

Using *Folsomia candida* to Test the
Toxicity of Weathered Petroleum-
impacted Field Soils before and after
Phytoremediation

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. I understand that my thesis may be made electronically available to the public.

Abstract

The Canadian Council of Ministers of the Environment (CCME) developed guidelines for petroleum hydrocarbon (PHC) impacted field soils based on the “worst case” scenario of a fresh petroleum spill (CCME, 2001b; CCME, 2008b). Therefore, when these guidelines are applied as remedial benchmarks, they may be too conservative to be used as realistic targets as they do not account for weathering, which has been shown to decrease the toxicity of PHCs in soil. Chronic toxicity tests were performed using weathered PHC-impacted field soil from three different field sites (ON1, AB1 and BC1) and *Folsomia candida*. The highest PHC concentration of soil obtained from ON1 (635 mg/kg F2 and 12,000 mg/kg F3) and AB1 (610 mg/kg F2 and 2,900 mg/kg F3) did not affect *F. candida* survival and reproduction. However, when *F. candida* were exposed to PHC-impacted soil obtained from the BC1 site, a LC₂₅ of 2,809 mg F2 + F3/kg was calculated for adult survival while an IC₂₅ of 1,030 mg F2 + F3/kg was calculated for juvenile production. The toxicity at BC1 was postulated to be caused by the F2 concentration (it was the only site with high F2).

Heat extraction and floatation methods were compared using the soil obtained from the ON1 field site. The number of adults obtained using the floatation method was always higher than the total number of adults obtained from the heat extraction method; however, only two of these results were statistically significant. This suggests that the floatation method is the best method to use to extract *Folsomia candida* and also indicates either method can be used with no significant effect on the conclusions.

Chronic toxicity tests usually focus on measuring sub-lethal endpoints; however, only juvenile production was included in the Environment Canada protocol (Environment Canada, 2005; Environment Canada, 2007a). The endpoints of weight, length and width were added to chronic toxicity tests on AB1 and BC1 soils to determine if they were suitable endpoints. The highest concentration tested for AB1 (610 mg F2/kg and 2,900 mg F3/kg) had no effect on the weight, length or width of the adults. However, the toxicity data obtained for the BC1soils provided an EC₂₅ of 421 mg F2 + F3/kg, 13,750 mg F2 + F3/kg and 17,425 mg F2

+ F3/kg for weight, length and width, respectively. The EC₂₅ of 421 mg F2 + F3/kg obtained for the weight of adults is lower than the IC₂₅ of 1,030 mg F2 + F3/kg obtained for juvenile production which indicating that weight is a more sensitive endpoint than juvenile production.

Avoidance-response tests involved placing a control and test soil on either side of a cylindrical container and adding 20 *Folsomia candida* to the midline (Environment Canada, 2007a; Liu *et al.*, 2010). The results using soil obtained from AB1 showed no trend between soil avoidance and increasing PHC concentration. However, the avoidance-response test, using soil obtained from BC1, indicated that *F. candida* avoidance increased with increasing petroleum concentration. These results show that avoidance-response tests were able to predict the outcome of the chronic toxicity tests. Overall, the above results indicate that the CCME guidelines are too conservative to apply to weathered PHC-impacted field soil when the impacts are primarily F3. Results also indicate that F2 and F3 concentrations of 250 mg/kg and 2,900 mg/kg, respectively would not adversely affect *F. candida* adult survival, juvenile production or adult weight.

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Dedication

To Luke, Jessie, Wendy and Gladys

This thesis never would have been finished without your support.

To my parents, Neil and Elaine, and my sister, Chelsey

This has been a long three and a half years and you have always been there for me.

and

To the memory of my Grandfather, Dick Aldis

1929 - 2013

I will always be your little chickadee.

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Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
AMTAG	Analytical Methods Technical Advisory Group
CCME	Canadian Council of Ministers of the Environment
CEC	Cation Exchange Capacity
DDT	dichlorodiphenyltrichloroethane
EC	Electrical Conductivity
EC _{1:2}	Electrical Conductivity of a soil slurry with 1 part soil and 2 parts water
EC _{25/50}	Effective Concentration required to decrease the endpoint by 25 or 50%
EC _e	Electrical Conductivity of a soil saturated with water
F2	PHC with carbon chain lengths from 11 to 16
F3	PHC with carbon chain lengths from 17 to 34
F4	PHC with carbon chain lengths > 34
IAA	Indole Acetic Acid
IC ₂₅	Concentration that Inhibits the endpoint by 25%
ISO	International Organization for Standardization
LC ₂₅	Lethal Concentration that decreases the population by 25%
OECD	Organisation for Economic and Co-operative Development
PEPS	PGPR Enhanced Phytoremediation System
PGPR	Plant Growth Promoting Rhizobacteria
PHC	Petroleum Hydrocarbon
PHC CWS	Canada Wide Standards for Petroleum Hydrocarbons in Soil
TPHCWG	Total Petroleum Hydrocarbons Criteria Working Group
UCM	Unresolved Complex Mixture
WHC	Water Holding Capacity

Chapter 1 Introduction

1.1 Background

Petroleum hydrocarbons (PHC) are heavily relied upon by our society because they are the primary basis for many fuels, plastics, clothing, and other products (Fingas, 2011). The demand for these products has led to an increase in transportation of PHC over long distances through marine and terrestrial environments (Fingas, 2011). Any resulting spills in these areas can be highly toxic to the surrounding plants, animals and microorganisms. As well, PHC impacts result from upstream production and downstream distribution of petroleum (Fingas, 2011).

Each year in Ontario approximately 1175 terrestrial petroleum spills that cause negative environmental impacts are reported to the Ministry of the Environment Spills Action Center (Ontario Ministry of the Environment, 2012). Unlike marine oil spills, such as the Exxon Valdez, these terrestrial spills are rarely publicized as they often occur at the refinery, well site, or other property owned by a petroleum company (Fingas, 2011; Peterson *et al.*, 2003). However, even small terrestrial petroleum spills will impact ecosystems through the loss of vegetation and alteration of soil properties (Hunt *et al.*, 1973). Methods that have been used to clean up terrestrial petroleum spills include removal of soil to landfill, landfarming, bioremediation and phytoremediation (Huang *et al.*, 2004; McCutcheon and Schnoor, 2003; Philp and Atlas, 2005).

The Canadian Council of Ministers of the Environment (CCME) have developed a set of guidelines for petroleum hydrocarbon (PHC) impacted soils that are protective of ecological receptors (e.g. plants and invertebrates) during fresh petroleum spills (CCME, 2001b). These guideline values are outlined in the PHC Canada Wide Standards and are used to screen for PHC-impacted field sites that have the potential to affect ecological receptors (CCME, 2008a). The use of “worst case” scenarios has made these guidelines highly conservative to ensure all potential PHC-impacted sites that could affect ecological receptors are identified. These guidelines have also been implemented as criteria for remediation of

PHC-impacted soils; however, they may be too conservative to apply as remediation criteria for sites that do not represent worst case scenarios (CCME, 2001b; CCME, 2008b).

One process that decreases the toxicity of PHC in impacted soils is weathering (Alexander, 1995; Maletic *et al.*, 2011). ‘Weathering’ is a term which incorporates all naturally occurring chemical and biological processes that are involved in the breakdown and alteration of organic chemicals (Alexander, 1995; Maletic *et al.*, 2011). These processes include volatilization, sorption and biodegradation (Alexander, 1995; Gallego *et al.*, 2010; Maletic *et al.*, 2011; Osuji *et al.*, 2006). Weathering has been shown to decrease the toxicity of spilled PHC and thus the Tier 1 PHC soil guidelines might be too conservative to apply to sites with weathered PHC residuals (Alexander, 1995; Maletic *et al.*, 2011). How soil toxicity changes with weathering and phytoremediation is the subject of this thesis.

1.2 Soil and Soil Properties

Soil is formed from the weathering of bedrock and contains numerous minerals and organic materials which make a suitable medium for plant growth (Brady and Weil, 2010; Hillel, 2008; Soil Classification Working Group, 1998). Soils are important in an agricultural capacity as they regulate water flow, moderate temperature, recycle nutrients, remove toxins and support plant growth (Brady and Weil, 2010; Hillel, 2008). The availability of usable soil is decreasing because of contamination by metals, pesticides, PHC and other chemicals which are toxic and can alter a soil’s properties (Brady and Weil, 2010; Philp and Atlas, 2005). Understanding the complexity of soil will aid in its preservation and in the interpretation of toxicity test results (Environment Canada, 2007a; Hillel, 2008). The complexity of soils has led them to be defined and classified by their parent material (i.e. source bedrock) and their physical and chemical characteristics (Brady and Weil, 2010; Hillel, 2008). Each of the soil properties described below can impact the survival and reproduction of soil invertebrates and plants (Domene *et al.*, 2011; Kaneda and Kaneko, 2002; Marshall and Kevan, 1967; Owojori *et al.*, 2009; Saitoh *et al.*, 2011; Sorenson and Holmstrup, 2005).

Soil texture describes the particle size distribution of three soil separates (categories): sand (0.05-2 mm), silt (0.002-0.05 mm), and clay (<0.002 mm) (Brady and Weil, 2010; Tan, 2009). These particles arrange in various ways with increasing clay and organic matter to form aggregates which make up the soil structure (Brady and Weil, 2010). As most soils are mineral soils (a soil which contains less than 20% organic matter) the ratio of the soil separates impacts the soil structure and porosity (Brady and Weil, 2010; Simpson, 1983; Tan, 2009). Increasing the sand content improves aeration, drainage and loosens the soil (Brady and Weil, 2010; Tan, 2009). Increasing the clay content elevate water holding capacity (WHC) and cation exchange capacity (CEC) of soil (Brady and Weil, 2010; Tan 2009). Silt exhibits some of the properties of both sand and clay, increasing the aeration, drainage, WHC and CEC; however, this increase is not as large as the increase caused by the sand and clay separately (Tan, 2009).

The cation exchange capacity is defined as the amount of cations (NH_4^+ , K^+ , Ca^{2+} , and Mg^{2+}) a soil can adsorb per kilogram (Brady and Weil, 2010; Tan, 2009). A soil with a high CEC indicates that the potential number of cations that can be sorbed by the soil is high (Brady and Weil, 2010; Simpson, 1983; Tan, 2009). The CEC of a soil increases when the sand content is low, and the silt and clay contents are high (Brady and Weil, 2010). The CEC will also be high when the organic matter content of the soil (i.e., biological material) is high (Brady and Weil, 2010). The relationship between soil CEC and fertility is relatively complex.

The bulk density of soil is defined by Equation 1 (Brady and Weil, 2010). Briefly, it is the weight of soil dried in the oven divided by the volume of dry soil, including both the soil solids and the associated pore space (Brady and Weil, 2010). Increasing compaction and a finer texture will decrease the pore spaces in the soil resulting in an increase in bulk density. Increasing the bulk density leads to compaction of soil and an associated decrease in oxygen and water content (Brady and Weil, 2010).

$$\text{Bulk density} = \frac{\text{soil weight}}{\text{soil volume}} \quad \text{Equation 1}$$

The total carbon content of a soil includes both inorganic and organic carbon. Inorganic carbon comprises the mineral contents of the soil, including clays and carbonates (Brady and Weil, 2010) whereas the organic carbon content of a soil comprises the living organisms, plant detritus, animal remains and droppings, and humus (Brady and Weil, 2010; Simpson, 1983; Tan, 2009). Soil humus is organic matter that cannot be decayed further; it comprises 60-80% of the soil organic matter content and is formed from the degradation and polymerization of biological molecules by microorganisms (Brady and Weil, 2010; Simpson, 1983; Tan, 2009). Biological molecules include proteins, lipids, fatty acids and amino acids from decaying organic matter (Brady and Weil, 2010; Simpson, 1983; Tan, 2009). Humus (e.g., fulvic acids) is made of high molecular weight compounds that can contain many aromatic rings, making them extremely resistant to degradation (Brady and Weil, 2010; Simpson, 1983; Tan, 2009). The presence of soil humus affects many of the soil properties; it accounts for 50-90% of the CEC, increases the WHC and buffers the pH of the soil (Brady and Weil, 2010; Simpson, 1983; Tan, 2009).

The water holding capacity (WHC), also known as field capacity, is proportional to the saturation of the soil minus the free water found in the large soil pores (Brady and Weil, 2010; Environment Canada, 2007a; Simpson, 1983). When the soil is moistened to its WHC, the small soil pores contain the maximum amount of water available to plants and microorganisms while the large soil pores are a source of aeration (Brady and Weil, 2010; Tiwari *et al.*, 1987). The WHC is elevated by increasing the amount of clay, decreasing the bulk density, and increasing the amount of organic material in the soil (Brady and Weil, 2010).

The pH of the soil varies between 3-7 in humid regions and 7-9 in desert regions (Tan, 2009). When the soil is more acidic, the major cations available are Al^{3+} and H^+ while Na^+ , K^+ , Ca^{2+} , and Mg^{2+} become available in basic soils (Simpson, 1983; Tan 2009). Generally, the pH value of the soils can affect the specific cations available for plant uptake, the CEC, and the microorganisms that are present in the soil (Brady and Weil, 2010; Simpson, 1983; Tan, 2009). The pH of the soil can increase with decreasing moisture content and decreasing clay content (Hillel, 2008).

Soil salinity is affected by the concentration of ions such as Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , SO_4^{2-} , NO_3^- , HCO_3^- and CO_3^{2-} in the soil solution (Hillel, 2008). Salinity is a measure of the ability of soil to conduct an electrical current which is often described by the electrical conductivity (EC; Grisso *et al.*, 2009). A soil is considered to be saline when it has an EC greater than 4 dS/m (Hillel, 2008). An increase in the EC of the soil often correlates to an increase in the CEC and a decrease in the organic carbon content, the total nitrogen and the percent moisture (Pan *et al.*, 2013; Grisso *et al.*, 2009). If there is an increase in the amount of sodium ions found in soil through the addition of salts or brine water, it can result in an increase in bulk density by dispersing the clay particles (Hillel, 2008). The dispersed clay particles will clog pores when water is present consequently affecting soil drainage (Hillel, 2008).

The soil temperature does not affect the pH, texture or bulk density of a soil but will impact the moisture content and subsequently microbial growth and activity as well as seed germination (Simpson, 1983; Tan, 2009). Decreasing the soil temperature will lower oxygen levels, slow microbial activity and influence the imbibition of water required for seed germination. Increasing the soil temperature to extremes will kill plants and microbes (Brady and Weil, 2010; Tan, 2009). The soil temperature is affected by both the organic matter and water content (Simpson, 1983). Increasing organic matter darkens the surface soil, decreasing the reflection of the sun's rays, resulting in an increase in temperature while increasing the moisture content moderates the soil temperature (Brady and Weil, 2010; Simpson, 1983).

1.3 Petroleum Hydrocarbons and their Effect on Soil Properties

Petroleum hydrocarbons (PHCs) are defined as a complex mixture of thousands of organic compounds that contain primarily carbon and hydrogen (CCME, 2001a; CCME, 2008b; CCME 2008d). PHCs are derived from biological molecules that have been in the soil for many millennia. PHCs can be found in sources such as petroleum and coal (CCME, 2008a). The PHCs found in petroleum are a complex mixture of more than 700 organic molecules (CCME, 2008a; CCME, 2008d; Petrov, 1987; Long, 1998). The PHC molecules found in these mixtures vary among the sources of petroleum. By definition, they can constitute up to 70% of the mass of petroleum; other compounds containing nitrogen, oxygen, sulphur, and

metals constitute the other 30% of the mass (CCME, 2008d; Goodger, 1975; Petrov, 1987; Long, 1998). PHCs enter soil environments either directly through deliberate or intentional releases or indirectly via inadvertent spills. Regardless of how PHCs enter the environment, they are known to be toxic to microbial, plant and animal life (Erlacher *et al.*, 2013; Fingas, 2011; George *et al.*, 2011; Onwurah *et al.*, 2007). In addition to direct and indirect toxicity to organisms, PHCs can also affect the soil properties described in section 1.2 (Adebiyi and Afedia, 2011).

Amending soil with PHCs has been shown to increase the bulk density of the soil (Izdebska-Mucha, 2008; Martinho *et al.*, 2010). Martinho *et al.* (2010) showed that the porosity of loam forest soil spiked with 7% diesel oil decreased by 3%. Izdebska-Mucha (2008) used a porosimeter to directly measure the pore space in the soil samples. They found that saturating soils of glacial till with diesel oil and “petrol” decreased the porosity of the soil by 9% and 1%, respectively (Izdebska-Mucha, 2008). Since the bulk density of soil is directly related to the pore size, it was concluded that the bulk density of soil generally increases with the addition of PHCs (see Equation 1; Brady and Weil, 2010; Izdebska-Mucha, 2008; Martinho *et al.*, 2010).

