 0 to day 16 because the data from day 0 to day 32 were not amenable to the models; however, the accumulation of total aliphatics for both day 0 to day 16 and day 0 to day 64 were modelled separately (Table 4-5).

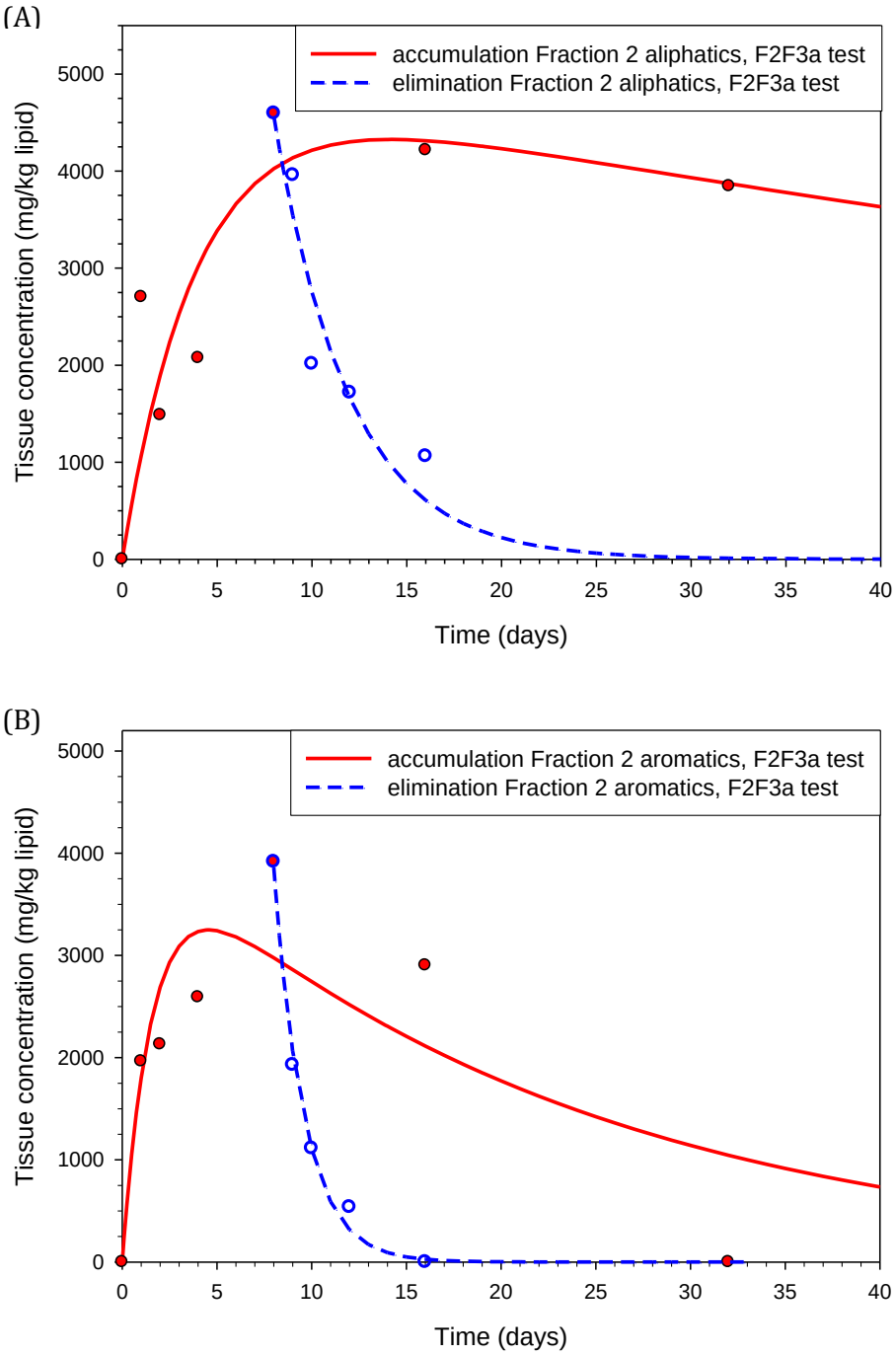


Figure 4-4: Accumulation and elimination of Fraction 2 (A) aliphatic and (B) aromatic compounds by *E. andrei* following exposure to soil contaminated with Fraction 2 and Fraction 3a.

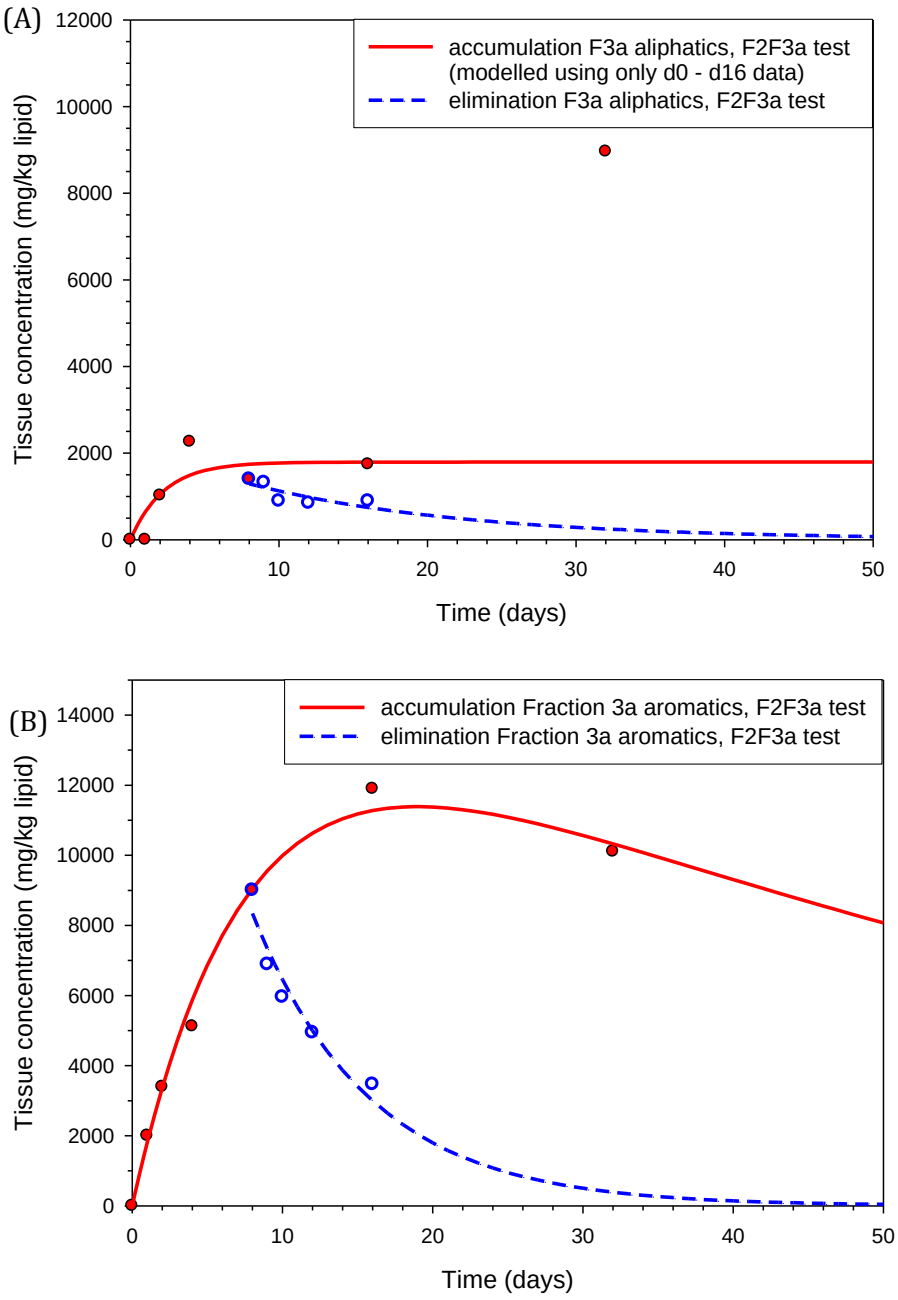


Figure 4-5: Accumulation and elimination of Fraction 3a (A) aliphatic and (B) aromatic compounds by *E. andrei* following exposure to soil contaminated with Fraction 2 and Fraction 3a. The *y*-axes are not to the same scale.