The total organic matter of a soil can be lower in soils with petroleum contamination than comparable un-impacted soils (Adebiyi and Afedia, 2011; Labud *et al.*, 2007; Wang *et al.*, 2010). The decrease in organic matter is thought to be due to the excess addition of carbon, with no corresponding increase in nitrogen, slowing the microorganisms ability to breakdown organic materials in the soil as well as PHCs (Adebiyi and Afedia, 2011; Osuji and Nwoye, 2007). Adebiyi and Afedia (2011) and Osuji and Nwoye (2007) showed that the total organic matter in PHC-impacted soil was 27.7% and 32%, respectively, lower than in the un-impacted soil. However, one study has shown that the percent organic matter in soil increases with the addition of petroleum (Kisic *et al.*, 2009). This study measured the total organic carbon content through digestion and converted it to organic matter while the studies by Adebiyi and Afedia (2011) and Osuji and Nwoye (2007) directly measured the organic matter content through oxidation with potassium dichromate and titration with iron sulphate.

When compared to un-impacted soil, petroleum contamination has been shown to increase the pH of the soil (Adebiyi and Afedia, 2011; Kistic *et al.*, 2009; Wang *et al.*, 2010). Kistic *et al.* (2009) determined that spiking soils with drilling fluid increased the soils pH from 6.4 to 6.8. Adebiyi and Afedia (2009) found that pH increased from 4.8 to 6.72 in soils impacted with waste oil from automobiles. An increase in the pH of soil is noted with the addition of PHCs; however, the extent of this increase is dependent on the source of petroleum (Adebiyi and Afedia, 2001; Kistic *et al.*, 2009).

Petroleum contamination has been shown to increase the EC of soil (Adebiyi and Afeda, 2011; Arocena and Rutherford, 2005). Crude oil has been found to contain brine, an artifact of the marine sediments thought to have formed the oil (Goodger, 1975). Adebiyi and Afeda (2011) found that waste automobile oil increased the EC from 0.24 dS/m to 0.804 dS/m. Although this is likely not a significant increase, it indicates that petroleum products have the potential to increase the EC of soil. Arocena and Rutherford (2005) looked at the impact of petroleum extraction waste from flare pits on the EC of the soil. Though the flare pits may contain waste products other than PHCs, they found that the EC increased from 0.28 dS/m to 4.5 dS/m because of the increase in soluble calcium, potassium, sodium and magnesium (Arocena and Rutherford, 2005). This drastic increase in EC was attributed to the possible presence of brine water in the flare pit (Arocena and Rutherford, 2005). The presence of PHC in the soil has also been shown to increase the Al^{3+} and Mn^{2+} ions which, in addition to increasing the EC, are also directly toxic to plants (Onwurah *et al.*, 2007).

Petroleum has also been found to affect the CEC, WHC, and temperature (Kowsar *et al.*, 1969; Ogboghodo *et al.*, 2004; Urum *et al.*, 2004). Urum *et al.* (2004) note that as the CEC and pH increases, the sorption of PHCs into the soil also increases. Ogboghodo *et al.* (2004) indicated that the WHC of an oil spiked soil decreased largely because the hydrophobic nature of PHCs decreased the wettability of the soil. Kowsar *et al.* (1969) showed that petroleum resins sprayed on soil as mulch increased the temperature of the silty clay loam soil by making it darker.

1.4 The Canada-Wide Standards for PHC in Soil

The Canadian Council of Ministers of the Environment (CCME) consists of ministers from the federal, provincial and territorial governments who are committed to protecting the Canadian environment by dealing with issues of national concern (CCME, 2011). One of the issues that the CCME has investigated is PHC-impacted soil. They noted that more than 100,000 sites, or approximately 60% of the contaminated sites in Canada, contain PHCs (CCME, 2001a; CCME, 2008b; CCME, 2008c). The CCME determined that there were inconsistencies in site management and remediation which led them, along with the Analytical Methods Technical Advisory Group (AMTAG), to prepare a standardized method called the Canada-Wide Standards (CWS) Reference Method for PHC in Soil (CCME, 2008b). This standard outlines the extraction of F1 with methanol and F2 to F4 with a 50:50 acetone:hexane solution and the determination of each fraction concentration using a gas chromatograph with a flame ionization detector; it also outlines the concentration of each fraction that indicated when a site is remediated (Table 1-1; CCME, 2001a; CCME 2001b). The PHC CWS was based on work performed by the US Total Petroleum Hydrocarbons Criteria Working Group (TPHCWG) with consideration to scientific, technical and socio-economic factors along with input from stakeholders (CCME, 2008b; CCME, 2008d). The PHCs were subdivided into four fractions based on their equivalent carbon number and physical and chemical properties: F1 (C₆-C₁₀), F2 (> C₁₀-C₁₆), F3 (> C₁₆-C₃₄) and F4 (> C₃₄⁺) (CCME, 2001a; CCME, 2008a; CCME, 2008b; CCME, 2008d).

The goal of the PHC CWS is to protect human health and the environment, while providing consistent and practical remediation targets (CCME, 2008b; CCME, 2008d). To achieve this goal, a three-tiered system was developed to manage PHCs. Tier 1 outlines the generic numerical remediation standards for each PHC fraction (Table 1-1); Tier 2 incorporates site specific information into adjustable parameters to obtain guideline values that are more applicable to a specific site; Tier 3 utilizes risk assessment to obtain site-specific remediation targets.

Table 1-1: CCME remediation criteria for the direct contact exposure pathway of ecological receptors for fine grained PHC-impacted soil with different end land uses in mg/kg (CCME, 2008d)

End Land Use	Fine soil (particle size \leq 75 μm) (mg/kg)				Coarse Soil (particle size $>$ 75 μm) (mg/kg)			
	F1	F2	F3	F4	F1	F2	F3	F4
Agricultural	210	150	1300	5600	30	150	300	2800
Residential/Parkland	210	150	1300	5600	30	150	300	2800
Commercial/Industrial	320	260	2500	6600	320	260	1700	3300

The generic Tier 1 PHC guidelines (Table 1-1) are considered by the CCME to be relatively conservative but practical while providing adequate protection of the human and ecological receptors at most sites (CCME, 2008b; CCME, 2008d). The governing exposure pathway in Tier 1 is ecological soil contact because of the sensitivity of vascular plants and invertebrates to PHC; as a result, most toxicity tests have incorporated these organisms (CCME, 2008a; CCME, 2008b; CCME, 2008d). Despite the efforts of the CCME to prepare scientifically sound and practical guidelines, they have acknowledged that these values were selected without enough information to support them and that the toxicity tests were largely performed by one group of researchers (CCME, 2001a; CCME, 2008b). The CCME have also acknowledged that they relied on the use of fresh Federated Crude oil despite the fact that it was not known whether this type of oil was representative of those found in Canada (CCME, 2008b). The CCME also noted that little attention was paid to weathering when deriving the Tier 1 guidelines because there was not enough data to prepare models that would include weathering effects (CCME, 2008a; CCME, 2008d).

1.5 Weathering of PHC

Weathering of PHCs is the result of three main natural processes in soil: volatilization, biodegradation, and sorption (Alexander, 1995; Gallego *et al.*, 2010; Maletic *et al.*, 2011; Osuji *et al.*, 2006). Volatilization is the loss of low molecular weight hydrocarbons (F1 to F2) to the air (Gallego *et al.*, 2010; Maletic *et al.*, 2011). The hydrocarbons within the range of C₆ to C₁₄ will largely volatilize resulting in a relative increase of the heavier F2 to F4 fractions (Pichtel and Liskanen, 2001). Biodegradation of PHCs can be carried out by indigenous microorganisms in soil, and involves the oxidation of mid-range molecular weight hydrocarbons (F1 to F3) to form organic acids and CO₂ (Maletic *et al.*, 2011; Osuji *et al.*, 2006). Hydrocarbons in the C₆ to C₃₂ range can be degraded by microorganisms that have an oxygenase or hydroxylase enzyme which adds a carboxyl or hydroxyl group to the terminal carbon atom (Beilen and Funhoff, 2007). The organic acids produced during the initial stages of biodegradation do not appear during sample analysis with a gas chromatograph (Cozzarelli, *et al.*, 1994). Sorption is the association of PHCs with organic matter or soil particles, often through diffusion into pore spaces or attachment through van de Waals forces (Brusseau, 1997; Maletic *et al.*, 2011; Yong and Rao, 1991). Sorption of PHCs

decreases their bioavailability to the point where the PHCs should be considered as inert as soil humus (Maletic *et al.*, 2011). Each of these weathering processes is able to decrease the amount and availability of the PHCs, shifting the PHC composition toward higher molecular weight hydrocarbons, which decreases the overall soil toxicity (Alexander, 1995; Maletic *et al.*, 2011).

1.6 The Plant Growth Promoting Rhizobacteria Enhanced Phytoremediation System

Landfilling, which involves digging up the contaminated soil and taking it to landfill, is currently the most common method of remediating PHC-impacted soil (Philp and Atlas, 2005). This method is not only costly, time consuming and difficult to apply in remote areas, but it can also increase the potential for the exposure of the workers and the local community (National Research Council, 1999; Philp and Atlas, 2005). The need for inexpensive, easily implemented and environmentally responsible remediation methods has led to the development of strategies such as landfarming, bioremediation, and phytoremediation (Marmiroli and McCutcheon, 2003; Philp and Atlas, 2005; Zimmerman *et al.*, 1991).

Landfarming involves spreading petroleum waste on soil within a defined area and physically mixing to promote PHC volatilization. Bioremediation employs the addition of microbes and soil amendments, such as fertilizer or organic matter, to PHC-impacted soil in an attempt to increase the indigenous populations of hydrocarbon-degrading bacteria (Bollag *et al.*, 1994; Friend, 1996; Mohan *et al.*, 2006; Philip and Atlas, 2005). This technology assumes that the soil contains hydrocarbon degrading bacteria, that the contaminants are in a form microorganisms are able to degrade and that the soil properties (pH, texture, moisture, etc.) can be optimized to support and encourage the growth of PHC-degrading microorganisms (Philp and Atlas, 2005). It is, however, difficult to meet all of these assumptions which is the likely reason for the poor efficacy of bioremediation (Atlas and Cerniglia, 1995; Bollag *et al.*, 1994; Philp and Atlas, 2005).

An improvement to bioremediation was to use plants to accumulate, volatilize, degrade or stabilize contaminants in soil, water or air. This process eventually became known as phytoremediation (Evans and Furlong, 2011; McCutcheon and Schnoor, 2003; Wei and

Zhou, 2008). The rhizosphere of plants is an approximately 2-mm region of soil around the root that is influenced by the root (Brady and Weil, 2010). The rhizosphere is able to stimulate and support the growth of indigenous PHC-degrading microorganisms by providing exudates such as organic acids, sugars and amino acids from plant roots which can be utilized by microorganisms in the rhizosphere (Brady and Weil, 2010; Olson *et al.*, 2003; Schwab and Banks, 1994). The efficacy of this technology is based on the production of high amounts of plant biomass, through maximizing the surface area of the roots and shoots, which increases the potential for rhizosphere degradation and accumulation (Evans and Furlong, 2011; Olson *et al.*, 2003; Schwab and Banks, 1994; Wei and Zhou, 2008). Several aspects of the remediation techniques described above were used to prepare the Plant Growth Promoting Rhizobacteria (PGPR) Enhanced Phytoremediation System (PEPS) (Huang *et al.*, 2004).

PEPS was designed to increase the rate of PHC remediation through the use of tilling and soil amendments, the treatment of seeds with PGPR (some of which are hydrocarbon degraders) and the growth of plants (Huang *et al.*, 2004). As was outlined earlier, the weathering of PHC involves volatilization, biodegradation, and sorption (Alexander, 1995; Gallego *et al.*, 2010; Maletic *et al.*, 2011; Osuji *et al.*, 2006). Tilling is a method employed to amplify the weathering of PHCs by improving volatilization and promoting the growth of microorganisms (Friend, 1996; Mohan *et al.*, 2006; Philp and Atlas, 2005). PGPR are used to increase plant biomass by mitigating the ethylene stress response (Glick *et al.*, 1994; Glick, 1995; Glick *et al.*, 1998; Hall *et al.*, 1996; Larcher, 2003). These naturally occurring bacteria decrease ethylene through the production of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and the phytohormone indole acetic acid (IAA; Figure 1-1; Glick *et al.*, 2008; Larcher, 2003).

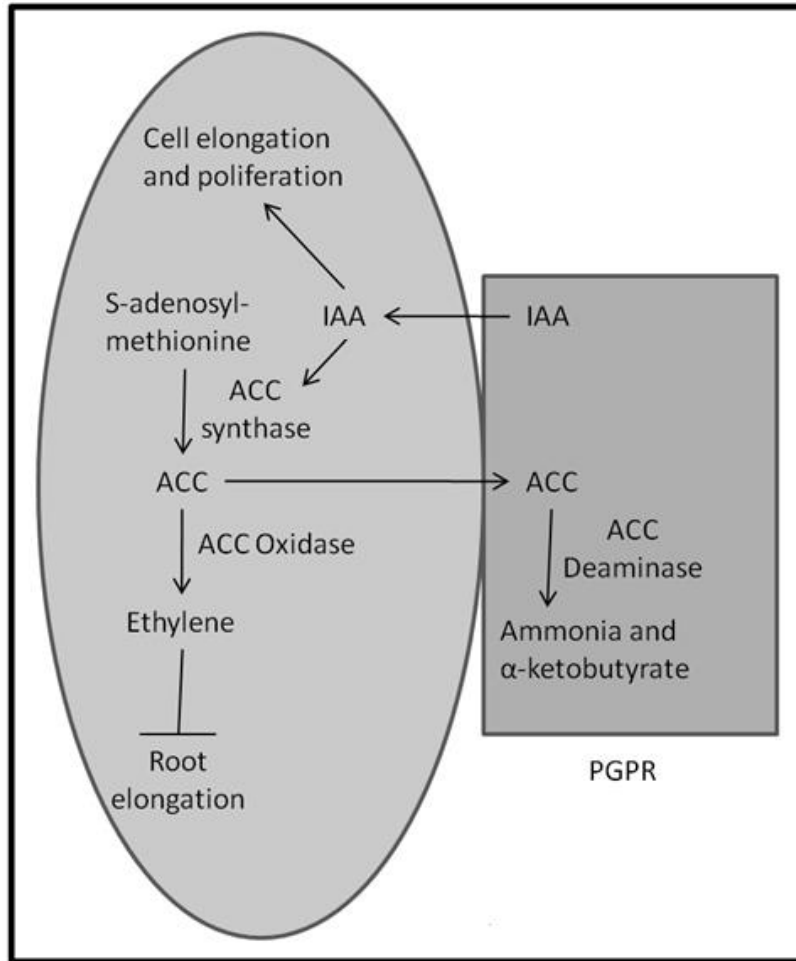


Figure 1-1: Effect of PGPR on the reduction of the ethylene stress response in plants (adapted from Glick *et al.*, 1998 with permission)

PGPR are in direct contact with the plant root and will take up ACC as it is exuded from the plant, inducing the plant to exude ACC to maintain equilibrium; the decreasing ACC in the plant decreases the ethylene response which leads to a decrease in the plant stress response (Figure 1-1; Glick *et al.*, 1998; Hall *et al.*, 1996). The ACC taken up by PGPR is converted into α -ketobuterate and ammonia through an enzyme called ACC deaminase; the ammonia that is produced can then be used as a nitrogen source for the PGPR (Glick *et al.*, 1994; Glick, 1995; Glick *et al.*, 1998). PGPR can also produce IAA, a plant hormone (auxin), which is then exuded by the bacterium and taken up by the plant (Larcher, 2003). IAA uptake by the plant results in root elongation and the production ACC by the plant-based enzyme ACC-synthase (Figure 1-1; Glick, 1995; Glick *et al.*, 1998; Larcher, 2003). Overall, the ACC deaminase within the bacterium decreases the plant ethylene stress response and allows for plant growth within contaminated soils (Figure 1-1; Glick, 1995; Gurska *et al.*, 2009). The species of PGPR currently being used in PEPS are the *Pseudomonads* UW3 and UW4; UW3 has been shown to also be capable of degrading PHCs (Huang *et al.*, 2004). The ability to degrade petroleum indicates that this PGPR will also directly aid in the weathering of PHC through biodegradation (Philp and Atlas, 2005). The plants used in PEPS are able to enhance biodegradation using root exudates to increase the population of indigenous microorganisms and enhance volatilization using roots to increase the porosity of the soil (Hutchinson *et al.*, 2003; Walton *et al.*, 1994). Plants are also able to intensify biodegradation using the rhizosphere to support indigenous hydrocarbon degrading microorganisms (Hutchinson *et al.*, 2003; Walton *et al.*, 1994). This indicates that plants are also able to naturally enhance the remediation of PHC contaminants, above those that have been noted in fields that contain no plants, by enhancing the weathering processes (Bollag *et al.*, 1994; 2013; McCutcheon and Schnoor, 2003).

1.7 Toxicity Testing using *Folsomia candida*

Toxicity testing identifies the lethal (e.g., using mortality tests) and sub-lethal (e.g., using reproduction, growth or avoidance tests) concentrations of a contaminant that adversely affect an organism (Newman, 2010; Landis *et al.*, 2011). This is routinely investigated using an exposure-concentration-response model where a contaminant or contaminant source is added to a controlled test vessel containing the test organism (Newman, 2010; Landis *et al.*, 2011). Standardized toxicity tests have been developed for soil-dwelling micro-arthropods (e.g., Collembola), invertebrates (e.g., earthworms), and terrestrial plants (e.g., oats) (van Gestel *et al.*, 1997; Environment Canada, 2005a; Environment Canada, 2007a; Environment Canada, 2007b). These tests use natural and artificial soils to determine if there is a toxic response of an organism to an impacted soil (van Gestel *et al.*, 1997; Environment Canada, 2005a; Environment Canada, 2007a; Environment Canada, 2007b).

Collembola is a large class of organisms that includes more than 6500 species worldwide (Hopkin, 1997; Rusek, 1998). They are wingless, soft-bodied arthropods that are commonly found in the upper layers of soil and leaf litter (Eisenbeis and Wichard, 1987; Hopkin, 1997; Rusek, 1998). Collembola range from 0.2 – 10 millimeters long and play an important role in organic matter decomposition, nutrient cycling and improvement of the structure of the soil (Behan-Pelletier, 2003; Hopkin, 1997; Rusek, 1998). Many members of the Collembola class have three common features: the furca, the cuticle, and the ventral tube (Figure 1-2). The furca is produced from the fusion of the two hind legs and is normally tucked up underneath their abdomen by a hook structure called the tenaculum (Eisenbeis and Wichard, 1987; Hopkin, 1997). Collembola use their furca to push against a substrate and launch them in a somersaulting motion into the air to escape predators (Eisenbeis and Wichard, 1987). This springing motion is the reason why members of the Collembola class are commonly called “springtails” (Eisenbeis and Wichard, 1987). On a microscopic level, the Collembola cuticle follows a hexagonal pattern which is formed from triangular protuberances, known as microtubercles and the ridges that interconnect them (Eisenbeis and Wichard, 1987; Hopkin, 1997). This structure along with the presence of a waxy layer allows air to become trapped around their body, creating a hydrophobic outer surface (Hopkin, 1997; Ghiradella and Radigan, 1974). The permeability of the cuticle varies during the springtails life cycle, but an increase in permeability prior to molting is known to occur (Schreiber and Eisenbeis, 1985). Molting is the process where the entire cuticle is replaced, including the lining of the gut (Thimm *et al.*, 1998). The ventral tube, formed from a pair of appendages, is where the majority of environmental exchanges occur (Eisenbeis and Wichard, 1987; Hopkin, 1997). The main function of the ventral tube is to transport water into the Collembola, although some solute uptake may also occur (Drummond, 1953; Hopkin, 1997; Schreiber and Eisenbeis, 1985).

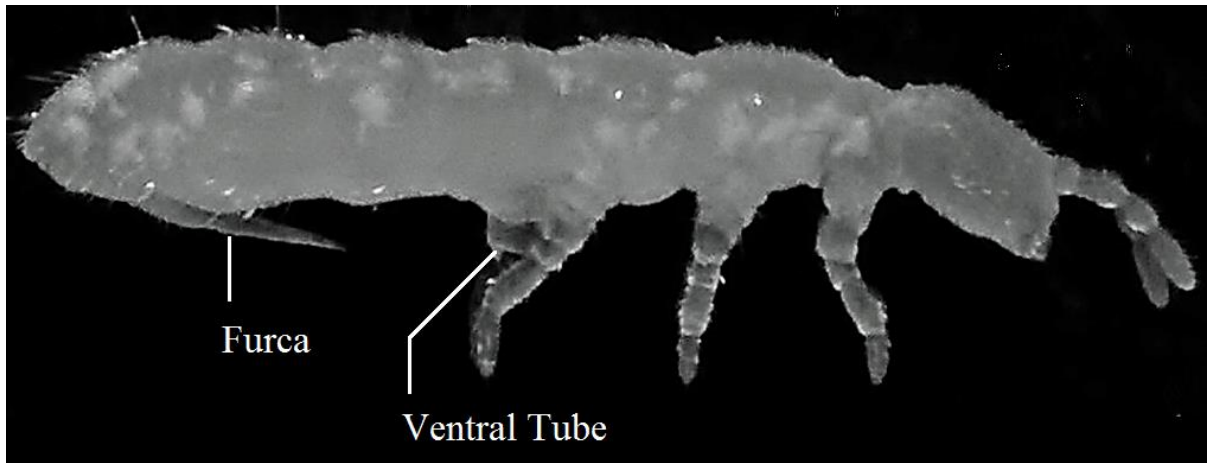


Figure 1-2. Three common features of Collembola: the ventral tube to collect water, the furca to spring away from predators and the retinaculum to hold the furca against the abdomen when not in use (the retinaculum is not visible on this photograph)

The ventral tube terminates in vesicles which allow for adherence to smooth surfaces (Eisenbeis and Wichard, 1987; Hopkin, 1997). The ventral tube also contains sensory organs, resembling hair-like structures, which allow Collembola to sense the moisture content, salinity, and pH of the substrate (Eisenbeis and Wichard, 1987; Hopkin, 1997). The species of Collembola that was used for this research (obtained from Stantec Consulting Ltd. [Guelph, Ontario] in conjunction with Frei University in the Netherlands) belongs to the order: Entomobryomorpha, superfamily: Isotomoidea, subfamily: Proistominae, species *Folsomia candida* (Environment Canada, 2007a).

Folsomia candida are eudaphic, meaning that they reside largely in the organic and topsoil (O and A) horizons of surface soil (Eisenbeis and Wichard, 1987; Hopkin, 2007; Kaersgaard *et al.*, 2004; Kolar *et al.*, 2008; Noel *et al.*, 2006). They are also found in flower pots and compost piles located in urban areas (Rombke *et al.*, 2006). *Folsomia candida* have an optimal temperature for reproduction of 21°C and can live under laboratory conditions for approximately 130 days (Snider and Butcher, 1973). They average 1.27 mm in length and can complete one life cycle in 28 days (Marshall and Kevan, 1967; Snider and Butcher, 1973). Most populations of *Folsomia candida* exhibit parthenogenesis (Fрати *et al.*, 2004; Marshall and Kevan, 1967; Pike and Kingcombe, 2009). This asexual form of reproduction might have stemmed from the presence of a gram negative α -proteobacteria, commonly known as *Wolbachia*, in the ovaries which results in male killing (Fрати *et al.*, 2004; Marshall and Kevan, 1967; Pike and Kingcombe, 2009; Rousset *et al.*, 1992; Vandekerckhove *et al.*, 1999; Werren *et al.*, 1995). *Folsomia candida* juveniles are born with adult characteristics and molt to increase in size (Marshall and Kevan, 1967). Maturity is reached at the sixth molt which is approximately 18 days after hatching (Snider and Butcher, 1973). *Folsomia candida* are commonly used in toxicity testing because of their parthenogenicity and their ease of culturing (Fountain and Hopkin, 2005).

1.8 Previous Toxicity Research using *Folsomia candida*

The first use of *Folsomia candida* in toxicity testing occurred in 1969 for a feeding study that involved spiking food with DDT (dichlorodiphenyltrichloroethane) to determine its toxicity (Butcher *et al.*, 1969). At the time there were no standard protocols for feeding studies or the use of *Folsomia candida*, and the results determined that DDT did not significantly affect mortality (Butcher *et al.*, 1969; Thompson and Gore, 1972). The lack of a significant toxic effect in the DDT experiment delayed the first attempt at preparing a standard protocol until 1972 when Thompson and Gore looked at the effect of 29 insecticides on the survival of *Folsomia candida* (Thompson and Gore, 1972). Thompson and Gore (1972) indicated that *Folsomia candida* were easy to culture and they concluded that these organisms fit the criteria for use in a bioassay. The authors also noted that because *Folsomia candida* live in the soil, they would be better suited for soil toxicity tests than the non-soil-dwelling organisms that were currently being used (Thompson and Gore, 1972). By 1981 *Folsomia candida* had become highly popular in the scientific community for use in pesticide feeding studies (Subagja and Snider, 1981). It was not until the 1990s that toxicity protocols for *Folsomia candida*, including survival and reproduction, were developed (Crommentuijn *et al.*, 1993).

In the 1980s researchers began using springtails for testing the toxicity of petroleum-impacted landfarmed soils (Neuhauser *et al.*, 1989; Pirhonen and Huhta, 1984). Neuhauser *et al.* (1989) estimated the number of Collembola, mites, and earthworms in a plot of soil before and after the spreading of liquid refinery waste oil. They found that all populations decreased rapidly after PHCs were applied to the soil and that higher initial oil applications led to slower population recovery rates (Neuhauser *et al.*, 1989). Pirhonen and Huhta (1984) carried out a similar test where the populations of Collembola, earthworms, and nematodes were estimated and then hydraulic oil or fuel oil was added to the soil. When the hydraulic oil was applied, the nematode population was unaffected while the earthworm population decreased to zero and did not begin to recover until a year and a half after the two year test was completed (Pirhonen and Huhta, 1984). When the fuel oil was applied, the nematode

population fully recovered less than two months after the treatment while the earthworm population dropped to 50% of the control for the duration of the two year test (Pirhonen and Huhta, 1984). The Collembola population showed no recovery and it remained at less than 10% of the control for the duration of the test, regardless of oil treatment (Pirhonen and Huhta, 1984). Both of these tests monitored the populations of naturally occurring organisms in the soil and showed that the toxicity to soil organism populations was greatest when the spill was fresh (Pirhonen and Huhta, 1984). As the oil in the soil weathered, the negative effects on the organisms decreased (Pirhonen and Huhta, 1984). These studies also indicated that Collembola may be more sensitive to some types of oils than other soil dwelling organisms, but it was not until approximately 20 years later that toxicity tests using Collembola were applied PHC-impacted soils in the laboratory (Pirhonen and Huhta, 1984; van Gestel *et al.*, 2011).

In 1999 the International Organization for Standardization (ISO), an organization devoted to the preparation of common industrial procedures, published a survival and reproduction toxicity test method specifically for *Folsomia candida* (ISO, 1999). One of the first applications of the ISO test method was as part of a test battery by van Gestel *et al.* (2011) to determine the toxicity of weathered mineral oil. The results of this test showed a significant negative correlation between *Folsomia candida* reproduction and oil concentrations of 50 to 3300 mg/kg (van Gestel *et al.*, 2011). Once the ISO test method was proven to be useful in an Ecological Risk Assessment test battery, Environment Canada and the Organisation for Economic and Co-operative Development (OECD) began updating the protocol by incorporating ideas and methods developed from the application of the ISO method (Achazi, 2002; Environment Canada, 2007a; Scott-Fordsmand and Krogh, 2004). The Environment Canada protocol was completed in 2007 with two key differences that were not included in the ISO method; the Environment Canada biological test method incorporated the use of contaminated field soils (in addition to the chemically-spiked artificial soils) and both sexually reproducing and parthenogenic species (Environment Canada, 2007a; ISO, 1999).

1.9 The Effects of Soil Properties on *Folsomia candida*

As mentioned in Section 1.7, *Folsomia candida* spend most of the time in contact with the upper layers of the soil (Eisenbeis and Wichard, 1987; Kaersgaard *et al.*, 2004; Kolar *et al.*, 2008; Noel *et al.*, 2006). *Folsomia candida* are known to respond to the soil properties such as moisture content, salinity and pH through sensory organs on their ventral tube (Eisenbeis and Wichard, 1987; Hopkin, 1997). Their growth and reproduction rates can also be significantly increased or decreased by changes to the moisture content as well as the temperature of the soil (Marshall and Kevan, 1967; Timmermans *et al.*, 2009; Waagner *et al.*, 2011). Soil properties which affect *Folsomia candida* are described below.

The bulk density, soil structure (i.e., aggregation of soil particles) and the soil texture impact habitat suitability and the avoidance response of *Folsomia candida* (Domene *et al.*, 2011; Natal-da-Luz *et al.*, 2008; Wickenbrock and Heisler, 1997). *Folsomia candida* prefer living in macropore spaces that are 4 mm in diameter; these pore spaces can be formed by earthworms (Wickenbrock and Heisler, 1997). Increasing the soils bulk density (i.e., increasing compaction) decreases the macropore space resulting in decreased living space for *Folsomia candida* (Brady and Weil, 2010). Soils with a finer texture are easily compacted with fewer macropore spaces and are often avoided by *Folsomia candida* (Natal-da-Luz *et al.*, 2008). When soils have a larger proportion of smaller particles (e.g., a soil high in kaolinite clay), they can provoke an avoidance response in *Folsomia candida* (Brady and Weil, 2010; Natal-da-Luz *et al.*, 2008; Domene *et al.*, 2011).

The organic matter content in the soil is also very important to Collembola survival (Castano-Meneses *et al.*, 2004; Saitoh *et al.*, 2011). Collembola feed on fungi, bacteria, fungal spores and decaying plant and animal material found in the soil organic matter (Castano-Meneses *et al.*, 2004). Collembola populations have been shown to increase with increasing organic matter (Saitoh *et al.*, 2011). Saitoh *et al.* (2011) determined that 105 Collembola can be supported per gram of organic carbon. In laboratory-prepared artificial soil, *Folsomia candida* will avoid soils with only 2% organic matter despite alterations in soil texture (Natal-da-Luz *et al.*, 2008).

Folsomia candida have been shown to be very sensitive to moisture content (Timmermans *et al.*, 2009; Domene *et al.*, 2011; Waagner *et al.*, 2011). A relative humidity below 99.4% inhibits *Folsomia candida* oviposition (Waagner *et al.*, 2011). However, adults exhibited normal behaviour until the relative humidity dropped to 98.2%, at which point a desiccation response was initiated by the springtails (Timmermans *et al.*, 2009; Waagner *et al.*, 2011). This desiccation response involves the production of the sugar compounds myoinositol, glucose and trehalose by the springtail in order to increase the movement of water vapor through the cuticle and into the springtail (Timmermans *et al.*, 2009). A delay in the molting process may also occur in an attempt to conserve water (Timmermans *et al.*, 2009). Mortality of adult *Folsomia candida* was noted below a relative humidity of 97% (Bayley and Holmstrup, 1999).

Folsomia candida have been shown to be adversely affected by low pH (Kaneda and Kaneko, 2002; Sorenson and Holmstrup, 2005). Sorenson and Holmstrup (2005) showed that no mortality was observed in adults when the pH decreased from 7.74 to 5.35 in a sandy loam soil; however, the lethal concentration to kill 50% (LC₅₀) of the juveniles occurred at a pH of 5.4. Kaneda and Kaneko (2002) studied the effect of pH on *Folsomia candida* in soils with a high organic matter content and volcanic ash parent material. It was determined that a drop in pH from 6.83 to 4.6 significantly decreased *Folsomia candida* growth (Kaneda and Kaneo, 2002).

Owojori *et al.* (2009) showed that *Folsomia candida* are sensitive to salinity. They worked with an electrical conductivity (EC_e) range of 0.08 to 1.62 dS/m (Owojori *et al.*, 2009). An EC_e of 1.03 dS/m resulted in decreased juvenile production rates while an EC_e of 1.62 ds/m resulted in complete inhibition of juvenile production (Owojori *et al.*, 2009). However, an EC_e of 1.62 dS/m had no effect on adult survival (Owojori *et al.*, 2009).

Soil temperature had a statistically significant effect on juvenile production in *Folsomia candida* (Table 1-2; Marshall and Kevan, 1967; Simpson, 1983; Tan, 2009). A change in temperature of a few degrees, either positive or negative, from the 22°C optimum has been shown to have a marked impact on the number of eggs produced, the percentage of eggs that hatch, the number of days required for hatching to occur, and the number of days between ovipositions in *Folsomia candida* (Table 1-2; Marshall and Kevan, 1967). At 22 °C more eggs were produced than at any other temperature and the viability of these eggs was also the highest (Table 1-2; Marshall and Kevan, 1967). In addition to the benefits of increased juvenile production, the ranges for the time between ovipositions and the time to hatch are narrower than at other temperatures (Table 1-2; Marshall and Kevan, 1967).

1.10 Alternate Test Methods, New Endpoints and Avoidance Tests for *Folsomia candida*

There are two sets of methods that can be used to remove the springtails from the soil: dynamic and mechanical (Edwards and Fletcher, 1971). The idea behind the dynamic method is to use heat to drive the organisms down through the soil to a catch vessel for enumeration (Edwards and Fletcher, 1971). The mechanical methods involve floating the organisms from the soil, using a solution with a density (specific gravity) greater than that of the organisms, and counting them (Edwards and Fletcher, 1971). There are advantages and disadvantages to each method. For example heat extraction collects only live, intact organisms and can be used for soils with high organic matter; however, it requires a consistent room temperature of 20 ± 1 °C and is difficult to use with soils that are easily compacted (i.e., those with a high bulk density) and/or with high clay content (Edwards and Fletcher, 1971). No method has been shown to be 100% efficient in removing springtails from the soil and the efficiency of each method varies with species and soil type (Edwards and Fletcher, 1971; Snider and Snider, 1997). The Environment Canada protocol recommends the use of the floatation and heat extraction methods which are briefly described below (Environment Canada, 2007a).

Table 1-2: Alterations in egg development and oviposition with temperature (adapted from Marshall and Kevan, 1967).