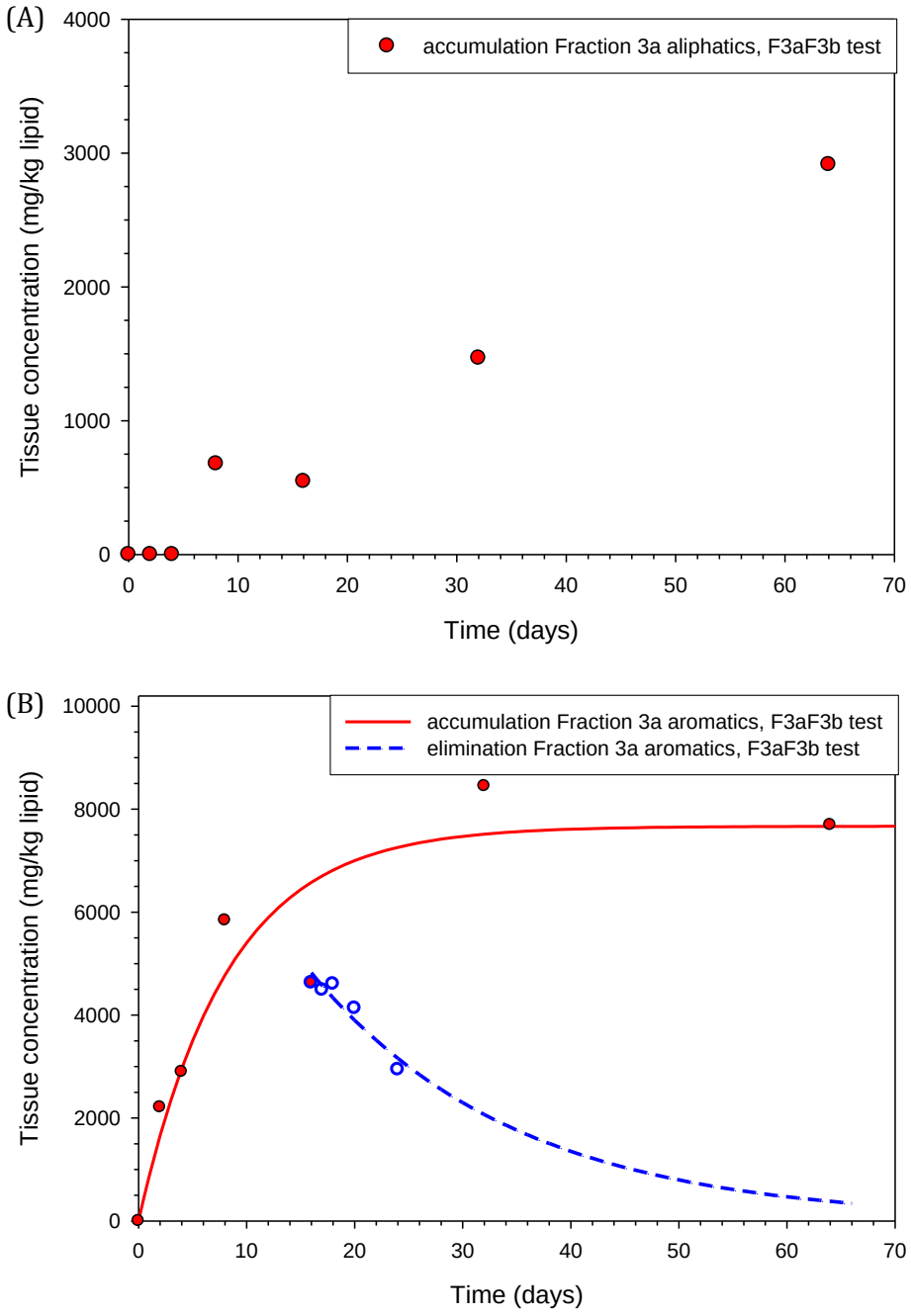


Figure 4-6: Accumulation and elimination of Fraction 3a (A) aliphatic and (B) aromatic compounds by *E. andrei* following exposure to soil contaminated with Fraction 3a and Fraction 3b. The *y*-axes are not to the same scale.

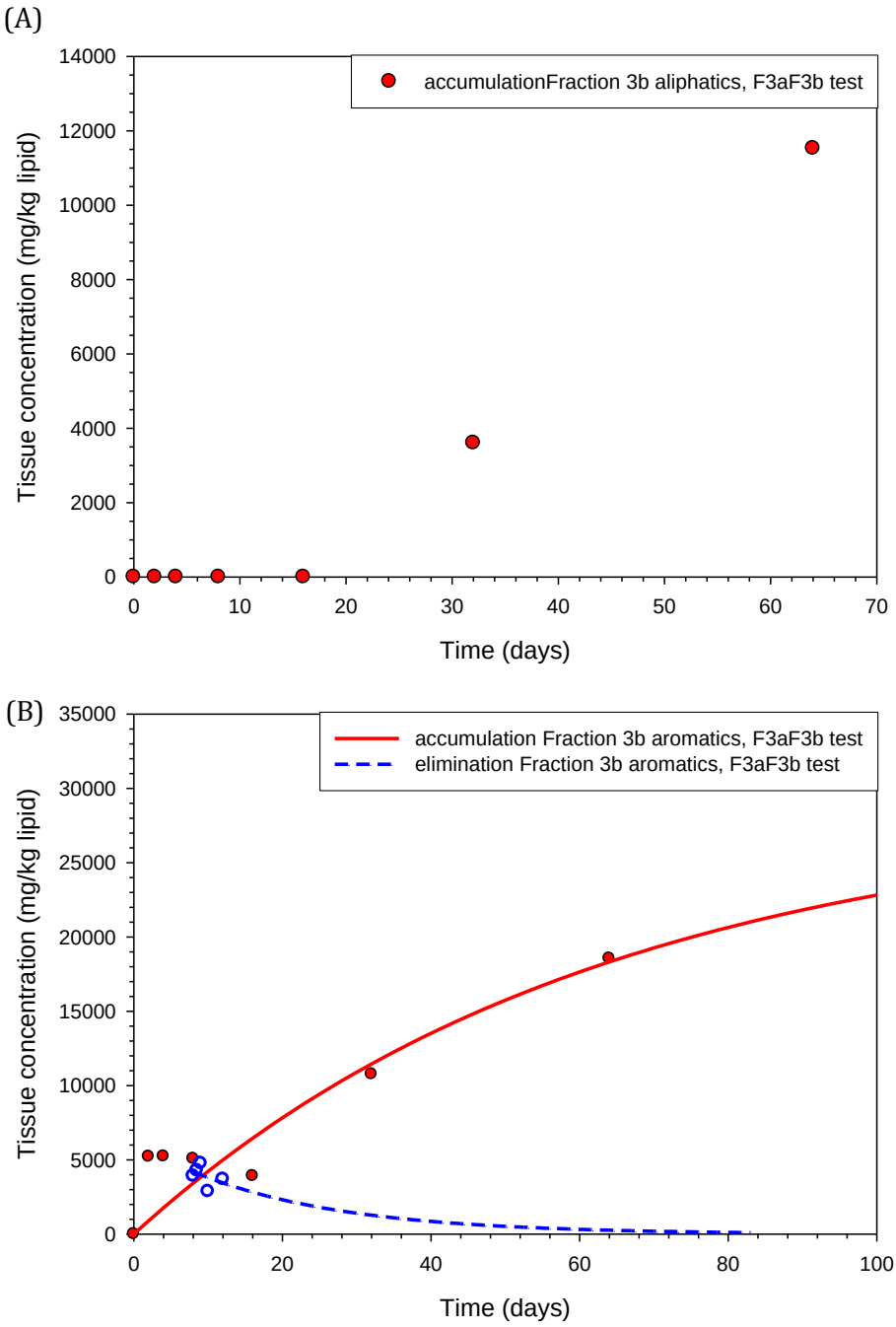


Figure 4-7: Accumulation and elimination of Fraction 3b (A) aliphatic and (B) aromatic compounds by *E. andrei* following exposure to soil contaminated with Fraction 3a and Fraction 3b. The *x*- and *y*-axes are not to the same scale.

In the F3aF3b accumulation test, very little F3a or F3b aliphatics were accumulated at the start of the test (Figure 4-6 and Figure 4-7). Though identifiable *n*-alkane peaks and a small UCM were noted in the GC-MS chromatographs following only two days of accumulation (Figure 4-1), the concentration of the accumulated total aliphatics remained below the level determined to be significantly different from control tissues and therefore the accumulation of aliphatics is considered to be nil.

Elimination of aliphatics and aromatics was monophasic and generally was modelled well by Equation 3-3, Chapter 3. As observed when tested individually, F2 aromatics were completely eliminated over the 8-d elimination phase. Very little elimination of F3b aromatics was noted in the F3aF3b test and, thus, the elimination model fit the data poorly (Figure 4-7). Elimination of F3a and F3b aliphatics could not be determined as there was no significant accumulation of these compounds during the 16-d period prior to the elimination phase.

Accumulation and elimination rate constants for total aliphatics or total aromatics fell between the values observed for the individual distillates (Table 4-5). Similar to what was observed in tests with individual distillates, higher accumulation rates were observed for aromatics resulting in a disproportionate accumulation, over the duration of this study, of aromatics over aliphatics by earthworms as compared to the proportions found in the soils. This is illustrated in Figure 4-8 for total aromatics and aliphatics for each binary combination.