Temperature (°C)	Number of Females	Days Between Ovipositions	Number of Eggs	Percentage Hatched	Number of Days to Hatch
18	4	13-15	15	46.7	15-16
20	4	14-16	35	71.5	10-15
22	5	10-13	41	87.8	9-10
24	*6	*11-13	*59	*88.9	11-13
28	N/A	N/A	N/A	0	N/A

* - not statistically different from the results obtained at 22 °C

N/A – no data collected

The floatation method involves adding water to the test vessel and allowing the springtails to float to the surface (because of their hydrophobic cuticle) to be counted (Environment Canada, 2007a). The heat extraction method involves removing the soil from the test container onto a screen and adding a heat source to force the springtails through the screen onto a dark surface for enumeration (Environment Canada, 2007a). Testing performed in the 1970s favored the heat extraction method while current methods appear to emphasize the floatation method (Edwards and Fletcher, 1971; Environment Canada, 2007a).

Research into chronic toxicity tests with other organisms indicated a focus on measuring sub-lethal endpoints (Environment Canada, 2005a; Environment Canada, 2007b). These endpoints have included weight and length in addition to reproduction (Environment Canada, 2007b). While the Environment Canada protocol only contains the sub-lethal end point of reproduction, other tests involving the toxicity of metals and pesticides to Collembola have measured the sub-lethal endpoint of growth in terms of weight and length (Environment Canada, 2007a; Folker-Hansen *et al.*, 1996; Petersen *et al.*, 1997). Methods of measurement have included the use of an electrobalance to measure the weight and a stereo microscope with monitor to manually measure the length (Folker-Hansen *et al.*, 1996; Hilligsoe and Holmstrup, 2003). By incorporating the end-points of weight and length into the chronic toxicity test protocols, these tests might provide more information on how PHCs affect soil invertebrates through increased sensitivity (Folker-Hansen *et al.*, 1996).

Acute toxicity tests use shorter exposure times and measure either lethal or sub-lethal effects (e.g., avoidance-response) of exposed organisms (Environment Canada, 2007b). Avoidance-response tests have been used extensively to assess the impact of contaminated soils on earthworms and the results of such tests have correlated with the results obtained in the longer chronic toxicity tests (Environment Canada, 2007b). One of the first uses of avoidance-response tests with Collembola was to assess the effects of the pesticide Betanal (Heupel, 2002). The method employed a cylindrical container with a divider (Heupel, 2002). Two different soils were placed on either side of the divider and ten collembolan individuals were initially placed on one side or the other to test avoidance of the organisms (Heupel, 2002). By 2009, avoidance-response

tests with *Collembola* had been shown to have the potential as a pre-screening test of PHC-impacted field sites; the results of the avoidance tests would determine if chronic toxicity tests needed to be performed (Natal-da-Luz *et al.*, 2008). The method was then adapted as follows: two different soils were placed on either side of a divider, the divider was removed, and 20 juveniles 10 to 12 days old were placed along the midline (Liu *et al.*, 2010). After 48 hours the divider was reinserted and water was added to determine the number of *Collembola* on each side (Liu *et al.*, 2010). The use of an acute toxicity test to screen a site for the concentration that *Folsomia candida* begin to avoid, would indicate the concentration where toxic effects would likely be seen in the chronic toxicity tests (Natal-da-Luz *et al.*, 2008). If this value was higher than the CCME remediation criteria then chronic toxicity tests would need to be performed for verification (Natal-da-Luz *et al.*, 2008).

The determination of a more efficient method for removal of the springtails from the soil, the addition of growth metrics as assessment endpoints and the inclusion of an acute avoidance-response test has the potential to improve and/or expand the scope of the current Environment Canada biological test method (Natal-da-Luz *et al.*, 2008). The inclusion of weight and length to the chronic toxicity test might improve the sensitivity of the test (Folker-Hansen *et al.*, 1996). Adding the acute avoidance-response test provides a potential screening tool for contaminated site soils and eliminates the need for chronic tests if the avoidance test shows an effect (Folker-Hansen *et al.*, 1996).

1.11 Summary and Hypothesis

Folsomia candida is affected by soil properties; therefore, the alterations of soil properties that occurs with the presence of PHC will likely impact *Folsomia candida* (Adebiyi and Afedia, 2011; van Gestel *et al.*, 2011). However, the composition of petroleum changes over time because of weathering processes. The CCME did not consider weathering in their derivation of the Tier 1 guidelines, an omission that was pointed out by the CCME itself (CCME, 2008a). The hypothesis for this research is that the toxicity of weathered PHC-impacted field soils will occur at higher concentrations than the CCME guidelines. The four objectives used to test this hypothesis were to: (1) use chronic toxicity tests with *Folsomia candida* to determine the approximate IC₂₅ (inhibitory concentration that decreases juvenile production by 25%) of

weathered PHC-impacted field soil at three different field sites, (2) compare the heat and floatation extraction methods to determine their efficiency at removing *Folsomia candida* adults and juveniles from soil, (3) determine if the sub-lethal endpoints (weight, length and/or width) will be a suitable addition to the chronic toxicity test protocol and (4) investigate the feasibility of using avoidance tests as a preliminary assessment method of the toxicity of weathered petroleum-impacted sites.

Chapter 2 Toxicity of Field Soil with Different Levels of Weathered PHC to *Folsomia candida* and a Comparison of Extraction Methods for Removal of *F. candida* from Soil

2.1 Introduction

Oil refineries receive crude oil and process the petroleum into products such as fuels, lubricating oils and plastics (Bjorlykke, 2010; Fingas, 2011). Although the efficiency of oil processing has increased there are still waste products from production (e.g., oil sludge and oil tank residue) (Knowlton and Rucker, 1979). One method of treating petroleum waste is known as land-farming (Knowlton and Rucker, 1979). Land-farming involves spreading waste onto soil and mixing it to increase aeration (Knowlton and Rucker, 1979). Land-farms have been used for disposal of oil sludge at refinery sites for over 50 years (Knowlton and Rucker, 1979).

Oil contains approximately 70% petroleum hydrocarbons (PHCs) by mass; it is a complex mixture of more than 700 organic molecules that contain only carbon and hydrogen (CCME, 2001a; CCME, 2008a; CCME, 2008b; CCME, 2008d; Petrov, 1987; Long, 1998). The Canadian Council of Ministers of the Environment (CCME) has determined soil guidelines for sites impacted with PHC; these are outlined in The Canada-Wide Standards (CWS) for PHC in Soil (PHC CWS; CCME, 2001a; CCME, 2008a; CCME, 2008b; CCME, 2008c). The PHC CWS were based on four hydrocarbon fractions similar to those outlined by the United States Total Petroleum Hydrocarbons Criteria Working Group (TPHCWG; CCME, 2008b; CCME, 2008d). These fractions were determined based on boiling points and equivalent carbon numbers: F1 (C6-C10), F2 (>C10-C16), F3 (>C16-C34) and F4 (>C34). The PHC CWS can be used as generic (Tier 1) remediation criteria for each fraction that can be applied to all PHC-impacted sites (Table 1-1; CCME, 2008b). Because Tier 1 values are designed to be protective of ecological receptors during the worst exposure scenarios, these guidelines were developed using data from toxicity tests with fresh (i.e. un-weathered) oil and did not consider the effect of weathering on PHC toxicity in soils.

Weathering of PHC results from three main natural processes in soil: volatilization, the loss of low molecular weight hydrocarbons; biodegradation, the oxidation of mid-range molecular weight hydrocarbons by indigenous soil microorganisms; and sorption, the binding of

high molecular weight PHC molecules with particles or organic matter within the soil (Alexander, 1995; Brusseau, 1997; Gallego *et al.*, 2010; Maletic *et al.*, 2011; Osuji *et al.*, 2006; Yong and Rao, 1991). Each of these weathering processes can either alter the composition of the petroleum in the soil or influence its bioavailability, thereby decreasing its toxicity (Alexander, 1995; Maletic *et al.*, 2011).

Folsomia candida (commonly referred to as “Springtails”) is a soil-dwelling invertebrate species that is commonly used in toxicity tests (Fountain and Hopkin, 2005). Their ability to reproduce parthenogenically, resulting in an all-female population, is an important consideration for use in toxicity tests (Fountain and Hopkin, 2005). The all-female population removes the consideration of male to female ratios, which is critical for toxicity tests with sexually reproducing invertebrates to obtain the optimal number of progeny (Environment Canada, 2007b; Frati *et al.*, 2004; Fountain and Hopkin, 2005; Marshall and Kevan, 1967; Pike and Kingcombe, 2009; Rousset *et al.*, 1992; Vandekerckhove *et al.*, 1999; Werren *et al.*, 1995). Two other features which make *F. candida* ideal for toxicity testing are their cuticle and their ventral tube. The cuticle is hydrophobic due to its waxy coating which repels water and the microscopic hexagonal pattern of protrusions which trap air around the body (Eisenbeis and Wichard, 1987; Ghiradella and Radigan, 1974; Hopkin, 1997). The hydrophobic outer cuticle can make the removal of *F. candida* from soil easier as the organisms will float in water (Environment Canada, 2007a). The ventral tube is where most of the environmental exchanges, such as water uptake, occur (Drummond, 1953; Eisenbeis and Wichard, 1987; Hopkin, 1997; Schreiber and Eisenbeis, 1985). The end of the ventral tube contains hair-like sensory organs which allow *F. candida* to sense the moisture content, salinity and pH of the substrate (Eisenbeis and Witchard, 1987; Hopkin, 1997).

Soil properties such as organic matter content and electrical conductivity (EC) influence the behavior of *Folsomia candida* (Bayley and Holmstrup, 1999; Domene *et al.*, 2011; Kaneda and Kaneo, 2002; Marshall and Kevan, 1967; Natal-da-Luz *et al.*, 2008; Owojori *et al.*, 2009; Waagner *et al.*, 2011). *Folsomia candida* prefer soils that contain $\geq 2\%$ organic matter, an electrical conductivity (EC_e) of < 1 dS/m, a low bulk density (e.g., < 1.6 Mg/m³) and a soil pH > 6.83 (Bayley and Holmstrup, 1999; Brady and Weil, 2010; Domene *et al.*, 2011; Kaneda and Kaneo, 2002; Marshall and Kevan, 1967; Natal-da-Luz *et al.*, 2008; Owojori *et al.*, 2009; Waagner *et al.*, 2011).

To test the toxicity of soil, a dose response model is often used where an organism is exposed to increasing concentrations of a contaminant (Newman, 2010; Landis *et al.*, 2011). The toxicity test is used to identify the concentrations of a contaminant that causes lethal and sub-lethal effects on an organism (Newman, 2010; Landis *et al.*, 2011). *Folsomia candida* were originally used in feeding studies with pesticides but, their successful use led to their inclusion in toxicity test protocols involving survival and reproduction (Butcher *et al.*, 1969; Crommentuijn *et al.*, 1993; Subagja and Snider, 1981; Thompson and Gore, 1972).

The Environment Canada protocol recommends *Folsomia candida* be removed from the soil at the end of a specified exposure period using either the floatation or the heat extraction methods (Environment Canada, 2007b). Originally, the floatation method employed the addition of a solution with a specific gravity greater than that of the organisms to allow *F. candida* to float to the surface for counting (Edwards and Fletcher, 1971). Because the outer surface of *F. candida* is hydrophobic, the current floatation method employs the addition of water to the soil (Eisenbeis and Wichard, 1987; Environment Canada, 2007b). The heat extraction method uses heat to drive the organisms, away from a heat source, out of the soil and into a vessel where they can be quantified (Edwards and Fletcher, 1971). Each extraction method has advantages and disadvantages. The floatation method does not stress the organisms with alterations in temperature and is most effective with low organic matter content soils; however, it extracts both living and dead organisms as well as molted cuticular tissues (Edwards and Fletcher, 1971). The heat extraction method collects only live, intact organisms, and can be used for soils with relatively high organic matter content. However, it requires a constant room temperature of 20 ± 1 °C and is difficult to use with high clay content soils (Edwards and Fletcher, 1971). In the 1970s, the heat extraction method was the preferred method for springtail quantification after chronic toxicity tests; however, current methods emphasize the floatation method (Environment Canada, 2007a).

A comparison of the heat extraction method and the floatation method will be performed to determine which extraction method is the most efficient and appropriate for use in toxicity tests. The hypothesis for this section of the research is that the heat extraction method is the most appropriate method for use in chronic toxicity tests with *Folsomia candida*. The main focus of this research is to determine an LC₂₅ (the lethal concentration that decreases the population by 25%) and IC₂₅ (the inhibitory concentration that decreases juvenile production by 25%) for

comparison to CCME guideline values. To determine an LC₂₅ and IC₂₅, a concentration-response curve is prepared by diluting a relatively high concentration of weathered PHC-impacted soil. The hypothesis for this section of the research is that the toxicity of weathered PHC-impacted field soils will occur at higher concentrations than the CCME guidelines. To test this hypothesis, experiments were conducted to expose *F. candida* to a PHC concentration gradient in soil to determine the approximate IC₂₅ of weathered-PHC soil at a land-farm site, and, in the process determine if the heat extraction method or the floatation method is the best method to remove *Folsomia candida* from the soil at the end of a chronic toxicity test.

2.2 Materials and Methods

2.2.1 *Folsomia candida* Cultures, Maintenance, and Preparation for Experiments

Cultures of *Folsomia candida* were obtained from Stantec Consulting Ltd. (Guelph, ON) and were placed on a Plaster of Paris and charcoal medium. The medium was prepared using 480 g DAP Plaster of Paris[®], 60 g activated charcoal (Catalog number C9157-500G, Sigma-Aldrich, Oakville, ON) and 500 mL of deionized water. Cultures were watered every three days with deionized water, fed with Fleischmann's[®] Traditional Activated Dry Yeast as required and sealed with Parafilm M[®] (Beemis, Neenah, WI) to maintain moisture (Environment Canada, 2007a). Cultures were maintained in a growth chamber at 20 ± 2 °C under cool white fluorescent lights at an intensity of 6.5-7.5 $\mu\text{moles}/(\text{m}^2 \cdot \text{s})$, a photoperiod of 12 hours and a relative humidity above 30% (Environment Canada, 2007a).

Synchronization of *Folsomia candida* cultures was performed to generate juveniles within two days of age for use in toxicity tests. Glass microscope slides were coated in the Plaster of Paris and charcoal medium (described above), and allowed to dry (adapted from Environment Canada, 2007a by Wan Lee, May 23, 2011). A moist paintbrush was used to collect and remove eggs from stock *Folsomia candida* cultures which were then placed onto the coated microscope slides (adapted from Environment Canada, 2007a by Wan Lee, May 23, 2011). These slides were then placed into Petri-plates which had been coated with the Plaster of Paris and charcoal medium and moistened to saturation using deionized water (Environment Canada, 2007a). Approximately 15 yeast pellets were placed on opposite sides of each microscope slide and eggs were allowed to hatch for 48 hours in the growth chamber. The slides along with any unhatched eggs were removed and all of the springtails that had hatched were allowed to develop for 10 days (Environment Canada, 2007a).

2.2.2 Soil for Chronic Toxicity Tests

Site soil was obtained from a refinery land-farm site in Ontario (ON1). The refinery was decommissioned 20 years ago and the land-farm had been left to weather. Soil was obtained from two points: an impacted section with high PHC content and an un-impacted section from just off site.

2.2.3 Initial Soil Preparation

2.2.3.1 Site Soil

All impacted and un-impacted soil from each container obtained was sieved through a 6-mm mesh screen to remove rocks, roots and other large debris (Environment Canada, 2007a). Soils that were too moist to sieve immediately were air dried overnight in a fume hood. Soils in containers were thoroughly homogenized then sub-samples were taken and tested for F2 to F4 by WEBi (Waterloo, Ontario). Based on the results obtained, control soil was thoroughly mixed and used to dilute the PHC-impacted soil. A sample of each dilution was sent to Maxxam for F2 to F4 analysis and these concentrations are reported in Table 2-1. The soil was stored at room temperature until required.

2.2.3.2 Artificial Soil

An artificial control soil, which was used for the chronic toxicity tests, was prepared by thoroughly mixing 10% air dried *Sphagnum* sp. peat moss (Belgian Nursery, Breslau, ON) that had been sieved through a 2mm mesh screen, 20% air dried kaolin clay (Dundee Pottery, New Dundee, ON) which had a particle size of <40 µm and 70% grade 70 air dried silica sand by mass (Barco 71, Opta Minerals, Waterdown, ON; Environment Canada, 2007a). The pH of the artificial soil was adjusted to 6.5-7.5 by adding 70 g of CaCO₃ (catalogue number C5929-500gG, Sigma-Aldrich, Oakville, ON; Environment Canada, 2007a). 1.7 L of Milli Q water were added to prepare an artificial soil with 20% moisture (see section 2.2.4.1). The soil was then left at room temperature for three days to allow for pH equilibration (Environment Canada, 2007a). The actual pH and moisture content were determined as described in section 2.2.4 and the soil was stored at 4°C until required.

2.2.4 Soil Properties required for use in Toxicity Tests

The pH, water holding capacity (WHC), percent moisture content and ideal moisture content of all soils were measured prior to their use in toxicity testing (Environment Canada, 2007a; Table 2-1). The electrical conductivity (EC) was also measured (Table 2-1).

Table 2-1: Soil properties for site ON1 analyzed by SGS laboratories (Guelph, ON), petroleum content (mg/kg), analyzed by Maxxam Analytics (Waterloo, ON) and in-house testing of WHC, moisture content, ideal moisture content, EC_e and water repellancy, of the PHC-impacted dilutions.

Site	Site Dilutions	Soil Properties															
		pH	Organic matter (%)	Total Nitrogen (%)	Total Phosphorous (ppm)	Organic Carbon (%)	% Sand	% Silt	% Clay	Cation Exchange Capacity	F2 (mg/kg)	F3 (mg/kg)	F4 (mg/kg)	WHC	Initial Moisture Content (%)	Ideal Moisture Content (% WHC)	EC _e (dS/m)
ON1	0 (control)	5.1	4.04	0.34	534	2.20	80	15	5	5.9	<10	95	51				1.84
	1	5.1	2.30	0.30	524	1.30	76	17	7	5.6	<10	180	130	52.0	9.6	33	2.00
	2	5.0	3.70	0.28	549	2.10	76	17	7	5.9	17	280	130	49.8	9.4	30	2.00
	3	5.8	4.50	0.24	581	2.50	78	17	5	6.7	<10	390	240	50.4	10.2	31	1.02
	4	5.8	4.70	0.27	584	2.60	79	16	5	7.9	99	1200	550	51.6	10.6	27	1.92
	5	6.3	5.10	0.23	611	2.90	79	17	4	8.8	62	1300	640	50.4	11.3	29	2.14
	6	6.7	6.70	0.30	766	3.70	76	17	7	9.4	330	3500	1700	46.7	13.5	29	2.74
	7	6.8	14.9	0.35	669	8.30	71	21	8	9.5	410	6200	3100	43.4	15.3	32	4.50
	8	6.6	8.70	0.36	861	4.80	71	21	8	10.3	520	5400	2000	46.6	13.7	34	4.88
	9	6.7	11.3	0.45	917	6.30	71	22	7	12.6	160	7900	3900	44.3	15.6	37	4.78
	10	6.6	16.2	0.60	1214	9.00	68	26	6	12.0	635	12000	5350	46.4	19.4	33	5.44
	Artificial Soil	7.5	4.00	0.05	55	2.20	76	8	16	8.73	<10	27	17	64.4	20.0	70	0.70

2.2.4.1 pH

The pH of the soils was tested as follows: 25 g of soil at the ideal moisture content (see section 2.2.4.3) was placed in a beaker and 50 mL of water was added and thoroughly mixed (adapted from Environment Canada, 2007a by Emma Shrive Stantec, May 12, 2011). The mixture was allowed to settle for 30 minutes and the pH of the solution was measured (adapted from Environment Canada, 2007a by Emma Shrive Stantec, May 12, 2011) using a pH meter (Fischer Scientific, Ottawa, ON). The pH of the Plaster of Paris was measured without allowing it to settle and using pH paper (Micro Essentials Laboratory, New York, NY) to avoid damage to the pH meter probe.

2.2.4.2 Water Holding Capacity

To obtain the WHC approximately 130 g of soil were dried in an oven at 105°C. Filter paper 18.5 cm in diameter with a course pore size (catalogue number 09-790-12G, Fisher Scientific, Ottawa, ON) was placed into a glass funnel with a 10 cm diameter (Environment Canada, 2007a). The filter paper was primed with approximately 10 mL of deionized water. The funnel and moistened filter paper were then weighed using a top-loading balance with 0.01 gram sensitivity (OHAUS TS400, M&L Testing Equipment (1995) Inc., Dundas, ON). The funnel was placed into a 500-mL Pyrex Erlenmeyer flask to support the funnel and collect the water that flowed through. A slurry of 100 g of the dried soil and 100 mL of deionized water was prepared and added to the funnel (Environment Canada, 2007a). Deionized water (10 ml) was used to rinse the vessel that contained the soil slurry. The funnel was then covered with aluminum foil to prevent evaporation (Environment Canada, 2007a). After three hours the aluminum foil was removed and the funnel containing the filter paper and wet soil was weighed (Environment Canada, 2007a). The WHC was calculated using the following equation:

$$WHC = \frac{(F) - (I + \textit{weight of dry soil})}{\textit{weight of dry soil}} \times 100\% \quad \text{Equation 2}$$

Where,

F = weight of funnel + wet filter paper + wet soil

I = weight of funnel + wet filter paper

2.2.4.3 Moisture Content

Briefly, the percent moisture was determined by weighing a soil sample before and after drying in a 105°C oven for 24 hours. The percent moistures were then calculated using Equation 3:

$$\frac{\text{weight of wet soil} - \text{weight of dry soil}}{\text{weight of dry soil}} \times 100\% \quad \text{Equation 3}$$

To determine the ideal moisture content for each field soil, water was added to the oven dried soil until soil clumps became 3-5 mm in diameter (Environment Canada, 2007a). The percent moisture was calculated as above and then expressed as a percentage of the WHC on a dry weight basis.

2.2.4.4 Electrical Conductivity (EC_e)

The EC_e can be obtained by preparing a soil slurry from 1 part soil to two parts water (EC_{1:2}) and multiplying by 2 (Chang, 2008). The EC_{1:2} was determined by drying approximately 50 g of soil at room temperature for at least 72 hours (adapted from Chang, 2008). The dried soil was then passed through a 2-mm sieve, and 15 g were added to a 50-mL falcon tube (catalog number CA21008-940, VWR International, Mississauga, ON) with 30 mL of deionized water (adapted from Chang, 2008). This was then shaken for 30 minutes at 120 rpm and the soil was allowed to settle overnight (adapted from Chang, 2008). The supernatant was then removed and the EC_{1:2} was measured using a conductivity meter (CON 510, Oakton Instruments, Vernon Hills, IL). The values obtained were multiplied by two to give the EC_e in dS/m (adapted from Chang, 2008).

2.2.5 Toxicity Tests

2.2.5.1 Soil Preparation

The chronic toxicity tests, as described below, were performed using the soils outlined in Section 2.2.3. The soil was prepared prior to testing by increasing the soils moisture content to the ideal moisture content (see Table 2-1 for ideal moisture contents). Equations 4, 5, and 6 were used to determine the amount of water to add to each soil:

$$D_w = W_w - (W_w \times MC)$$

Equation 4

Where,

D_w = the dry weight of the soil

W_w = the wet weight of the soil required for the test

MC = the desired soil moisture content

$$P_w = \left[WHC \times \left(\frac{P_{WHC}}{100} \right) \right] - MC$$

Equation 5

Where,

P_w = the percentage of water to add to the soil

WHC = the water holding capacity

P_{WHC} = the ideal moisture content

MC = the initial moisture content of the soil

$$V_W = (P_W \times M)/100 \quad \text{Equation 6}$$

Where,

V_W = volume of water to add to the soil (mL)

P_W = the percentage of water to add to the soil

M = the total dry weight of soil required for the test

The calculated amount of deionized water required to obtain the ideal soil moisture was added to the soil and thoroughly mixed in. The soil was then allowed to sit for approximately 20 minutes to ensure all water was absorbed.

2.2.5.2 Chronic Toxicity Tests

The juvenile *Folsomia candida* and soil were prepared according to Section 2.2.1 and 2.2.5.1. Thirty grams of each soil were added to three 125-mL clear glass jars with opaque lids (Maxxam Analytics, Waterloo, ON). The total weight of the jar and soil was measured and recorded. The soil in the jars was then gently compressed to a consistent density and the soil surface was made even (Environment Canada, 2007a). The jars were then sealed and placed into the growth chamber at $20 \pm 2^\circ\text{C}$ overnight (Environment Canada, 2007a). The jars were removed from the chamber and 10 juveniles were added along with eight yeast pellets (approximately 8 mg; Environment Canada, 2007a). The jars were resealed and placed back into the growth chamber at $20 \pm 2^\circ\text{C}$ for 28 days (Environment Canada, 2007a).

Once a week for the duration of the test, the test jars were aerated and watered as follows. Eight jars were randomly selected, weighed and deionized water was added until the initial total jar weight (the previously recorded initial weight of the jar and soil at its optimum water content) was reached (Environment Canada, 2007a). The amount of water required, was recorded for each jar and an average was obtained (Environment Canada, 2007a). The average amount of water was then added to all remaining jars in that test (Environment Canada, 2007a). On weeks 2 and weeks 3 the yeast was removed from all jars and replaced

with 10 mg of fresh yeast due to the presence of mould. After 28 days all of the jars were removed from the chamber and the number of surviving springtails was measured using the heat extraction method or the floatation method (Environment Canada, 2007a).

2.2.5.3 Heat Extraction

In preparation for heat extraction, the heat extraction units were prepared using two 4.5 ounce polypropylene cups (catalogue number 14955103, Fisher Scientific, Ottawa, ON). The bottom of one cup was removed and a 7-mesh round PVC needlework canvas was cut and glued with non-toxic glue into the lip groove of the cup (Environment Canada, 2012). The other cup was filled with approximately one centimeter of the Plaster of Paris/charcoal media (Environment Canada, 2012).

The Plaster mix in the cup was moistened to saturation with deionized water. The cup containing the needlework canvas was turned upside down and a double layer of cheesecloth was placed over the needlework canvas (Environment Canada, 2012). The cup with the needlework canvas was then placed on top of the cup containing the Plaster of Paris and they were joined together with Parafilm-M[®] (Environment Canada, 2012).

Once the toxicity test was finished the test jars were removed from the growth chamber and the soil was placed into an extraction unit. The test jars were filled with water and any remaining live springtails present were counted and the total number was saved to add to the number obtained from the heat extraction (Environment Canada, 2012). The extraction units were then placed under lamps with 60 watt light bulbs set 30 cm away from the soil surface (Environment Canada, 2012). There were four extraction units per lamp and the soil was watered every hour for the first 36 hours of the extraction. After 24 hours, the lamps were lowered to 15 cm above the soil surface (Environment Canada, 2012). When the soil temperature had reached 36°C (after approximately 48 hours) the lights were turned off and the soil from the extraction unit was placed into a beaker (Environment Canada, 2012). Water was added to the beaker and any live springtails present were counted to obtain an extraction efficiency value (Environment Canada, 2012).

2.2.5.4 Flootation

Water was added to the container containing the soil and the slurry was swirled and immediately decanted into a second container. More water was added to cover the bottom of the second container along with several drops of dye (Color Coat Red, Becker Underwood, Ames, IA) for easier visualization. The number of adults and juveniles were counted and recorded. The contents of the secondary container were then discarded. More water was then added to the container containing the soil, to cover approximately 1 cm above the soil, and the process was repeated until no soil was left in the container. If necessary a stir stick was used to aid in the mixing of the water and soil slurry.

2.2.6 Statistical Analysis

Statistical differences within tests were determined using the Student's t-test with a two tailed distribution and equal variance.

2.3 Results

The 11 dilutions prepared from ON1 soil and used in toxicity tests were initially assessed for F2, F3 and F4 (Table 2-1). The lowest concentration of weathered PHCs in the soil was in the site control (dilution 0) which had an F2, F3 and F4 concentration of 10 mg/kg, 95 mg/kg and 51 mg/kg, respectively. While, the highest concentration (dilution 10) had an F2, F3 and F4 concentration of 635 mg/kg, 12,000 mg/kg and 5,350 mg/kg, respectively (Table 2-1). F4 is the least toxic of the fractions tested (ESG International, 2003) and the F4 concentration did not exceed the 5,600 mg/kg CCME guideline in dilution 10. Because the F4 concentration at this level was unlikely to have effects on the organisms, it was excluded from the analysis. Both the F2 and F3 concentrations exceeded the soil quality guidelines of 150 mg F2/kg and 1,300 mg F3/kg at 12,635 mg F2 + F3/kg total (dilution 10: F2 = 635 mg/kg and F3 = 12,000 mg/kg); therefore, both fractions were used in the data analyses.

2.3.1 ON1 Chromatogram

The representative chromatogram was prepared by WEBi and can be used to determine the concentration of each PHC fraction in the sample. The chromatogram was generated by injecting a sample of the PHC-impacted field soil, that had been extracted with a 1:1 Acetone:Hexane solution and passed through a silica gel column to remove biogenics, into a gas chromatograph with a flame ionization detector. Each hydrocarbon has a specific retention time with the lower molecular weight hydrocarbons volatilizing first and the higher molecular weight hydrocarbons volatilizing after an increase in temperature. Hydrocarbon standards of C₁₀, C₁₆ and C₃₄ were used to determine the retention time of the borders of F2 (C₁₀ to > C₁₀ and C₁₆) and F3 (> C₁₆ and C₃₄ to > C₃₄). The area under the UCM (Unresolved Complex Mixture) in the sample chromatogram between these retention times is proportional to the concentration of that fraction in the sample.

The representative chromatogram obtained for ON1 dilution 10 soil (Figure 2-1) indicates that the F2 section of the UCM (approximately 1 to 4 minutes) contains all hydrocarbons in the F2 range (C₁₀ to C₁₆), while the F3 section of the UCM (approximately 4 to 11 minutes) is largely composed of high molecular weight F3 hydrocarbons (approximately C₃₀ to C₃₄). The ON1 chromatogram also indicates that the distribution of F2 and F3 is approximately 5% and 95%, respectively, which indicates F3 is the predominant fraction.

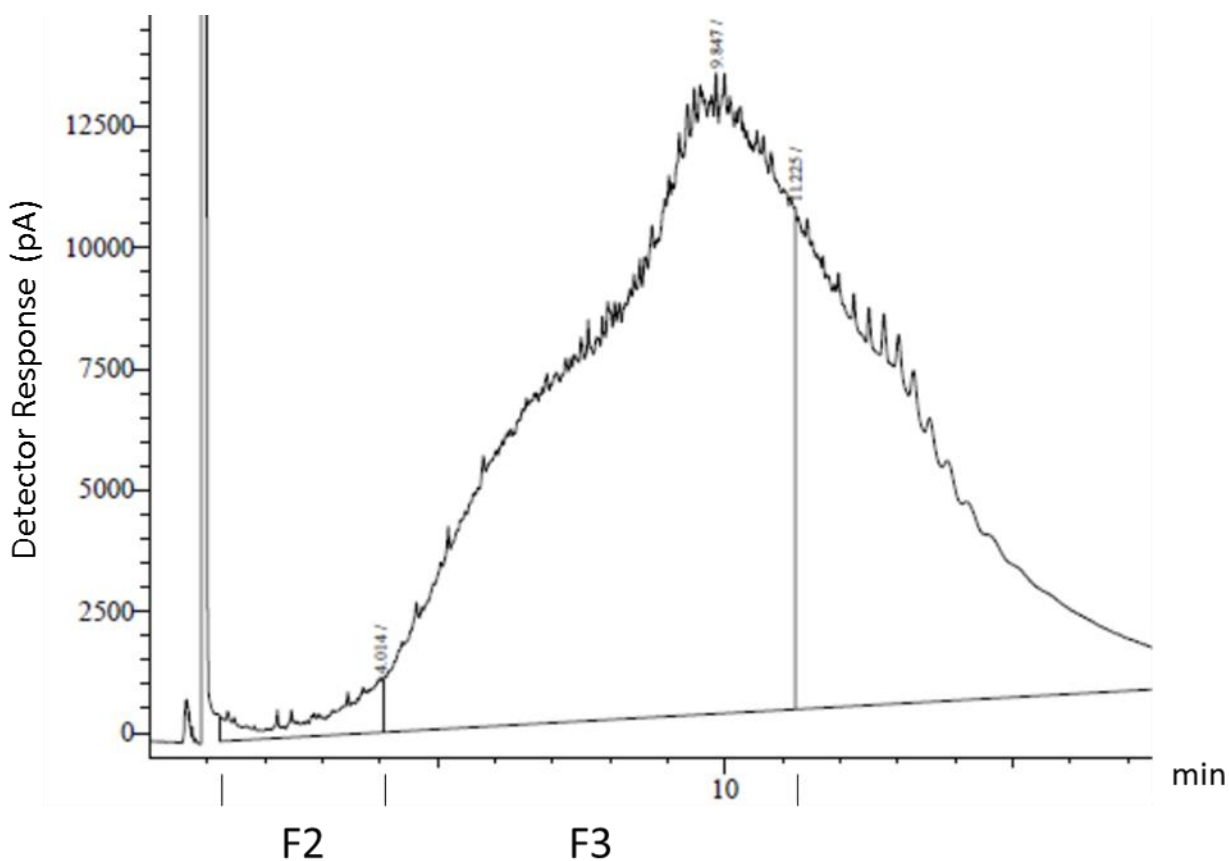


Figure 2-1: ON1 Chromatogram

It is important to note that the large peak found at approximately 1 minute is a solvent peak and the vertical line at approximately 4 minutes represents the retention time of the C_{16} standard while the vertical line at approximately 11.25 minutes represents the retention time of the C_{34} standard. The horizontal line across the bottom of the UCM represents the area that was used to calculate the concentration of the F2 and F3 fractions.

2.3.2 ON1 Chronic Toxicity Tests

Chronic toxicity tests were used to determine an LC₂₅ for survival and IC₂₅ for reproduction, using soils obtained from ON1, to compare to the CCME Tier 1 guidelines. The results for the endpoints of survival and reproduction were plotted on the y axis against the total F2 to F3 petroleum content on the x axis (Figure 2-2 to Figure 2-5).