Based on the models, the time to reach a maximum body burden as well as the time during the exposure period for which tissue concentrations were 80% or greater of the maximum were determined (Table 4-6). Aromatics always reached their maximum tissue concentrations before aliphatics.

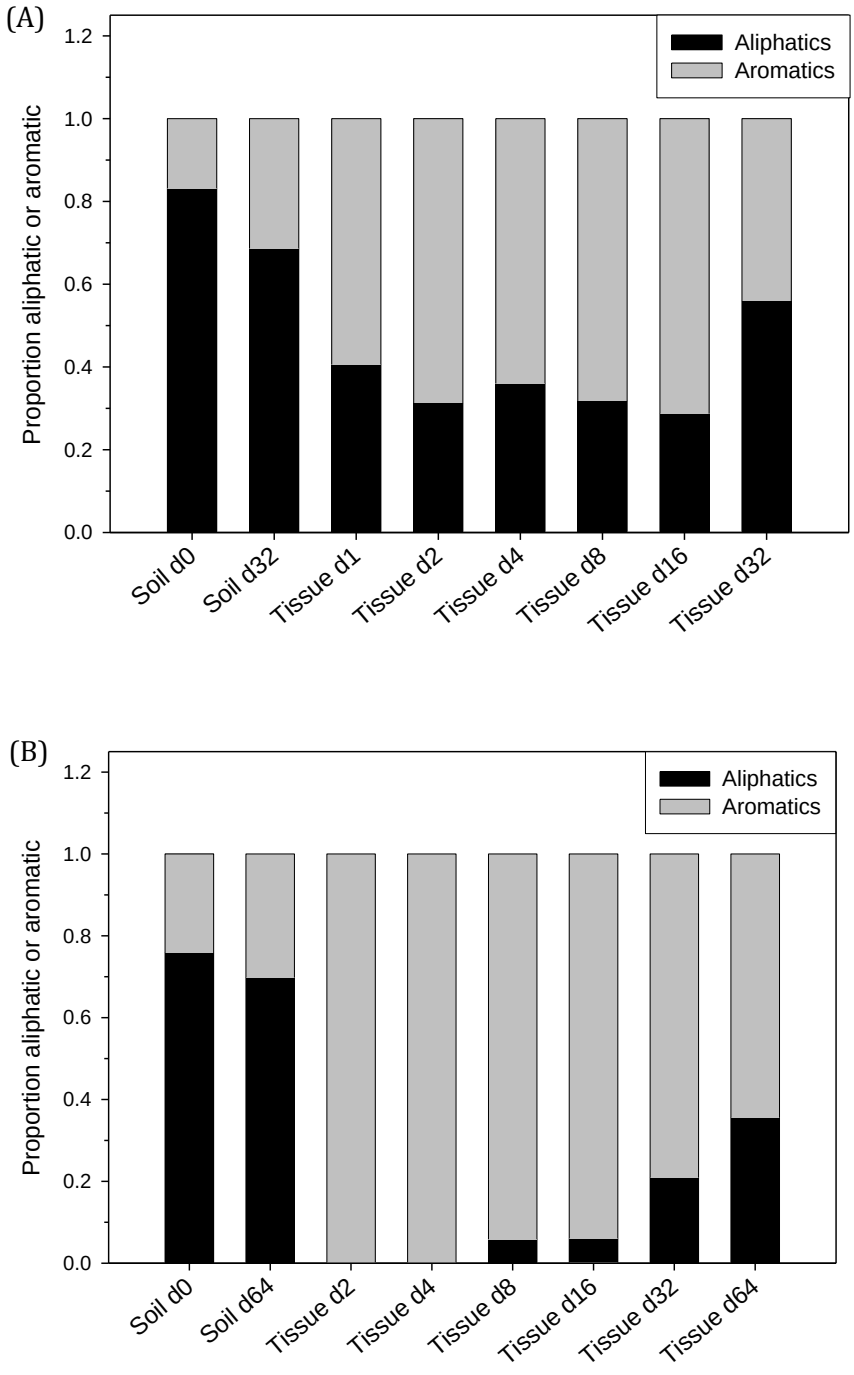


Figure 4-8: Proportion aliphatic and aromatic compounds in soils and *E*. andrei tissues in toxicokinetic studies with binary combinations of petroleum distillates (A) F2 and F3a and (B) F3a and F3b

Table 4-6: Time (days) to reach maximum body burden and time during which ≥80% of maximum is achieved for tests with binary combinations of distillates F2, F3a, and F3b

Distillate	Aliphatics		Aromatics	
	≥80% maximum	Maximum	≥80% maximum	Maximum
F2F3a test				
F2	5.3-46.1	14.2	1.9-11.2	4.6
F3a	---[1]	---[1]	8.1-41.5	19.0
Total	27.6	51.4	5.5-30.4	13.2
F3aF3b test				
F3a	---[1]	---[1]	13.2	24.6[2]
F3b	---[1]	---[1]	100.6	187.2[2]
Total	---[1]	---[1]	38.3	71.3[2]

[1]unable to determine

[2] determined as 95% of maximum for data fit by standard first-order accumulation curves

4.3.3.3. Biota-soil accumulation factors

Biota-soil accumulation factors were determined for aliphatics and aromatics in each distillate range as well as for total aliphatics and aromatics (Table 4-7). BSAFs were greater than one for F2 aliphatics and aromatics, F3a aromatics, and total F2F3a aromatics. Bioaccumulation of F2 aliphatics in the F2F3a test and F3a aromatics in the F3aF3b test were all below two, indicating minimal bioaccumulation.

Table 4-7: Biota-soil accumulation factors (BSAFs) for *Eisenia andrei* following exposure to binary combinations of petroleum distillates F2, F3a, and/or F3b. BSAFs were determined for each individual distillate using the maximum measured tissue concentration or the maximum modelled tissue concentration.

		BSAF (kg OC/kg lipid)			
		Aliphatics		Aromatics	
		measured	model	measured	model
F2F3a	F2	1.25	1.15	7.24	6.01
	F3a	0.6	0.12	3.66	3.50
	Total	0.68	0.76	3.90	3.63
F3aF3b	F3a	0.17	n/a	1.71	1.55
	F3b	0.12	n/a	0.57	0.88
	Total	0.12	n/a	0.70	0.73

n/a not applicable

4.4. DISCUSSION

4.4.1. Comparison of binary tests to tests of individual distillates

4.4.1.1. Measured versus nominal soil concentrations

The presence of a second distillate generally did not affect the measured concentration, as percent of the nominal concentration, of a distillate in the soil during treatment preparation. The percent nominal concentrations for F3a alone, F3b alone (Table E 2, Chapter 2, Appendix E), and F2, F3a, or F3b in combination with a second distillate (Table 4-3), were not significantly different from each other ($p > 0.05$). Only the percent nominal concentration of F2 when spiked into soil alone was significantly less ($p \leq 0.016$) than the others.

This difference in measured F2 concentration when spiked into soil alone or in combination with F3a resulted in non-equitoxic concentrations of F2 and F3a in the F2F3a toxicity test. Equitoxic concentrations were based on single LC50 values for each distillate reported as nominal concentrations. In order for the actual concentrations to remain in equitoxic proportions, the loss of distillate upon spiking (i.e., % nominal) for each distillate would need to remain constant regardless of whether the distillate was added to the soil alone or with another distillate. The lower

loss of F2 when added to soil with F3a than alone resulted in a larger contribution of F2 to the total toxic unit of the mixture.

4.4.1.2. Comparison of the toxicokinetics of individual and binary combinations of distillates

Rate constants for tests with individual distillates (Table 3-2, Chapter 3) and binary combinations of distillates (Table 4-5) followed the same pattern: 1) they were always in the order F2 > F3a > F3b; 2) they were consistently greater for accumulation than for elimination; and 3) they were consistently greater for aromatics than aliphatics. As with distillates when tested individually, a disproportionate accumulation of aromatic compounds as compared to aliphatic compounds was observed (Figure 4-8). This is in agreement with others who have noted that the majority of body burdens following PHC exposure will be from the aromatic fraction (Verbruggen et al. 2008).

Rate constants for the distillates in binary combination were less than those for distillates tested individually (Table 4-5 and Table 3-2, Chapter 3). The effect of the different rate constants on the accumulation curves can be seen in Figure 4-9 and Figure 4-11, where, for comparative purposes, the accumulation curves for each distillate from individual and binary accumulation tests were standardized to the same initial soil concentration of distillate. This standardization assumes that the accumulation pattern remains constant regardless of the initial soil concentration, though this may not necessarily be the case (Jager et al. 2000).

Fractions 2 and 3a aliphatics and aromatics in the F2F3a toxicokinetic test had similar shaped accumulation curves as observed when these distillates were tested individually. Unlike in the other tests, a peak could not be modelled for F3a aromatics in the F3aF3b test, though it is possible that one occurred. Several possible processes were described in Chapter 3 explaining the accumulation kinetics observed for the individual distillates, including loss of distillate from the soil and changes in the bioavailability/concentration of distillate in the pore water. These processes are likely also occurring in the tests with binary combinations of distillates. It is difficult to determine to what extent the loss of distillate from the soil is affecting the accumulation curves; little to no loss of distillate was observed in the first 16 d of the test with F2F3a and the data were very variable in the F3aF3b test resulting in little confidence in the determined k_s values.