2.3.2.1 ON1 Chronic Toxicity Test Results with Floatation

F. candida adult survival for springtails placed in artificial soil was on average 9.4 ± 0.43 springtails per test unit ($n = 6$) while adult survival for springtails placed in the reference control soil, dilution 0 (146 mg/kg total F2 to F3), was on average 7.2 ± 1.35 (Figure 2-2). The difference between the averages of the artificial soil and reference control soil used to dilute the site soil was not statistically significant ($p = 0.131$). There were no statistical differences between adult survival at each dilution ($p \geq 0.05$; Figure 2-2). An estimate of the LC₂₅ could not be determined because the data were not monotonic (i.e., did not show a decrease with increasing PHC concentration in the different dilutions) with respect to adult survival (Figure 2-2). Therefore, the highest exposure concentration of 12,635 mg F2 + F3/kg was determined to have no effect on *F. candida*.

The average juvenile production for *F. candida* in the site soil treatments was 739 ± 128 for artificial soil and 489 ± 48 for dilution 0 (146 mg F2 + F3/kg) (Figure 2-3) which were not statistically different ($p = 0.116$). The differences between the number of juveniles obtained from the other concentrations were also not statistically different ($p = 0.073$; Figure 2-3) indicating that there was no relationship between juvenile production and increasing petroleum concentration ($R^2 = 0.00005$). Therefore, the highest concentration at ON1, 12,635 mg/kg, was determined to have no effect on *F. candida*.

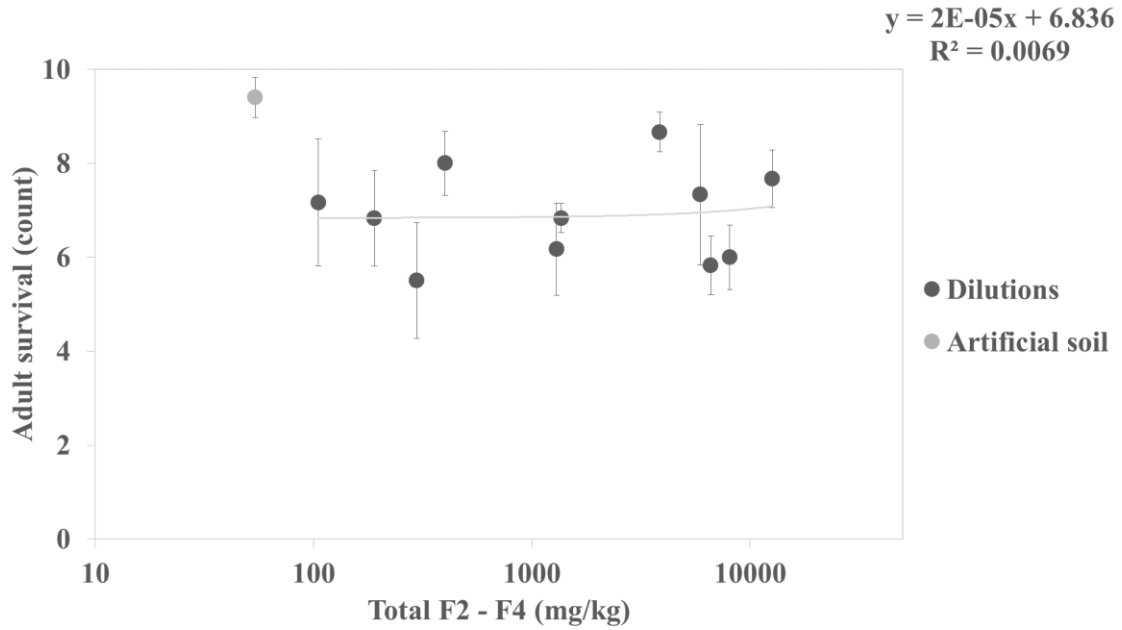


Figure 2-2: Average number of *Folsomia candida* adults extracted with floatation for each treatment of chronic toxicity tests with ON1, a reference control soil and an artificial control soil.

Adult survival counts were plotted relative to the total F2 plus F3 concentration of each dilution; standard error bars are shown; no statistical differences are noted (n= 6) for all points.

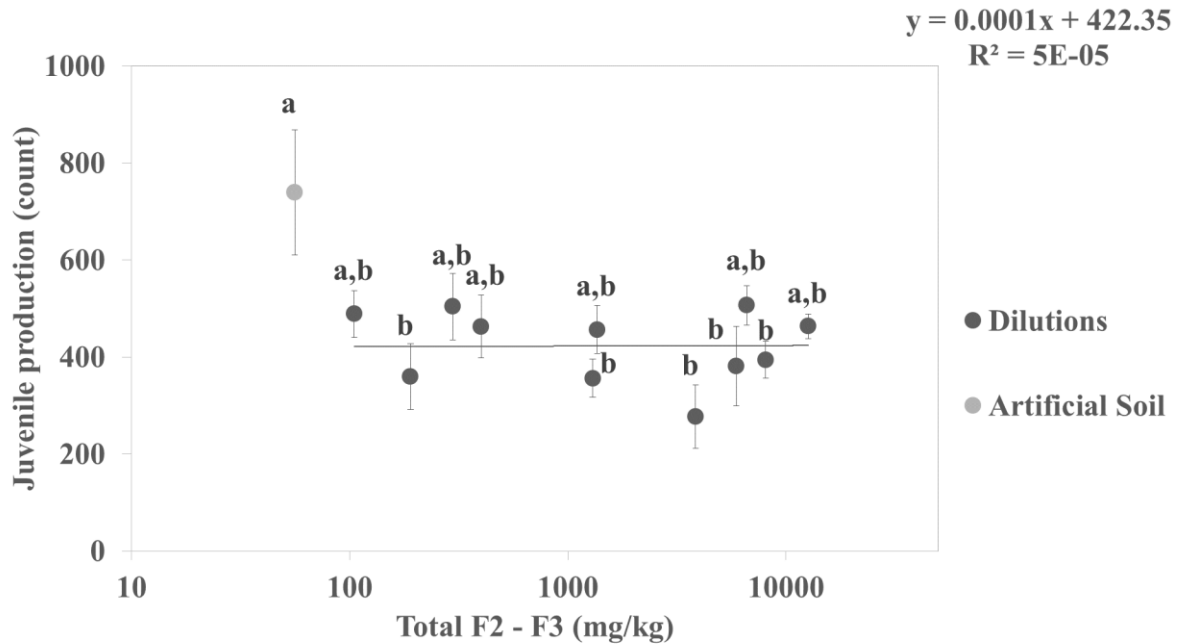


Figure 2-3: Average number of *Folsomia candida* juveniles extracted with floatation for each treatment of chronic toxicity tests with ON1, a reference control soil and an artificial control soil.

Juvenile production counts were plotted relative to the total F2 to F3 concentration of each dilution; standard error bars are shown; statistical differences are noted with a's and b's; n= 6 for artificial soil and dilution 8; n=5 for dilutions 0,3,4,5,7,10; n=4 for dilutions 1,2,6,9.

2.3.2.2 Results with Heat Extraction

As a comparison to the above data using floatation, toxicity tests were then performed with heat extraction. Table 2-2 summarizes the mean *F. candida* adult survival for Collembola extracted from chronic toxicity tests on ON1 with heat extraction and heat extraction plus floatation. Extraction efficiencies varied for each treatment and ranged from 49 to 97%. The initial room temperature was also noted to be 24°C when the heat extractions performed here were completed. (Note: as will be presented later in this thesis, the initial room temperature for heat extraction in Chapter 3 was 20°C and the extraction efficiency was 100%). Because of the low efficiency (see Table 2-2), the total adult survival of *F. candida* used in toxicity tests with ON1 were plotted in Figure 2-4. The site control, dilution 0 (146 mg/kg total F2 to F3), had an average adult survival of 5.33 ± 1.31 and there were no statistical differences between each point ($p \geq 0.0680$). The trend line (Figure 2-4) indicates a slight increase in toxicity with increasing petroleum concentration which indicates an LC₂₅ can be determined using the equation of the line. The LC₂₅ was estimated to be 35,960 mg/kg.

2.3.2.3 Comparison of Heat Extraction and Floatation Results from Chronic Toxicity Tests on ON1

The adult springtails from chronic toxicity tests on ON1 were removed from the soil using either heat extraction or floatation. The efficiency of the floatation method in comparison with the heat extraction method was determined (Table 2-2). The methods yielded comparable results with only two statistical differences: dilution 5 (1,362 mg F2 + F3/kg) and dilution 10 (12,635 mg F2 + F3/kg) ($p = 0.0364$ and $p = 0.0067$, respectively). The total adult counts obtained from each method were plotted in Figure 2-5 and trend lines were used to determine a probable LC₂₅. For heat extraction the LC₂₅ was determined to be 35,960 mg F2 + F3/kg. However, an LC₂₅ for the floatation method could not be determined using the formula for the trend line because the data are not monotonic. Therefore, 12,635 mg F2 + F3/kg was determined to have no effect on *F. candida*.

Table 2-2: Adult counts (n = 6) from chronic toxicity tests with heat extraction and with heat extraction and floatation

Sample	Experiment 1: Heat Extraction				Experiment 2: Floatation	
	PHC F2 to F3 (mg/kg)	Adults from heat extraction (#)	Total adults (heat extraction + floatation (#))	Average Extraction Efficiency (%)	Adults from floatation (#)	Efficiency of floatation vs total adults from heat extraction (%)
Artificial soil	54	9.17 ± 0.75	10.00 ± 0.73	91.65 ± 3.37	9.50 ± 0.43	95
Dilution 0 (control)	105	5.17 ± 1.25	5.33 ± 1.31	97.50 ± 5.59	7.17 ± 1.35	116
Dilution 1	190	4.83 ± 1.22	6.17 ± 0.87	72.20 ± 10.91	6.83 ± 1.01	111
Dilution 2	297	4.17 ± 0.61	5.83 ± 0.71	#81.07 ± 4.88	5.50 ± 1.23	94
Dilution 3	400	3.67 ± 0.80	5.50 ± 1.06	#69.92 ± 8.39	8.00 ± 0.68	145
Dilution 4	1299	4.67 ± 1.45	5.50 ± 1.50	82.22 ± 9.20	6.17 ± 0.98	112
Dilution 5	1362	3.67 ± 0.95	4.33 ± 0.99	83.67 ± 7.64	*6.83 ± 0.31	154
Dilution 6	3850	4.17 ± 0.83	6.67 ± 0.84	#59.49 ± 7.91	8.67 ± 0.42	130
Dilution 7	6610	2.50 ± 0.76	5.67 ± 1.36	#49.24 ± 13.65	5.83 ± 1.30	103
Dilution 8	5920	4.20 ± 0.87	5.60 ± 1.64	71.98 ± 10.70	7.33 ± 1.50	131
Dilution 9	8060	3.83 ± 0.48	6.33 ± 0.61	#62.65 ± 9.46	6.00 ± 0.68	95
Dilution 10	12635	2.83 ± 0.48	4.33 ± 0.76	#70.28 ± 7.82	*7.67 ± 0.61	117

Note:

* indicate a statistically significant increase in adults from heat extraction to floatation (p = 0.0364 and p = 0.0067, respectively)

indicate statistical differences in efficiencies from dilution 0

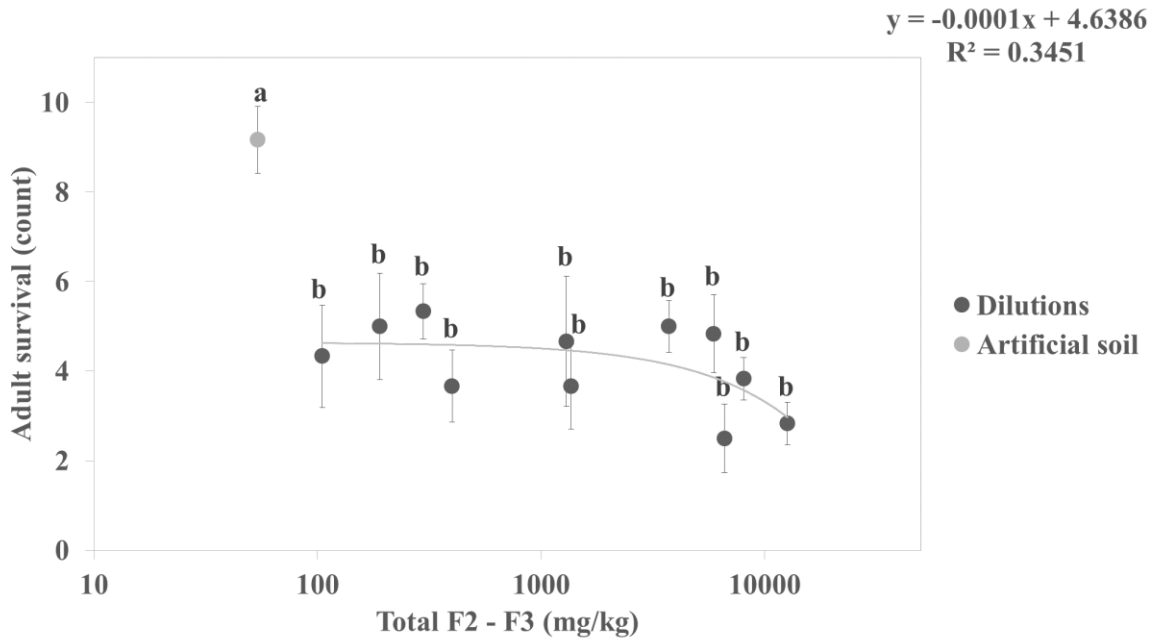


Figure 2-4: Average total number of *Folsomia candida* adults extracted with heat extraction for each treatment of chronic toxicity tests with ON1, a reference control soil and an artificial control soil.

Adult survival counts were plotted relative to the total F2 to F3 concentration of each dilution; standard error bars are shown; no statistical differences are noted; n= 6 for all points.

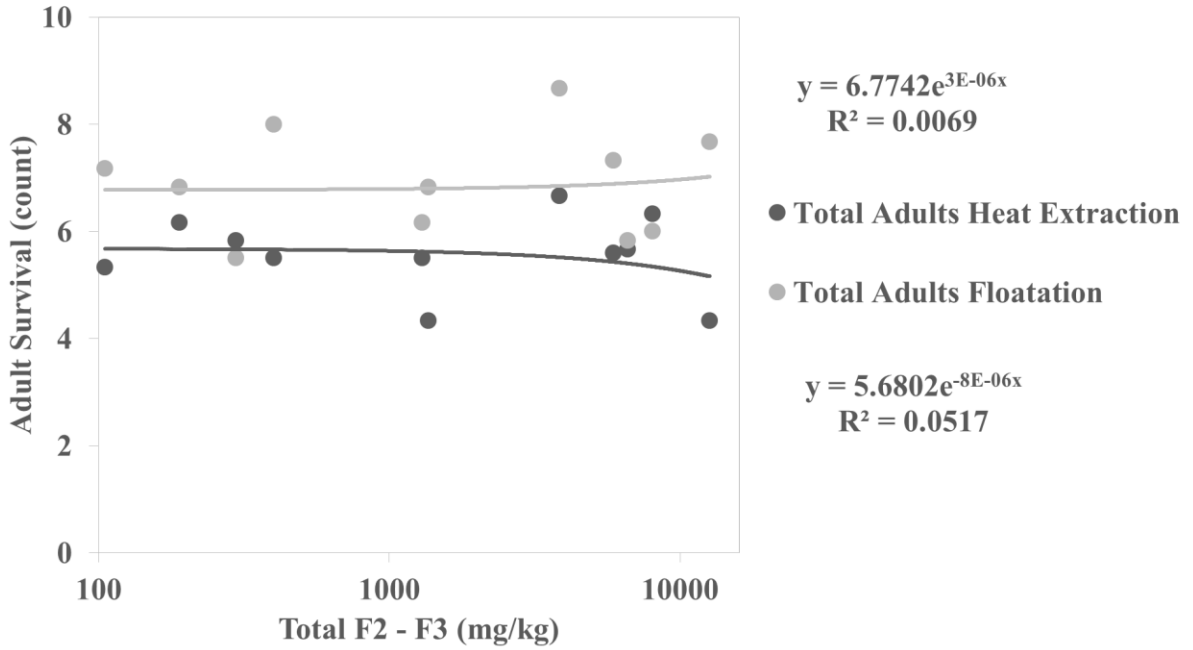


Figure 2-5: Average number of *Folsomia candida* juveniles extracted with floatation for each treatment of chronic toxicity tests with ON1, a reference control soil and an artificial control soil.

Juvenile counts were plotted relative to the total F2 to F3 concentration of each dilution; n= 6 for all points.

2.4 Discussion

A weathered PHC concentration study was performed to determine an LC₂₅ and IC₂₅ for comparison with the CCME criteria. When chronic toxicity tests with ON1 were completed, two methods of *Folsomia candida* extraction, heat extraction and floatation, were used as there were difficulties in maintaining initial room temperature during heat extraction. The findings indicate that the heat extraction and floatation methods of removal of *Folsomia candida* from soil are equivalent. Importantly, there was no effect of a PHC concentration of 12,635 mg F2 + F3/kg on *F. candida* adult survival or juvenile production. The PHCs in the ON1 soil are primarily F3 (F3:F2 ratio of greater than 9:1) indicating that weathered PHC-impacted field soil with a high F3:F2 ratio is not likely toxic to *F. candida*.

2.4.1 Heat extraction versus floatation methods

Heat extraction and floatation methods are used to remove organisms from soil at the end of chronic toxicity tests (Edwards and Fletcher, 1971; Environment Canada, 2007a). The floatation method is currently the most commonly used method to remove *Folsomia candida* from the soil (Environment Canada, 2007a). The efficiency of the heat extraction method was compared to the efficiency of the floatation method, by comparing the number of adults obtained, to determine the most appropriate method to retrieve *Folsomia candida* from the soil.

The adult survival counts obtained from the heat extraction are listed in Table 2-2. The heat extracted soil was then placed in a beaker and the floatation method was performed. The extraction efficiency for the reference control soil (dilution 0; 97.50 ± 5.59) and the artificial soil (91.65 ± 3.37) were not statistically different ($p = 0.212$). Six of the dilutions (2, 3, 6, 7, 9 and 10; Table 2-2) had statistically different efficiencies when compared with the reference control soil (dilution 0; Table 2-2). It is not known what factors influenced the extraction efficiency; however both soil properties, initial room temperature and room ventilation may have had an effect.

Based on the extraction efficiencies, the heat extraction alone was unable to remove all surviving *Folsomia candida* from soil after the chronic toxicity tests. The total surviving adults from both the heat extraction and the floatation extraction of the soil after heat extraction were added. The result was compared with the number of adults obtained from the floatation method

(Table 2-2). In almost all cases the number of adults obtained from the floatation method was higher than the total number of adults obtained from the heat extraction method; however, only two of these cases were statistically different. These results indicate that the floatation method is the best method of removing *F. candida* from soil after chronic toxicity tests; however, when total adults are considered, both methods provide equivalent results.

2.4.2 Concentration curve of weathered PHC-impacted soil

Weathering alters the composition of PHCs through volatilization, degradation and sorption to soil particles (Alexander, 1995; Gallego *et al.*, 2010; Maletic *et al.*, 2011; Osuji *et al.*, 2006). PHCs with carbon chain lengths from 6 to 14 will largely be lost to the air through volatilization (Pichtel and Liskanen, 2001). PHCs with carbon chain lengths from 6 to 32 can be degraded by microorganisms (Beilen and Funhoff, 2007). PHC compounds with carbon chain lengths greater than 32 can diffuse into pore spaces within soil particles (Brusseau, 1997; Maletic *et al.*, 2011; Yong and Rao, 1991). All of these weathering processes are able to render PHCs less biologically available and therefore, less toxic (Maletic *et al.*, 2011).

ON1 soil was obtained from a decommissioned land-farm that had been weathered for approximately 20 years. Maletic *et al.* (2011) determined that weathering soil impacted with diesel and crude oil for 9 years resulted in a NOEC of 35,000 mg/kg total PHC. Since PHC-impacted soils at ON1 had weathered for more than twice as long as those of the Maletic *et al.* (2011) site and the highest concentration was only 12,636 mg/kg, it was suspected that ON1 soils would show no toxicity. The highest concentration of soil obtained from ON1, 12,635 mg F2 + F3/kg, had no effect on *F. candida* which agrees with expected results. The weathered PHC-impacted field soil obtained from ON1 had a high F3:F2 ratio indicating that the CCME guidelines for F3 can be raised from 1,300 mg/kg to 12,000 mg/kg without having an effect on *F. candida*. However, F2 has been shown to be more toxic than F3 (ESG, 2003) so it may be possible that the high concentration of F3 may be moderating the toxicity of F2.

2.5 Conclusions

It was predicted that the heat extraction would be the best method of removing *Folsomia candida* from the soil. Results indicate that, when total adults are accounted for, both heat extraction and the floatation method provide equivalent results. Even though there are several advantages to

using heat extraction, such as obtaining only live organisms, the labor intensive protocol and the number of factors that may affect the extraction (i.e. temperature and soil properties) suggest that heat extraction is likely not the most practical method of extraction.

The main hypothesis for this research was that the toxicity of weathered PHC-impacted field soils will occur at higher concentrations than the CCME guidelines. Because there was no effect of weathered PHC-impacted field soil from ON1, the CCME guidelines are not appropriate to apply to weathered PHC-impacted soil. If the CCME guidelines are to be used as remedial benchmarks for weathered PHC-impacted soil, they will need to be raised to accommodate the significant decrease in the toxicity of PHCs that occurs through weathering.

Recommendations and Further Research

Based on this research, it is recommended that the floatation method be used to remove *Folsomia candida* from soil after chronic toxicity tests. It is also recommended that if CCME guidelines are to be used as remediation criteria, the values should be raised to account for the decrease in toxicity resulting from weathering on PHCs.

There was no effect of the weathered PHC-impacted field soil obtained from ON1 which indicates that the F2 criteria can be raised from 150 mg/kg to 635 mg/kg and the F3 criteria can be raised from 1,300 mg/kg to 12,000 mg/kg without having an effect on *Folsomia candida*. These results indicate that a significant increase in the CCME guidelines may be warranted; however, they are based on the use of a single PHC-impacted field site with a high F3:F2 ratio. More PHC-impacted field sites need to be investigated to determine an appropriate increase in CCME guidelines that can be applied to these sites.

Chapter 3 Toxicity of Weathered Petroleum-Impacted Site Soil to *F. candida* and an Investigation of New Endpoints and Analyses for Collembola

3.1 Introduction

Approximately 30% of the mass of petroleum is made up of compounds containing nitrogen, oxygen, sulfur and metals, while the other 70% is made up of organic molecules called petroleum hydrocarbons (PHCs; CCME, 2001a; CCME, 2008a; CCME, 2008b; CCME, 2008d; Goodger, 1975; Petrov, 1987; Long, 1998). There are more than 700 types of PHC molecules, each of which contain only carbon and hydrogen (CCME, 2001a; CCME, 2008a; CCME, 2008b; CCME, 2008d; Goodger, 1975; Petrov, 1987; Long, 1998). PHCs are the primary source for products such as fuels, plastics and clothing; increasing demand for these products has led to increased drilling operations (Bjorlykke, 2010; Fingas, 2011). These increased drilling operations and decreasing oil resources has led to the development of safe and efficient methods of drilling through the use of drilling fluids and muds (Bjorlykke, 2010). Drilling muds suspend and remove rock cuttings from the head of the drill, cool the drill bit and act as a counterweight if a high pressure pocket is encountered during drilling (Bjorlykke, 2010). One type of drilling mud is diesel invert; a water-in-oil emulsion composed of diesel oil #2, a salt solution, an emulsifier and a weighting agent (Bennett, 1984; Boyed *et al.*, 1985; Jacques *et al.*, 1992; Macyk, 2005). Diesel oil #2 is considered the most toxic part of diesel invert as it contains 30-60% aromatics, more than 30 ppm benzene and PHCs (Boyed *et al.*, 1985; Jacques *et al.*, 1992).

More than 100,000 sites across Canada are contaminated with petroleum hydrocarbons (CCME, 2001a; CCME, 2008b; CCME, 2008c) many of which contain diesel invert and drilling mud waste. Inconsistencies in management and remediation of these sites led the Canadian Council of Ministers of the Environment (CCME) along with the Analytical Methods Technical Advisory Group (AMTAG) to develop a standardized protocol called the Canada-Wide Standards for PHC in Soil (PHC CWS; CCME, 2001a; CCME, 2008a; CCME, 2008b; CCME, 2008c). This standard was based in part on work performed by the US Total Petroleum Hydrocarbons Criteria Working Group (TPHCWG) with consideration of scientific, technical and socio-economic factors along with input from stakeholders (CCME, 2008b; CCME, 2008d). The TPHCWG subdivided PHCs into four fractions based on their physical and chemical

properties as well as their equivalent carbon numbers: F1 (C6-C10), F2 (C10-C16), F3 (C16-C34) and F4 (C34-C50⁺). The PHC CWS document outlines the generic Tier 1 screening guidelines for each fraction that can be applied to all sites (Table 1; CCME, 2008b). The CCME considers these guidelines to be attainable, but conservative enough to provide adequate human and ecological protection at most sites; however, the CCME also admitted that there were issues with the derivation of these guidelines (CCME, 2001a; CCME, 2008b; CCME 2008d). One of these issues was using fresh (i.e. un-weathered) oil to develop the guideline values without considering how weathering alters PHC toxicity.

Weathering of PHCs is the result of three main natural processes in soil: volatilization, biodegradation and sorption (Alexander, 1995; Gallego *et al.*, 2010; Maletic *et al.*, 2011; Osuji *et al.*, 2006). Volatilization is the loss of low molecular weight hydrocarbons to the air (Gallego *et al.*, 2010; Maletic *et al.*, 2011). Biodegradation involves the oxidation of mid-range molecular weight hydrocarbons by indigenous microorganisms (Maletic *et al.*, 2011; Osuji *et al.*, 2006). Sorption is the binding of PHCs with soil particles or organic matter within the soil matrix (Brusseau, 1997; Maletic *et al.*, 2011; Yong and Rao, 1991). Each of these weathering processes results in a decrease in the availability of the low to mid-range PHC and a subsequent compositional change (i.e. proportional increase in the higher molecular weight hydrocarbons) and a lowering of toxicity (Alexander, 1995; Maletic *et al.*, 2011).

Folsomia candida, a common invertebrate species used in soil toxicity testing (Fountain and Hopkin, 2005), have three features that make them ideal for toxicity testing: their cuticle, ventral tube and parthenogenic nature (i.e. asexual reproduction). Microscopically, the cuticle has raised ridges that form a hexagonal pattern with triangular projections at the corners (Eisenbeis and Wichard, 1987; Hopkin, 1997). This structure along with the presence of a waxy layer allows air to become trapped around their body, creating a hydrophobic outer surface (Hopkin, 1997; Ghiradella and Radigan, 1974). The ventral tube is where the majority of environmental exchanges, largely water uptake, occur (Drummond, 1953; Eisenbeis and Wichard, 1987; Hopkin, 1997; Schreiber and Eisenbeis, 1985). The end of the ventral tube contains hair-like sensory organs which allow *F. candida* to sense the moisture content, salinity and pH of the substrate (Eisenbeis and Witchard, 1987; Hopkin, 1997). *F. candida* are likely parthenogenic because of the presence of an alpha-proteobacteria in the ovaries; this bacteria results in male killing which results in an all-female population (Fрати *et al.*, 2004; Marshall and

Kevan, 1967; Pike and Kingcombe, 2009; Rousset *et al.*, 1992; Vandekerckhove *et al.*, 1999; Werren *et al.*, 1995).

Previous research suggests that petroleum may be either directly toxic to *Folsomia candida* or indirectly toxic through the alteration of soil properties (Adebiyi and Afedia, 2011; Arocena and Rutherford, 2005; Erlacher *et al.*, 2013; Fingas, 2011; George *et al.*, 2011; Izdebska-Mucha, 2008; Kisc *et al.*, 2009; Labud *et al.*, 2007; Martinho *et al.*, 2010; Onwurah *et al.*, 2007; Wang *et al.*, 2010). *F. candida* have been shown to avoid soils with a high bulk density and an organic matter content of less than 2% (Domene *et al.*, 2011; Natal-da-Luz *et al.*, 2008). Their survival and reproduction are negatively affected by a humidity level lower than 99.4%, soil acidity (pH < 6.83), extreme deviations from their optimum temperature (22°C) and an electrical conductivity (EC) of more than 1.03 dS/m (Bayley and Holmstrup, 1999; Kaneda and Kaneo, 2002; Marshall and Kevan, 1967; Owojori *et al.*, 2009; Waagner *et al.*, 2011). The presence of PHCs in soil have been shown to increase the bulk density, pH and EC while lowering the organic matter when compared with un-impacted controls (Adebiyi and Afedia, 2011; Arocena and Rutherford, 2005; Izdebska-Mucha, 2008; Kisc *et al.*, 2009; Labud *et al.*, 2007; Martinho *et al.*, 2010; Osuji and Nwoye, 2007; Urum *et al.*, 2004; Wang *et al.*, 2010).

The first application of toxicity test protocols involving survival and reproduction were as part of a toxicity test battery, using several toxicity tests, to determine the toxicity of weathered mineral oil (van Gestel *et al.*, 2011). Once the method involving survival and reproduction was shown to be useful as part of an ecological risk assessment test battery, Environment Canada decided to update the protocol (Environment Canada, 2007a). The heat extraction method is one of the methods recommended by Environment Canada to remove *F. candida* from the soil at the end of the chronic toxicity test (Environment Canada, 2007a). The heat extraction method uses the application of heat to the soil, encouraging the organisms to migrate away from the heat source and out of the soil at which point the organisms can be collected in a vessel for quantification (Edwards and Fletcher, 1971). In the 1970s, this extraction method was the preferred method for springtail quantification after chronic toxicity tests while current methods emphasize the floatation method (Edwards and Fletcher, 1971; Environment Canada, 2007a). The floatation method uses water to separate the soil from the hydrophobic *F. candida* which float to the surface of the container permitting quantification (Environment Canada, 2007a).

Chronic toxicity tests using other organisms have focused on measuring sub-lethal endpoints such as weight and length (Environment Canada, 2005a; Environment Canada, 2007b; Folker-Hansen *et al.*, 1996; Petersen *et al.*, 1997). Weight has been used for toxicity testing with cadmium using *F. candida*, but this endpoint has not been applied to toxicity tests with PHC-impacted soil (Crommentuijn *et al.*, 1993). It is postulated that by including weight and body length data along with the adult survival and juvenile production endpoints of the chronic toxicity tests, more detailed information on how petroleum affects soil invertebrates can be obtained (Folker-Hansen *et al.*, 1996).

Acute toxicity tests use shorter exposure times and measure either lethal effects on survival or sub-lethal effects like avoidance behaviour (Environment Canada, 2007a). Avoidance-response tests have been used extensively with earthworms and have been shown to have the same trends as the results obtained in the longer chronic reproduction tests (Environment Canada, 2007a). Heupal (2002) used avoidance tests with *Collembola* to test the toxicity of pesticides. By 2004, avoidance tests with *Collembola* had been shown to have potential as a pre-screening test of PHC-impacted soils to determine if chronic toxicity tests were necessary (Natal-da-Luz *et al.*, 2008). An acute avoidance-response test would be used to determine the lowest PHC concentration at which *F. candida* will avoid in the weathered PHC-impacted soils. This avoidance concentration indicates the PHC concentration where reproductive and mortality effects are likely to begin (Natal-da-Luz *et al.*, 2008). If the measured PHC concentration higher than the CCME guidelines, then chronic toxicity tests would not need to be performed (Natal-da-Luz *et al.*, 2008).

The properties of soil can be altered by the presence of petroleum and, by consequence, there might be measurable effects on *F. candida* (Adebiyi and Afedia, 2011; van Gestel *et al.*, 2011). However, weathering processes have been shown to decrease the concentration of the lighter, more toxic PHC petroleum fractions which has been shown to decrease toxicity (Alexander, 1995; Maletic *et al.*, 2011). The CCME did not consider weathering in their derivation of the Tier 1 guidelines, an omission that was pointed out by the CCME itself (CCME, 2008a). The hypothesis for this research was that the toxicity of weathered PHC-impacted field soils will occur at higher concentrations than the CCME guidelines. The three objectives used to test this hypothesis were to (1) use chronic toxicity tests with *Folsomia candida* to determine the approximate LC₂₅ and IC₂₅ of weathered-PHC soil at two different field sites and compare the

results to the CCME Tier 1 guidelines, (2) determine if the sub-lethal endpoints (weight, length and/or width) will be a suitable addition to the chronic toxicity test protocol as these endpoints may be more sensitive and better able to detect effects and (3) investigate the feasibility of using avoidance-response tests as a preliminary assessment method for toxicity screening of weathered petroleum-impacted sites.

3.2 Materials and Methods

3.2.1 *Folsomia candida* Cultures, Maintenance, and Preparation for Experiments

Cultures of *Folsomia candida* were obtained from Stantec Consulting Ltd. (Guelph, ON) and were placed on a Plaster of Paris and charcoal medium. The medium was prepared using 480 g DAP Plaster of Paris[®], 60 g activated charcoal (Catalog number C9157-500G, Sigma-Aldrich, Oakville, ON) and 500 mL of deionized water. Cultures were watered every three days with deionized water, fed with Fleischmann's[®] Traditional Activated Dry Yeast as required and sealed with Parafilm M[®] (Beemis, Neenah, WI) to maintain moisture (Environment Canada, 2007a). Cultures were maintained in a growth chamber at 20 ± 2 °C under cool white fluorescent lights at an intensity of 6.5-7.5 $\mu\text{moles}/(\text{m}^2 \cdot \text{s})$, a photoperiod of 12 hours and a relative humidity above 30% (Environment Canada, 2007a).