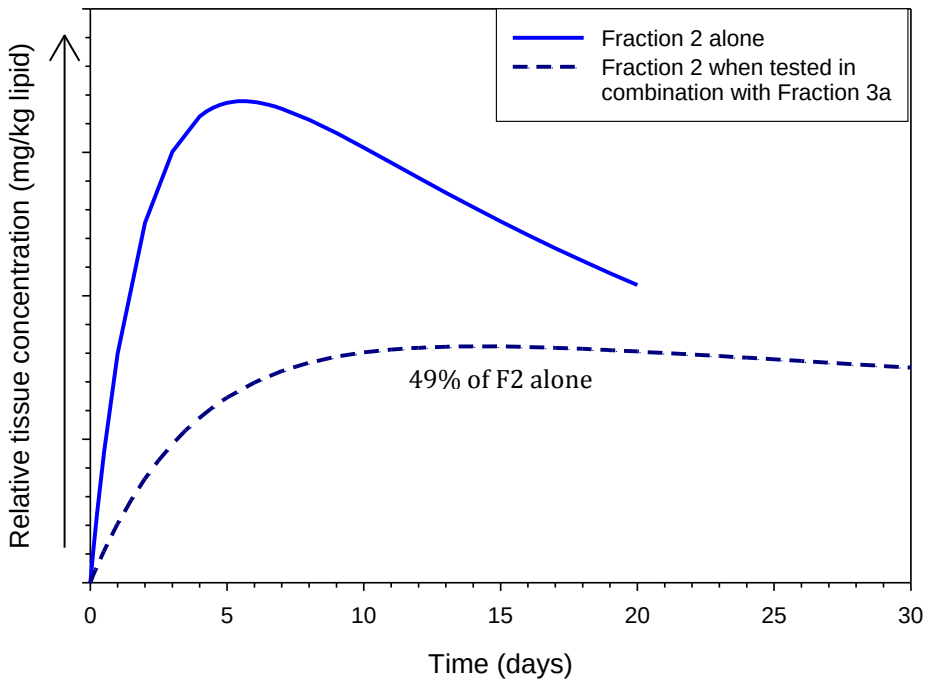
The accumulation of F3a and F3b aliphatics in the F3aF3b test was different from that observed in the tests with individual distillates. Accumulation of aliphatics was below the level considered

significantly above background for the first four (F3a) to 16 (F3b) days. The reason for this is unknown. It is possible that the presence of F3b acted as a sorbent for F3a, thus reducing accumulation (see below), but this would not explain the lack of initial accumulation of F3b. It is also possible that the concentration of petroleum hydrocarbons in the soil had sublethal effects (i.e., changes in behaviour or feeding) leading to decreased accumulation; however, a similar decrease in the accumulation of F3a and F3b aromatics would then have been expected.

From Figure 4-9 to Figure 4-11 and Table 4-6 and Table 3-5 (Chapter 3), it is noted that there is a delay in the time to reach the maximum tissue concentration. For F2, this is 2 and 9 d for aromatics and aliphatics, respectively, as compared to when F2 is tested alone. For F3a, the time to reach the maximum tissue concentration of aromatics is 6-12 d greater for binary combinations compared to F3a tested alone, while for F3b aromatics it takes more than 100 d longer to reach the maximum tissue concentration when F3a is present.

For many distillates the presence of a second distillate decreases the accumulation of the aliphatic and aromatic fractions by 44-71%. Only the accumulation of F3a aliphatics and aromatics in the F2F3a test was very similar to F3a alone (Figure 4-10). This is reflected in the BSAFs determined for F2 and F3a (Table 4-7 and Table 3-4, Chapter 3): of those BSAFs found to be greater than one, a decrease compared to when the distillate was tested alone was observed for F2 aliphatics and aromatics in the F2F3a test as well as for F3a aromatics in the F3aF3b test but not for F3a aromatics in the F2F3a test. The modelled accumulation of Fraction 3b aromatics in the F3aF3b test also did not show a large decrease in accumulation compared to F3b alone and accumulation curves were similar.

(A)



(B)

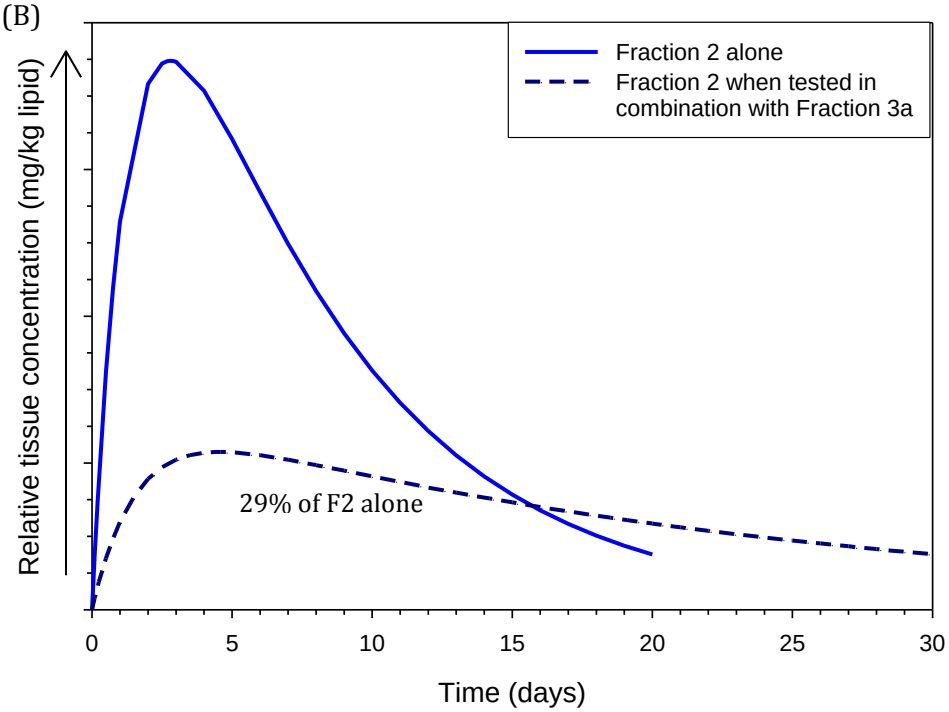


Figure 4-9: Model curves of the accumulation of Fraction 2 (A) aliphatic and (B) aromatic compounds by *E. andrei* exposed to soil contaminated with Fraction 2 alone or in combination with Fraction 3a. Curves are standardized to the same initial soil concentration to illustrate differences in the accumulation of the distillate alone and when it is in combination with another distillate. The *y*-axes are not to the same scale.

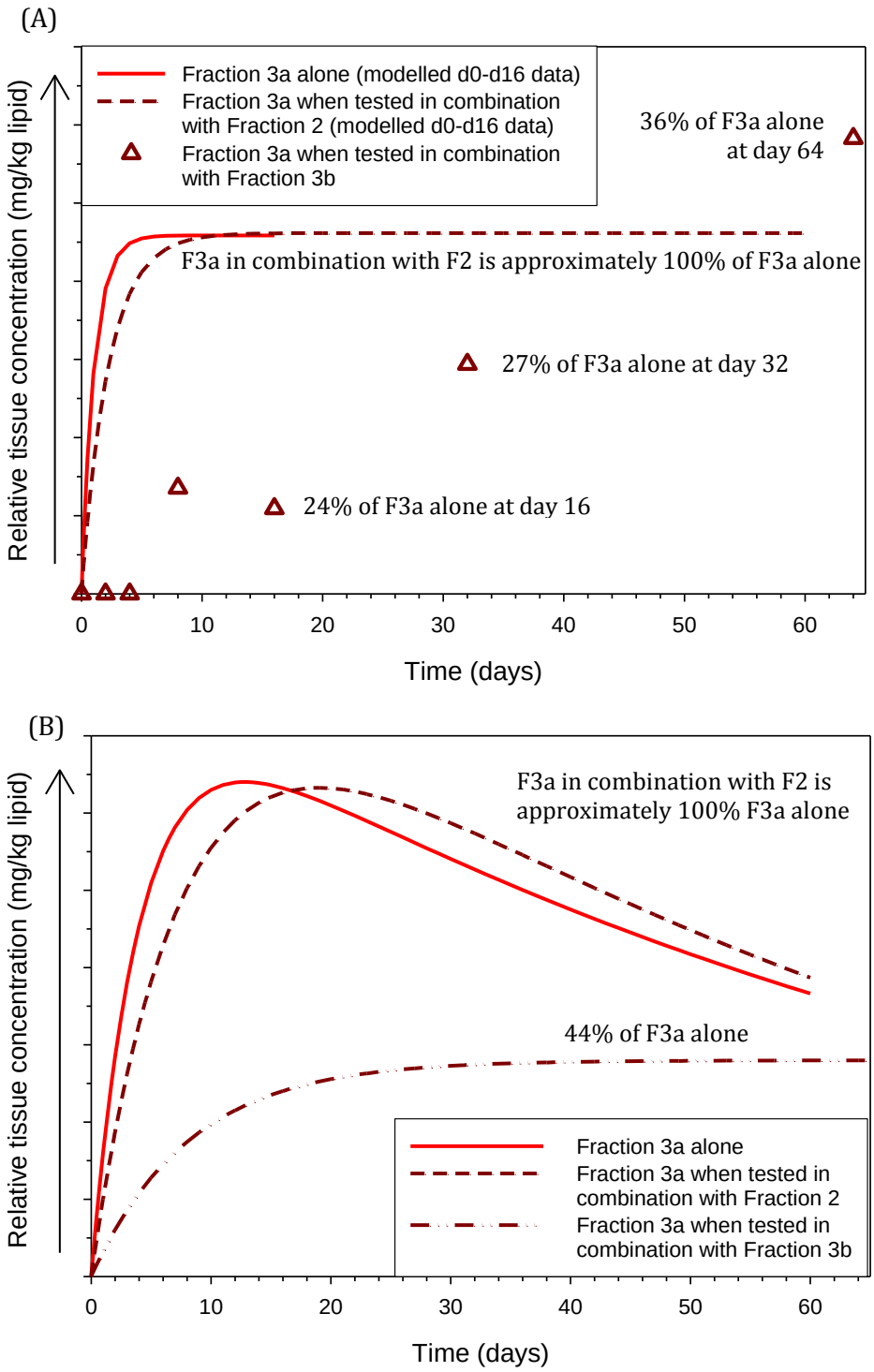


Figure 4-10: Model curves of the accumulation of Fraction 3a (A) aliphatic and (B) aromatic compounds by *E. andrei* exposed to soil contaminated with Fraction 3a alone or in combination with Fraction 2 or 3b. Curves are standardized to the same initial soil concentration to illustrate differences in the accumulation of the distillate alone and when it is in combination with another distillate. The *y*-axes are not to the same scale.

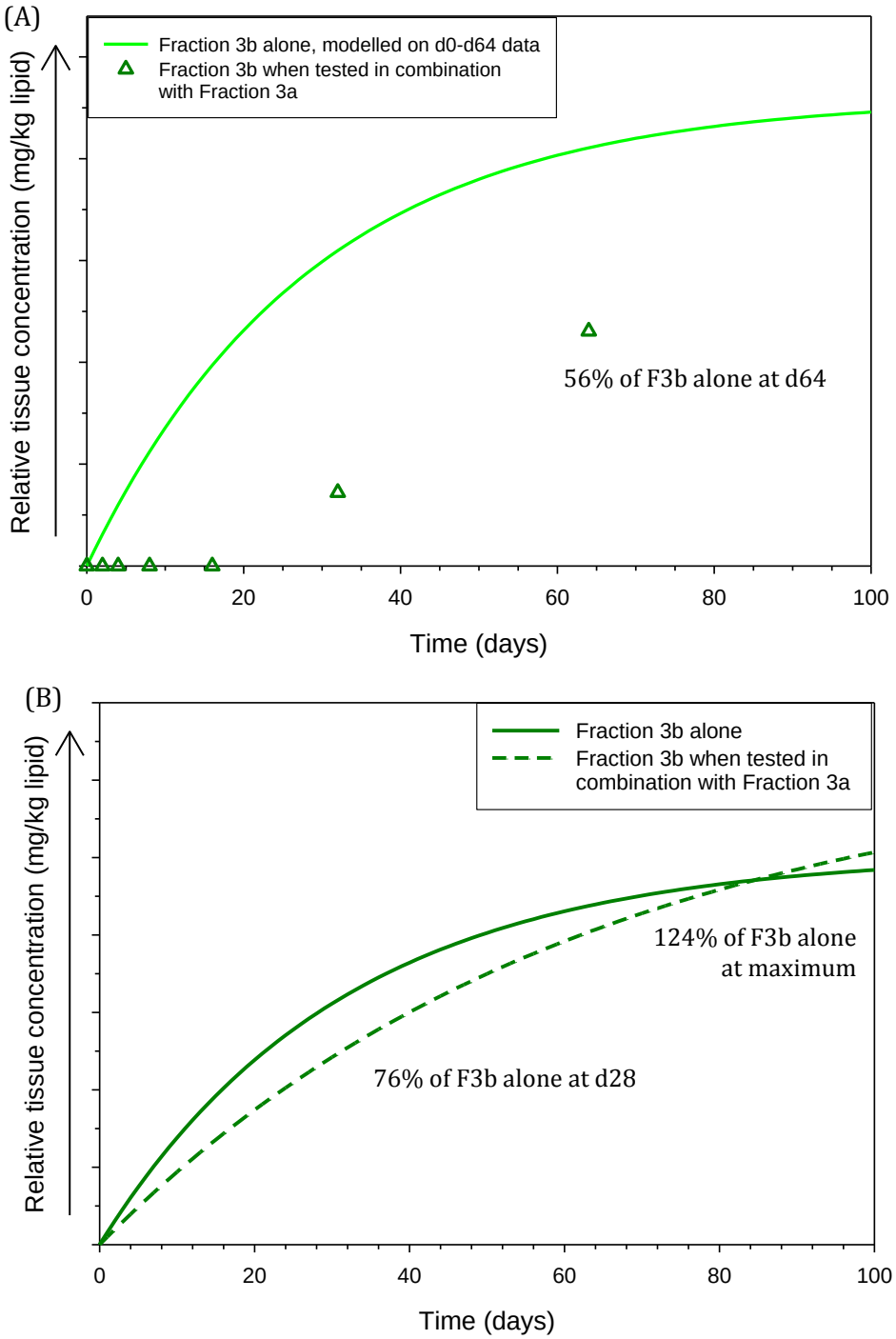


Figure 4-11: Model curves of the accumulation of Fraction 3b (A) aliphatic and (B) aromatic compounds by *E. andrei* exposed to soil contaminated with Fraction 3b alone or in combination with Fraction 3a. Curves are standardized to the same initial soil concentration to illustrate differences in the accumulation of the distillate alone and when it is in combination with another distillate. The *y*-axes are not to the same scale.

The presence of a non-aqueous phase liquid (NAPL) has been shown to act as an additional sorption phase for PAHs (Chen et al. 2008; Jonker et al. 2003) and PCBs (Jonker and Barendregt 2006) when present at concentrations above the critical separate phase concentration (CSPC) (Jonker et al. 2003). In fact, oil NAPLs may act as supersorbents for these compounds (Chen et al. 2008; Jonker and Barendregt 2006; Jonker et al. 2003). For soils and sediments, the CSPC is estimated to occur at concentrations of approximately 15% oil in organic carbon (w/w) (Jonker et al. 2003). This is equivalent to 8,385 mg oil/kg dry weight soil for the soils used in the present study.

NAPLs are likely present in both binary toxicokinetic tests. While the total concentration of petroleum hydrocarbons (2.3% on a measured concentration basis) is below the CSPC in the F2F3a toxicokinetic test, the concentration of F3a in the stock soil used in preparing the test soil was 21.5% (on a nominal concentration basis). It is possible that oil remained as a separate phase around soil aggregates after the mixing of amended soil. In the F3aF3b test, total petroleum hydrocarbon concentration is at the CSPC (15.9% on a measured concentration basis), indicating the presence of a NAPL in the amended soil. As well, both the F3a and F3b stock soils used in creating the final amended soil were above the CSPC (18 and 53.7% on a nominal concentration basis, respectively).

Sorption to a NAPL can lead to a decrease in the bioavailability of compounds to earthworms (Petersen et al. 2011; Quiñones-Rivera et al. 2003) as observed in many cases in the current study. The observed decrease in the accumulation of F2 in the presence of F3a (Figure 4-9) may be due to the preferential sorption of F2 into a separate F3a oil phase surrounding soil aggregates. As the F3a stock soil used for both the F3a and the F2F3a toxicokinetic studies were at similar concentrations (13 and 12 mg/g dry weight nominal, respectively) and above the CSPC, and both were used to make test soil of the same concentration (1,700 mg F3a/kg dry weight nominal), accumulation of F3a in both tests is expected to be similar. The addition of F2 at a concentration and using a methodology (direct addition to soil previously amended with F3a) that did not result in the formation of an F2 NAPL appears to not affect the accumulation of F3a. In addition, F3a is not expected to sorb into F2 to any great extent even if F2 was present at higher concentrations due to the less lipophilic nature of F2 in comparison to F3a.

The results of the F3aF3b toxicokinetic test also suggest that the presence of a NAPL decreased accumulation. A decrease in the accumulation of F3a aliphatics and aromatics (Figure 4-10) could be due to the sorption of the less lipophilic F3a into F3b present as a NAPL. The observed decrease in the accumulation of F3b aliphatics, and to a lesser extent aromatics (Figure 4-11), is harder to explain as the more lipophilic F3b constituents would be expected to associate more closely with the F3b NAPL; as F3b was tested at the same concentration individually as well as in the binary test, similar accumulation of F3b in both tests is expected. Decreases in the accumulation of distillate fractions in the F3aF3b test may reflect both the effect of the presence of a NAPL as well as sublethal effects of the contaminants on earthworm behaviour or health (Petersen et al. 2011) above what may have occurred in tests with individual distillates. In the F2F3a test, a decrease in bioavailability is believed to be the cause of the decrease in the accumulation of F2 as any effect on earthworm health and/or behaviour would have resulted in a similar decrease in the accumulation of F3a which was not observed. The observed decrease in accumulation of both F3a and F3b hydrocarbons by *E. andrei* in the F3aF3b test, on the other hand, may be due to a combination of both the presence of a NAPL and lower accumulation due to changes in behaviour and/or health. At the concentration of F3b used, soils were observed to be more hydrophobic and earthworms tended to be found together in a "ball" in the soil; this may reduce exposure to the soil. Despite this, the difference in the magnitude of the decrease in accumulation between F3b aliphatics and aromatics suggest that avoidance of the soil by earthworms is not solely responsible for lower accumulation. Further studies are required to investigate this.

4.4.2. Accumulation and its effect on mixture toxicity

Though the toxicities of constituents of petroleum hydrocarbons are expected to act in an additive manner (King et al. 1996), less-than-additive toxicity with earthworms was observed in the current study and by others (ESG 2003). This less-than-additive toxicity was not thought to be due to interactions among the distillates within the organism. Rather, from the toxicokinetic studies with individual distillates (Chapter 3), it was thought that this effect might be due to a temporal separation in the accumulation of hydrocarbons between distillates. While there may be some separation between when maximum concentrations were reached (Table 4-6) this could not explain the observed lack of toxicity at soil concentrations above the LC50 for individual distillates.

A delay in the time to reach a maximum body burden for each distillate was also observed in the binary toxicokinetic studies. This might explain, at least in part, the less-than-additive toxicity

observed, especially in instances where a threshold effect concentration is not achieved during the test duration. This may be the case in the F3aF3b toxicity test, as increased mortality was observed up to day 42, the longest duration tested. This, however, cannot explain the less-than-additive toxicity observed with F2 and F3a in combination. One test with F2F3a was extended to 42 d and no additional mortality was observed after 28 d, indicating that the threshold effect concentration for this combination is approximately 28 d. The LC50s for the two F2F3a tests were both greater than 1 TU at 28 d, therefore some other mechanism must be responsible for the less-than-additive toxicity.

It is believed that the observed decrease in the accumulation of some distillates when a second distillate is present is the main cause of the observed less-than-additive toxicity. In the F2F3a toxicokinetic tests, a 51-71% decrease in the accumulation of F2 aliphatic and aromatic compounds was observed when F3a was present, but no similar decrease in the accumulation of F3a compounds was noted. Therefore, the LC50 of the mixture is expected to occur at least at the concentration of F3a = 1 TU. The concentration of F3a at the 28-d LC50 for F2F3a was, in fact, approximately the same as or less than the LC50 observed for F3a alone (Table 4-4). If it is assumed that the accumulation of F2 compounds in the binary test is, on average, 39% of that when F2 is tested alone, the LC50 for the mixture can be adjusted accordingly. When this is done, the LC50 (and 95% confidence interval) of the mixture is 1.84 (1.67-2.04) and 1.10 (1.00-1.22) TU for F2F3a test 1 and test 2, respectively. Considering the uncertainty inherent with the estimates of soil concentration (estimates derived from relationships based on samples taken from a limited number of concentrations and tests) as well as variability, these results suggest that the observed toxicity of the distillates is additive if based on internal tissue concentrations and not on external soil concentrations.

The situation with the F3aF3b test is more complex. As the duration of the test increases, the LC50 of the binary mixture decreases and starts to approach 1 TU, although it is still significantly greater than 1 TU at day 42. However, no threshold effect concentration was reached during the 42 d duration of the toxicity test suggesting that the LC50 would be yet lower at the threshold concentration. The toxic units for the mixture, however, are based solely upon the concentration of F3a as F3b is not lethal to earthworms at the concentration tested (10,000 mg F3b/kg nominal). Accumulation of F3a was less in the presence of F3b than when tested alone (Figure 4-10). If the toxic units of the F3aF3b test 2 are adjusted for the average accumulation of F3a when in

combination with F3b (35.4% of F3a alone at day 32 or 40.0% of F3a alone at day 64), the 42-d LC50 of the mixture becomes 0.56 (0.53-0.59) TU or 0.63 (0.59-0.66) TU using the adjusted average accumulation based on day 32 or 64 data, respectively. These are below the expected LC50 = 1 TU for additive toxicity; however, the contribution of F3b to the toxicity has not been considered in this calculation. F3b, and especially F3b aromatics, are readily accumulated during the exposure period used and will contribute to the body burden and total toxic units even if at concentrations below those causing mortality.

Therefore, the decrease in accumulation of distillates when in the presence of petroleum hydrocarbons as a NAPL appears to be the cause of the observed less than additive toxicity of the binary mixtures for both F2F3a and F3aF3b.

4.4.3. Applicability of toxicokinetic results to toxicity tests and environmental samples

The concentration of F2 and F3a in the F2F3a test and F3a in the F3aF3b test is higher at the LC50 for the mixture than the concentrations tested in the toxicokinetic tests. This is because the toxicokinetic tests were conducted at sublethal concentrations and the mixtures were found to be less toxic, on a soil concentration basis, than expected. The concentration of a compound can affect its toxicokinetics (Jager et al. 2000) and therefore, the accumulation of distillates in the toxicity tests may not be strictly comparable to that observed in the toxicokinetic study. However, a similar decrease (but of unknown magnitude) in the bioavailability, and thus accumulation, of the distillates as observed in the toxicokinetic study due to sorption by a NAPL is assumed, as the concentrations of the distillates in the soil were the same (in the case of F3b in the F3aF3b test) or are greater than those tested in the toxicokinetic study. In fact, in the F2F3a toxicity and toxicokinetic tests, the ratios of F2 to F3a were approximately the same (0.24-0.30 w/w). Therefore, it is assumed that the accumulation of F2 is affected similarly in the two tests. In the F3aF3b test, since the concentration of F3b was the same in both tests, the ratio of F3a to F3b is much lower in the toxicokinetic study (0.16 w/w) as compared to its value at the 42-d LC50 for the mixture (0.74 w/w). Therefore, the results may not be as comparable. The effect of "toxic" stress on the accumulation of petroleum hydrocarbons in the toxicity tests is unknown.

The ratios of distillates at the LC50s of the binary toxicity tests, nevertheless, are similar to those in crude oil, if one compares the concentration of the lighter distillate to the total concentration of all higher molecular weight distillates. The assumption here is that the lower

molecular weight distillate will partition into the more lipophilic NAPL of higher molecular weight distillates and not associate to any extent with lower molecular weight compounds. The ratio of F2 to F3a in the F2F3a binary toxicity tests and that of F2 to (F3a+F3b+F4) in crude oil from which the distillates were derived are 0.3 and 0.49 (w/w), respectively. Those for F3a to F3b in the F3aF3b binary toxicity test and for F3a to (F3b+F4) in crude are 0.74 and 0.58 (w/w), respectively. These ratios of test concentrations to crude oil are similar and, therefore, it is expected that there may be similar less-than-additive toxicity observed at contaminated sites if concentrations are such that a NAPL is present.

4.4.4. Implications for the PHC CWS and assessment of contaminated sites

The PHC CWS regulates petroleum hydrocarbons based on the concentration of individual distillates in the soil and does not take into consideration any interactions among distillates when they are present concurrently. The present study indicates that the presence of petroleum hydrocarbons over multiple distillate ranges may affect the bioavailability of the hydrocarbons, resulting in a decrease in toxicity. Therefore, the PHC CWS ecological value for each distillate can be considered conservative, at least with regard to earthworm toxicity. There is evidence suggesting that similar interaction effects do not necessarily occur with other organisms (i.e., plants (ESG 2003)).

The results of the current study also demonstrate that for assessments at contaminated sites, concentrations of PHCs above the Tier 1 PHC CWS for the protection of ecological receptors may not equate to toxicity even at sites contaminated with fresh petroleum if the bioavailability is affected by the presence of a NAPL. This should be taken into consideration when interpreting toxicity test results at sites with high levels of contamination; the test results may indicate no toxicity, but the presence of petroleum hydrocarbons above the critical separate phase concentration for the site should be sufficient reason to require remediation.

4.5. CONCLUSIONS

The current study investigated the toxicity and toxicokinetics of PHC CWS distillate F2 and two subfractions (F3a and F3b) of PHC CWS distillate F3 with earthworms. The results of this investigation indicate that interactions may occur among distillates that reduce the bioavailability and thus toxicity of the distillates to earthworms. This is thought to occur when the more lipophilic

distillate is at a concentration resulting in a NAPL into which the less lipophilic distillate will preferentially partition. The lower bioavailability of (some of) the petroleum constituents results in an apparent less-than-additive toxicity of the petroleum distillates to earthworms on a soil concentration basis. However, adjustment of the soil concentrations to reflect the decrease in bioavailability generally indicates that toxicity is additive, in agreement with what is expected for petroleum hydrocarbons.

The degree of sorption to a NAPL is likely dependent on the amount of NAPL present as well as to its properties, such as lipophilicity. Thus, the bioavailability of petroleum hydrocarbons, and thus their toxicity on a soil concentration basis, will be dependent on the size and nature of a present NAPL. Measurements of toxicity related to lipid-adjusted internal concentrations (i.e., body burden) is preferable than to the bulk soil.

Chapter 5 **GENERAL CONCLUSIONS**

5.1.1. Summary of conclusions

Following the development and endorsement of the Canada-wide Standards for Petroleum Hydrocarbons in soils, data gaps in our knowledge with respect to the interaction between the CCME petroleum hydrocarbon distillates, soils, and soil organisms and the resultant toxicity of the PHCs were identified. During the course of this doctoral research, some of these data gaps have been addressed through the following hypotheses and objectives.

1. Hypothesis: the toxicity of the lower molecular weight constituents of F3 is sufficiently different from the toxicity of the higher molecular weight constituents to recommend separate regulation of the two subfractions under the PHC CWS.

Objectives

   a. Fractionate F3 into two subfractions, F3a (ECN >C16-C22) and F3b (ECN >C22-C34), and characterize these subfractions.
   b. Determine the toxicity of F3, F3a, and F3b in lethal and/or chronic toxicity tests with three species of soil organisms (one plant (*Elymus lanceolatus*) and two invertebrates (*Eisenia andrei* and *Orthonychiurus folsomi*)) known to be sensitive to F3 toxicity.
   c. Determine the toxicity of F2 to earthworms, to provide data required for subsequent comparisons and studies.
   d. Determine the relationship between nominal and measured concentrations for each distillate tested using select test concentrations.

2. Hypothesis: the toxicokinetics of PHC distillates F2, F3a, and F3b are not the same and therefore toxicity values for each distillate derived from fixed-duration tests are not directly comparable.
3. Hypothesis: the toxicokinetics of aliphatic and aromatic PHCs do not differ.

Objectives

   a. Determine the accumulation and elimination kinetics of the aliphatic and aromatic fractions of each distillate at one treatment level with one species (earthworms) over extended exposure durations.
   b. Determine the concentration of aliphatic and aromatic fractions of each distillate in soil over the exposure duration.

c. Interpret the toxicity results for each distillate considering the results of the toxicokinetic study and determine if it is valid to compare toxicity values derived for different distillates using the test system described in Chapter 2.

4. Hypothesis: the toxicity of binary combinations of petroleum distillates are concentration-additive.

Objectives

a. Determine the toxicities of binary combinations of consecutive petroleum distillates (F2F3a and F3aF3b) using the toxic unit approach.
b. Determine the toxicokinetics of binary combinations of F2F3a and F3aF3b for both aliphatic and aromatic fractions.

The results of the binary toxicity study were interpreted in light of the observed toxicokinetics to determine if the combinations of petroleum distillates were concentration-additive.

In Chapter 2, the first hypothesis regarding the toxicity of lower versus higher molecular weight subfractions of F3 was addressed. The conclusion was to reject the hypothesis; the toxicities of the lower molecular weight subfraction of F3, F3a, were not sufficiently different from the higher molecular weight subfraction, F3b, to recommend regulation of the two subfractions separately. The toxicity data for F3, F3a, F3b, and F2 suggest that as the boiling point range of the distillate increases, the exposure duration required to reach the threshold effect concentration increases as well. Lower toxicity values with increased exposure duration are expected especially with the highest molecular weight distillate, F3b, and had the test durations been sufficient to achieve threshold effect concentrations in all tests, the magnitude of the difference in toxicities between F3a and F3b likely would have been even less than noted in this study. This provides additional support to the recommendation not to regulate the two subfractions separately.

Based on previous studies with the CCME distillates (ESG 2003) and the results of the toxicity studies (Chapter 2 (Cermak et al. 2010)) which indicate that threshold concentrations were not achieved, as well as an expectation of differing loss of distillate from the soil over the test duration due to the physico-chemical properties of each distillate (Chapter 2 (Cermak et al. 2010)), investigations into the toxicokinetics of the distillates with the earthworm *Eisenia andrei* were conducted (Chapter 3). The results of these investigations confirmed that, in the test system used,

each distillate had both different toxicokinetics with earthworms and rates of loss from soil. Loss from the soil via volatilization and/or biodegradation was greatest for the lowest boiling point distillate, F2, and progressively decreased as the boiling point range of the distillate increased. Rates of accumulation from soil by earthworms also decreased as the boiling point range of the distillate increased, confirming the need for extended test durations for higher molecular weight distillates (i.e., F3a and F3b) to reach threshold effect concentrations.

A variety of accumulation curves were observed for the aliphatic and aromatic fractions of F2, F3a, and F3b. Some exhibited peak-shaped accumulation curves (F2 aliphatics and aromatics, F3a aromatics) while others had more typical first-order accumulation curves. The peak-shaped curves were attributed to changes over time in the amount of contaminant bioavailable for accumulation, due to loss from the soil (volatilization and/or biodegradation), sequestration into non-accessible sites in the soil, and/or depletion in pore water due to fast microbial degradation coupled with slow desorption from soil (Chapter 3).

The results from the toxicokinetic studies (Chapter 3) were compared to the results from the toxicity tests with F2, F3a, and F3b (Chapter 2 (Cermak et al. 2010)). Depending on the distillate tested, earthworms had significantly different internal exposure scenarios, ranging from highly acute and transient (F2) to very slow accumulation with an extended period of time required to reach maximal tissue concentrations (F3b). Durations of standard toxicity tests are generally not sufficiently long in duration for the toxicity of higher molecular weight distillates to be expressed. Therefore, the second hypothesis is not rejected and the data confirm that, for toxicity tests following the same or similar methodology, resultant toxicity values are not strictly comparable. Methodologies need to be developed to mitigate these differences or to account for them when calculating toxicity values.

The toxicokinetic results also demonstrated that there was a significant difference in the rate of accumulation between aliphatic and aromatic compounds by earthworms (Chapter 3). Despite higher concentrations of aliphatics versus aromatics in soil, earthworms accumulated disproportionally more of the aromatics than aliphatics. Therefore, the third hypothesis is rejected. Contrary to what is implicitly assumed in the PHC CWS, accumulation of aliphatics and aromatics by earthworms is not proportional to their concentration in the soil and the proportion accumulated for each chemical class differs among distillates.

The results from the toxicokinetic and toxicity studies suggest that though both aromatic and aliphatic compounds will contribute to the toxic body burden for narcosis, the aromatic compounds are the main contributors to the toxicity of each distillate, especially for F3a and F3b. Aromatics are accumulated to a greater degree and at a higher rate than aliphatics. Both aliphatics and aromatics are accumulated to maximal tissue concentrations within 7 d for F2, and are found at approximately the same concentration within tissues; therefore, for F2, both might contribute equally to toxicity. F3a reached a threshold effect concentration for lethality by 35 d, which agrees with the time to achieve the maximum concentration of aromatics. F3a aromatics constitute the largest proportion of tissue PHC residues during this duration, suggesting that aromatics are the main contributors to toxicity. Earthworms accumulated F3b PHCs but the exposure duration used in the toxicity tests (28-d), though longer than usually used in standard tests (14-d (EC 2004)) was less than the time required to achieve maximal tissue concentrations for either aromatics or aliphatics. It is noted, however, that aromatics were the main contributors to tissue concentrations in tests with F3b.

Studies have indicated that aromatic compounds are more toxic than aliphatic compounds with various species (Feuston et al. 1994; Khan et al. 1986; Payne et al. 1995; van Overbeek and Blondeau 1954; Vandervaart Cook 2002), including one using a similar crude oil and earthworms (Vandervaart Cook 2002). However, considering the differences in the accumulation of the two chemical classes, it is possible that the observed differences in toxicity may be due to toxicokinetics and not intrinsic toxicity. That being said, it is obvious that the aromatic fraction will be accumulated more quickly and will therefore exert its toxicity sooner.

Therefore, based on the data for toxicity and toxicokinetics for earthworms and assuming that similar disproportionate accumulation of aliphatics and aromatics occurs with other species, it is recommended that regulatory standards for ecological effects consider the aliphatic and aromatic fractions of petroleum hydrocarbons separately.

While this research was conducted solely with earthworms, similar disproportionate accumulation of aromatics over aliphatics and similar lower rates of accumulation with increasing boiling point range of the distillate likely occurs with other soil organisms as the physico-chemical properties of aliphatics and aromatics (e.g., water solubility, $\log K_{ow}$, $\log K_{oc}$) that affect their

bioavailability to and accumulation by earthworms likely also affect their bioavailability to other species of soil organisms. Similar losses of lower boiling point constituents from soil are also expected. Due to differences in physiology, organism size, and routes of exposure, the shapes of the toxicokinetic curves and time to reach maximum tissue concentrations may differ between species; therefore, it is advisable to conduct further studies with other species to determine how toxicity is affected by toxicokinetics. In addition, though this research was focussed on petroleum hydrocarbons, the need to consider loss of contaminant from soil, toxicokinetics, and test duration with other contaminants remains.

Using analytical data for soil concentrations from all studies (Chapters 2-4), relationships between the nominal soil concentration and measured concentration were determined for both soil amended with distillates individually (discussed in Chapter 2 (Cermak et al. 2010)) and in binary combinations (Chapter 4). For most distillates, there was no significant difference between the measured concentration reported as a percentage of nominal regardless of whether the distillate was amended to soil alone or in combination with another distillate with the exception of F2 when added alone. In this case, the amount of F2 measured in soils, as a percentage of the nominal concentration, was significantly lower.

The relationships between nominal and measured concentrations for F2 and F3 developed in this doctoral research (Chapter 2 (Cermak et al. 2010)) differs from that observed in previous work with these distillates (ESG 2003) that was used in the derivation of the 2001 ecological soil contact values for the PHC CWS (CCME 2000; CCME 2001a). The previous work had greater loss of both distillates despite using the same methodology in preparing soils (ESG 2003). If the previous values are incorrect, the 2001 PHC CWS ecological soil contact values for F2 and F3 may be too conservative. The CCME recognized this for F3 in its review of the PHC CWS in 2005 and adjusted the F3 values in the 2008 version of the PHC CWS; however, no similar adjustment was made for F2 (CCME 2008). The larger number of measurements made for F2 in this doctoral research suggests that the lower loss of F2 observed in the present research is more reflective of the true loss of F2 during soil treatment preparation.

The fourth hypothesis on the toxicity of binary combinations of distillates to earthworms was explored in Chapter 4. Earlier studies had indicated that, despite the known narcotic and assumed additive-nature of petroleum hydrocarbons, the toxicities of binary combinations of CCME

distillates were less-than-additive in lethal toxicity tests with *E. andrei* (ESG 2003). Lethal toxicity studies conducted with binary combinations of F2, F3a, and F3b confirm this (Chapter 4); however, toxicokinetic studies with these distillates individually (Chapter 3) suggest that different accumulation patterns for each individual distillate may result in earthworms having temporally-separated internal exposures for each distillate, which could translate into the observed less-than-additive toxicity. Therefore, a toxicokinetic study of binary combinations of F2, F3a, and F3b was undertaken to provide insight into this.

The toxicokinetics of the binary combinations of distillates did indicate some separation in exposure to the individual distillates, but this could not explain the lack of toxicity observed at soil concentrations above the LC50 for individual distillates. A pronounced decrease in the accumulation of aliphatics and/or aromatics for one and sometimes both of the distillates in each binary test as compared to when the distillates were tested alone was observed, however, presumably because of the presence of a NAPL surrounding at least some soil aggregates. This resulted in a decrease in tissue concentrations at soil concentrations that were lethally toxic when the distillates were tested alone. When this decrease in accumulation was taken into account when calculating the toxic units at the LC50 for a mixture, the results were closer to being in agreement with concentration-additive toxicity. It is concluded that the toxicity of binary combinations of distillates is concentration-additive if based on internal tissue concentrations and not on external soil concentrations, and thus the fourth hypothesis is not rejected.

The above research highlights the importance of understanding the toxicokinetics of PHCs, especially with regard to internal exposure scenarios, for interpreting and understanding the toxicity of these substances, and for determining the comparability of the toxicity values. With an understanding of toxicokinetics and exposure, standard toxicity test protocols may be adjusted to minimize differences in the exposure scenarios for test organisms, or at least used to better interpret the results and state the limitations of the tests. Regulators using data from standard toxicity tests need to understand these limitations when developing environmental standards, especially when using data for highly volatile or highly hydrophobic substances or developing standards for a contaminant with such a diverse range of physico-chemical properties as petroleum hydrocarbons.

5.1.2. Implications for the ecological regulations and the assessment of contaminated sites

The results of this research have several implications for ecological regulations and the assessment of contaminated sites.

With regard to the PHC CWS, the CCME voiced concerns regarding the applicability of the ecological standards for F3 and whether differences in toxicity between its lower (F3a) and higher (F3b) ECN constituents may be significant. It was determined that the difference in toxicity between these two subfractions of F3 was not sufficiently different to recommend regulating the two separately.

The toxicity and toxicokinetic studies (Chapters 2 (Cermak et al. 2010) and 3) with the individual distillates demonstrate how sufficient exposure durations are needed in order to determine the intrinsic toxicity of PHC distillates, especially for those with higher ECN ranges. Soils that may appear to be non-toxic or less toxic in standard toxicity tests may in fact be toxic or more toxic when test durations are extended. In fact, toxicity results for different distillates with earthworms using standard static, fixed duration toxicity tests are not truly comparable for petroleum hydrocarbons. It is recommended that further research be conducted to determine how test duration affects the toxicity values of higher ECN distillates. In addition, toxicokinetics studies should be conducted with other species to assist with the interpretation of toxicity test results.

The toxicokinetic study (Chapter 3) also demonstrates the disproportionate accumulation of aromatics over aliphatics by earthworms, indicating that the toxicity observed in Chapter 2 (Cermak et al. 2010) is mainly attributable to the aromatic fraction. It is recommended that aromatic and aliphatic PHCs be regulated separately. Further research into the toxicity of petroleum products with aromatic contents greater and less than 20% should be conducted.

The toxicity results (Chapter 2 (Cermak et al. 2010)) also highlighted the difference in sensitivity to PHCs among species and among various endpoints within a species. When estimating the risk associated with contamination, test species and endpoints should reflect a range in sensitivities to decrease the uncertainty associated with estimation of risk. Use of insensitive species and/or endpoints could result in toxic soils being classified as nontoxic. Use of nonthreshold concentration data and/or reliance on data from insensitive endpoints or species for

PHC contamination, as well as for other organic contaminants, might lead to an underestimation of risk and the development of remediation standards that are not truly protective of the terrestrial environment.

The PHC CWS do not consider interactions among different distillates when present together at contaminated sites. While the results of binary toxicity and toxicokinetic tests (Chapter 4) support that toxicity of PHCs is concentration-additive on an internal tissue concentration basis, on a soil concentration basis the toxicity may appear to be less-than-additive. The less-than-additive toxicity is due to a decrease in the bioavailability of PHCs, presumably due to the presence of a NAPL. Therefore, under similar situations in the field, the PHC CWS ecological standards can be considered conservative, at least with regard to earthworm toxicity. This may not be true for other species. During contaminated site assessments, the impact of a NAPL on soil toxicity should be taken into consideration when interpreting toxicity test results.

5.1.3. Additional recommendations

In addition to recommendations explicit to PHCs provided in subsection 5.1.2, the following recommendations are applicable to contaminants in general and are not specific to petroleum hydrocarbons.

The results of Chapters 2 and 3 demonstrate the importance of toxicokinetics and test duration on the interpretation and comparability of standard toxicity test results for highly volatile/degradable or highly hydrophobic contaminants. At a minimum, threshold effect concentrations should be used when comparing toxicity values to ensure that test durations are of sufficient duration to make meaningful comparisons. However, the use of threshold toxicity values does not provide information on the shape of the accumulation curve and how loss of contaminant and/or changes in bioavailability affects body burdens. Peak-shaped accumulation curves may indicate non-constant exposure. A description of the toxicokinetics of the contaminant with the species and test system of interest can provide information about how to modify the test protocols to mitigate differences in internal exposure scenarios.

In addition, the results of Chapters 3 and 4 highlight the importance of tissue concentrations (or body burdens) as the preferred metric of exposure. Soil exposures did not always correlate with the observed tissue concentrations, especially when multiple distillates were present in the soil.

Interaction among contaminants in the soil, particularly if one forms a NAPL, or between contaminants and soil particles (e.g., organic matter) can affect the bioavailability of the contaminant and thus its accumulation, as can fast biodegradation of a contaminant coupled with slow desorption from soil into the porewater. This may result in different toxicity values on a soil concentration basis. Internal tissue concentrations can provide a more consistent and comparable method for presenting toxicity data. Studies should be conducted to correlate tissue concentrations in soil organisms to toxicity to allow for the use of this parameter in assessments and regulatory settings.

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