Synchronization of *F. candida* cultures was performed to generate even-aged juveniles for use in toxicity tests. Glass microscope slides were coated in the Plaster of Paris[®] and charcoal medium (described above) and allowed to dry (adapted from Environment Canada, 2007a by Wan Lee, May 23, 2011). A moist paintbrush was used to collect and remove eggs from stock *F. candida* cultures which were then placed onto the coated microscope slides (adapted from Environment Canada, 2007a by Wan Lee, May 23, 2011). These slides were then placed into Petri-plates which had been coated with the Plaster of Paris and charcoal medium and moistened to saturation using deionized water (Environment Canada, 2007a). Approximately 15 yeast pellets were placed on opposite sides of each microscope slide and eggs were allowed to hatch for 48 hours in the growth chamber. The slides along with any unhatched eggs were removed and all of the springtails that had hatched were allowed to develop for 10 days or 16-24 days for use in either chronic toxicity tests or avoidance tests, respectively (Environment Canada, 2007b).

3.2.2 Soils for Chronic Toxicity and Avoidance Tests

Soil samples were collected from two sites which had been previously been impacted with diesel invert. The PHC-impacted soil was excavated from the site of the petroleum spill and spread on a treatment pad for remediation. One of the sites was in Southwestern Alberta (AB1) which had been left to weather for six years and had undergone phytoremediation for two years at the point of soil collection. The other site was in Northeastern British Columbia (BC1) which had been

weathered for 10 years and had undergone phytoremediation for 3 years at the time of soil collection (Table 3-1).

Soil was obtained at each site from four points: an un-impacted control soil (C) as well as points which had previously been determined to have a high (H), a medium (M) and a low (L) total petroleum content (Table 3-1). The un-impacted control soil for BC1 was collected from plots designated as remediation controls while the control soil for AB1 was a subsurface soil sample collected from off of the treatment pad (Table 3-1).

3.2.3 Initial Soil Preparation

3.2.3.1 Site Soils

Site soils were sieved through a 6-mm mesh screen to remove rocks, roots, and other large debris (Environment Canada, 2007a). Soils that were too moist to sieve immediately were air dried overnight in a fume hood. The sieved soil was then thoroughly homogenized, placed into storage containers and stored at room temperature until required.

3.2.3.2 Artificial Soil

An artificial control soil, which was used for the chronic toxicity tests, was prepared by thoroughly mixing 10% air dried *Sphagnum* sp. peat moss (Belgian Nursery, Breslau, ON) that had been sieved through a 2-mm mesh screen, 20% air dried kaolin clay (Dundee Pottery, New Dundee, ON) which had a particle size of <40 µm and 70% grade 70 air dried silica sand by mass (Barco 71, Opta Minerals, Waterdown, ON; Environment Canada, 2007a). The pH of the artificial soil was adjusted to 6.5-7.5 by adding 70 g of CaCO₃ (catalogue number C5929-500gG, Sigma-Aldrich, Oakville, ON; Environment Canada, 2007a). 1.7 L of Milli Q water were added to prepare an artificial soil with 20% moisture (see section 3.2.5.1). The soil was then left at room temperature for three days to allow for pH equilibration (Environment Canada, 2007a). The actual pH and moisture content were determined as described in section 3.2.4.1 and the soil was stored at 4°C until required.

Table 3-1: Soil properties for site AB1 and BC1 analyzed by SGS laboratories (Guelph, ON); petroleum content (mg/kg) analyzed by Maxxam Analytics (Waterloo, ON); and in-house testing of WHC, moisture content, ideal moisture content, EC_e and water repellancy, of the low (L), medium (M) and high (H) PHC-impacted site points as well as the un-impacted site specific reference control soil (C).

		Soil properties and PHC content															
Site	Site points	pH	Organic matter (%)	Total Nitrogen (%)	Total Phosphorous (ppm)	Organic carbon (%)	% Sand	% Silt	% Clay	Cation Exchange Capacity	F2 (mg/kg)	F3 (mg/kg)	F4 (mg/kg)	WHC	Initial Moisture Content (%)	Ideal Moisture Content (%WHC)	EC _e (dS/m)
AB1	C	6.58	1.10	0.01	156	0.60	55	32	13	9.99	12	38	15	46.32	12.0	35	0.079
	L	7.74	5.10	0.07	413	2.80	52	29	19	21.56	150	1200	42	59.22	20.0	41	0.700
	M	7.64	13.50	0.11	505	7.50	51	31	18	23.27	340	2600	84	75.29	31.0	48	1.182
	H	7.69	7.90	0.10	458	4.40	52	31	17	21.33	610	2900	88	75.18	28.0	43	1.270
BC1	C	7.69	2.50	0.10	393	1.40	29	45	26	17.03	<10	<10	<10	56.91	15.0	40	1.064
	L	7.90	2.20	0.05	486	1.20	27	46	27	15.13	80	580	49	53.56	6.0	37	0.620
	M	7.81	2.90	0.07	492	1.60	33	49	18	15.11	3500	3200	110	48.07	4.3	45	1.022
	H	7.80	3.20	0.07	486	1.80	29	48	23	14.39	5200	4800	120	50.35	6.2	41	0.780
Artificial soil		7.46	4.00	0.05	55	2.20	76	8	16	8.73	<10	27	17	64.4	20.0	70	0.700

3.2.4 Soil Properties required for use in Toxicity Tests

The pH, water holding capacity (WHC), percent moisture content and ideal moisture content of all soils were measured prior to their use in toxicity testing (Environment Canada, 2007a; Table 3-1). The electrical conductivity (EC) was also measured (Table 3-1).

3.2.4.1 pH

The pH of the soils was tested as follows: 25 g of soil at the ideal moisture content was placed in a beaker and 50 mL of water was added and thoroughly mixed (adapted from Environment Canada, 2007a by Emma Shrive Stantec Consulting Ltd., May 12, 2011). The mixture was allowed to settle for 30 minutes and the pH of the solution was measured (adapted from Environment Canada, 2007a by Emma Shrive Stantec Consulting Ltd., May 12, 2011) using a pH meter (Fischer Scientific, Ottawa, ON). The pH of the Plaster of Paris[®] was measured without allowing it to settle and using pH paper (Micro Essentials Laboratory, New York, NY) to avoid damage to the pH meter probe.

3.2.4.2 Water Holding Capacity

To obtain the WHC approximately 130 g of soil were dried in an oven at 105°C. Filter paper 18.5 cm in diameter with a coarse pore size (catalogue number 09-790-12G, Fisher Scientific, Ottawa, ON) was placed into a glass funnel with a 10 cm diameter (Environment Canada, 2007a). The filter paper was primed with approximately 10 mL of deionized water. The funnel and moistened filter paper were then weighed using a top-loading balance with 0.01 gram sensitivity (OHAUS TS400, M&L Testing Equipment (1995) Inc., Dundas, ON). The funnel was placed into a 500-mL Pyrex Erlenmeyer flask to support the funnel and collect the water that flowed through. A slurry of 100 g of the dried soil and 100 mL of deionized water was prepared and added to the funnel (Environment Canada, 2007a). Deionized water (10 ml) was used to rinse the vessel that contained the soil slurry. The funnel was then covered with aluminum foil to prevent evaporation (Environment Canada, 2007a). After three hours the aluminum foil was removed and the funnel containing the filter paper and wet soil was weighed (Environment Canada, 2007a). The WHC was calculated using the following equation:

$$WHC = \frac{(F) - (I + \textit{weight of dry soil})}{\textit{weight of dry soil}} \times 100\% \quad \text{Equation 7}$$

Where,

F = weight of funnel + wet filter paper + wet soil

I = weight of funnel + wet filter paper

3.2.4.3 Moisture Content

Briefly, the percent moisture was determined by weighing a soil sample before and after drying at 105°C in an oven for 24 hours. The percent moistures were then calculated using Equation 8:

$$\frac{\textit{weight of wet soil} - \textit{weight of dry soil}}{\textit{weight of dry soil}} \times 100\% \quad \text{Equation 8}$$

To determine the ideal moisture content for each field soil, water was added to the oven dried soil until soil clumps became 3-5 mm in diameter (Environment Canada, 2007a). The percent moisture was calculated as above and then expressed as a percentage of the WHC on a dry weight basis.

3.2.4.4 Electrical Conductivity (EC_e)

The EC_e of soil can be obtained by preparing a soil slurry from 1 part soil to two parts water (EC_{1:2}) and multiplying by 2 (Chang, 2008). The EC_{1:2} was determined by drying approximately 50 g of soil at room temperature for at least 72 hours (adapted from Chang, 2008). The dried soil was then passed through a 2-mm sieve, and 15 g were added to a 50-mL falcon tube (catalog number CA21008-940, VWR International, Mississauga, ON) with 30 mL of deionized water (adapted from Chang, 2008). The falcon tube was then shaken for 30 minutes at 120 rpm and the soil was allowed to settle overnight (adapted from Chang,

2008). The supernatant was then removed and the EC_{1:2} was measured using a conductivity meter (CON 510, Oakton Instruments, Vernon Hills, IL). The values obtained were multiplied by two to give the EC_e in dS/m (adapted from Chang, 2008).

3.2.5 Toxicity Tests

3.2.5.1 Soil Preparation

The chronic toxicity and the avoidance tests, as described below, were performed using the soils outlined in Section 3.2.3. The soil was prepared prior to testing by increasing the soils moisture content to the ideal moisture content (see Table 3-1 for ideal moisture contents). Equations 9, 10, and 11 were used to determine the amount of water to add to each soil:

$$D_W = W_W - (W_W \times MC)$$

Equation 9

Where,

D_W = the dry weight of the soil

W_W = the wet weight of the soil required for the test

MC = the desired soil moisture content

$$P_W = \left[WHC \times \left(\frac{P_{WHC}}{100} \right) \right] - MC$$

Equation 10

Where,

P_W = the percentage of water to add to the soil

WHC = the water holding capacity

P_{WHC} = the ideal moisture content

MC = the initial moisture content of the soil

$$V_W = (P_W \times M)/100$$

Equation 11

Where,

V_W = volume of water to add to the soil (mL)

P_W = the percentage of water to add to the soil

M = the total dry weight of soil required for the test

The calculated amount of deionized water required to obtain the ideal soil moisture was added to the soil and thoroughly mixed in. The soil was then allowed to sit for approximately 20 minutes to ensure all water was absorbed.

3.2.5.2 Chronic Toxicity Tests

The juvenile *F. candida* and site soil were prepared according to Section 3.2.1 and 3.2.5.1. Thirty grams of each soil were added to a minimum of three 125-mL clear glass jars with opaque lids (Maxxam Analytics, Waterloo, ON). The total weight of each jar and soil was measured and recorded. The soil in the jars was then gently compressed to a consistent density and the soil surface was made even (Environment Canada, 2007a). The jars were then sealed and placed into the growth chamber at $20 \pm 2^\circ\text{C}$ overnight (Environment Canada, 2007a). The jars containing the test soil were then removed from the chamber and 10 juveniles were added along with eight yeast pellets (approximately 8 mg) to each jar (Environment Canada, 2007a). The jars were resealed and placed back into the growth chamber at $20 \pm 2^\circ\text{C}$ for 28 days (Environment Canada, 2007a).

Once a week for the duration of the test, the test jars were aerated and watered as follows. Six jars were randomly selected, weighed and deionized water was added until the initial total jar weight (the previously recorded initial weight of the jar and soil at its optimum water content) was reached (Environment Canada, 2007a). The amount of water required, was recorded for each jar and an average was obtained (Environment Canada, 2007a). This average amount of water was then added to all remaining jars in that test (Environment

Canada, 2007a). On weeks 2 and 3 the yeast was checked to determine if there was enough to last until the next week. If the yeast had been largely eaten in any container, extra yeast (between 4 and 8 mg) was added to all containers in the test. After 28 days all of the jars were removed from the chamber and the number of surviving springtails was measured using the heat extraction method (Environment Canada, 2007a).

3.2.5.3 Heat Extraction

In preparation for heat extraction, the heat extraction units were prepared using two 4.5 ounce polypropylene cups (catalogue number 14955103, Fisher Scientific, Ottawa, ON). The bottom of one cup was removed and a 7-mesh round PVC needlework canvas was cut and glued, with non-toxic glue, into the lip groove of the cup (Environment Canada, 2012). The other cup was filled with approximately one centimeter of the Plaster of Paris/charcoal media (Environment Canada, 2012).

The Plaster/charcoal mixture in the cup was moistened to saturation with deionized water. The cup containing the needlework canvas was turned upside down and a double layer of cheesecloth was placed over the needlework canvas (Environment Canada, 2012). The cup with the needlework canvas was then placed on top of the cup containing the Plaster of Paris® and they were joined together with Parafilm-M® (Environment Canada, 2012).

Once the toxicity test was finished the test jars were removed from the growth chamber and the soil from each jar was placed into an extraction unit. The test jars were filled with water and any remaining live springtails present were counted and the total number was recorded to add to the number obtained from the heat extraction (Environment Canada, 2012). The extraction units were then placed under lamps with 60 watt light bulbs set 30 cm away from the soil surface (Environment Canada, 2012). There were four extraction units per lamp and the soil in each test unit was watered every hour for the first 36 hours of the extraction. After 24 hours, the lamps were lowered to 15 cm above the soil surface (Environment Canada, 2012). When the soil temperature had reached 36°C (after approximately 48 hours) the lights were turned off and the soil from the extraction unit was placed into a beaker (Environment Canada, 2012). Water was added to the beaker and any

live springtails present were counted to obtain an extraction efficiency value (Environment Canada, 2012). A small ruler was added to the bottom of the cup with the plaster and springtails and several photographs of each extraction vessel were taken using a 6.1 Megapixel Nikon D50 with an 18-55 mm lens (Henry's, Waterloo, ON) so that length and width measurements could be made. Several adult springtails were then removed from the cup, placed into vials and cold killed at -20°C for 24 hours to determine their weight. The vials were then kept in the freezer until weight analysis could be performed. Water was then added to the bottom of the cup containing the plaster and springtails and decanted into a second container. More water was added to cover the bottom of the second container along with several drops of dye (Color Coat Red, Becker Underwood, Ames, IA) for easier visualization of the springtails. The number of adults and juveniles were counted and recorded. This process was repeated until no springtails were present in the cup.

3.2.5.4 Avoidance-response Tests

The avoidance-response test was developed based on the work of Liu *et. al* (2010) in combination with the Environment Canada biological test methods (Environment Canada, 2007a). The purpose of preparing this method was to create a standardized protocol for use as an initial site assessment tool to determine if petroleum toxicity is likely to occur on a PHC-impacted soil site.

After the *Folsomia candida* juveniles and soil were prepared according to section 2.2.1 and 2.2.5.1, the avoidance test units were set up. An 8.9 cm diameter cylindrical plastic test vessel (catalogue numbers 16-89-NPPC and 89WRM-UCp, taral[®] plastics, Union City, CA) was divided into two sections (right and left) using a physical barrier that had been modified to fit into the container (Liu *et al.*, 2010). Half of the container was filled with 30 g of PHC-impacted test soil while the other half was filled with 30 g of the un-impacted control soil. The soil on each side was then gently compressed to a consistent density and the soil surface was leveled. The dividers were then removed and the test vessels were lightly tapped until the two soil types made physical contact with each other (Liu *et al.*, 2010). The test

vessels were then allowed to acclimate in the growth chamber for 24 hours at 20 ± 2 °C (Environment Canada, 2007a; Liu *et al.*, 2010).

An age synchronization was performed to decrease the possibility of behavioral variability with age and to obtain larger specimens. Immediately following the age synchronization, twenty 16 - 24 day old adults were placed along the midline of each test vessel (where the divider had been). The test vessel was then returned to the growth chamber at 20 ± 2 °C for 48 hours (Liu *et al.*, 2010).

At the completion of the test, the divider was reinserted along the midline and the soil on each side was removed and placed into separate beakers (Liu *et al.*, 2010). The floatation method was used to quantify the *F. candida* in each test vessel by adding 50 mL of deionized water to each beaker containing the test soils; the mixture was then stirred thoroughly (Environment Canada, 2007a). The soil was allowed to settle and an initial count of the floating springtails was performed (Environment Canada, 2007a). Soil was repeatedly stirred, allowed to settle and counted until the number of springtails recovered was consistent with the previous count.

3.2.6 Data Analysis

3.2.6.1 Photographic Analysis of Length and Width

Photographs taken at the end of the chronic tests were used to analyze springtail length and width. Each photograph was uploaded to a computer, enlarged to 200% and the number of pixels/mm of the ruler in the photograph was measured digitally. The number of pixels in the length and width of 5 randomly selected uncurled springtails (or every springtail that could be counted if the total number of adults recovered was less than 5) was then determined. The number of pixels obtained for the length and width was then converted to millimeters. The average length and width per springtail in each replicate was then calculated.

3.2.6.2 Weight Analysis

The springtail samples that were cold killed at -20°C for a minimum of 24 hours were kept at -20°C until weight analysis could be performed. Prior to weighing, frozen test vials were

thawed to room temperature. The vials were then placed in a 60°C oven. After 48 hours the vials were removed from the oven and placed in a desiccator. Vials were removed one at a time and the springtails were placed into 5 x 3.5 mm tin capsules (catalogue number D1002, Isomass Scientific, Calgary, AB). The tin capsule with the springtails was then weighed on a CAHN C-31 Microbalance ($\pm 0.1 \mu\text{g}$, CAHN Instruments Inc., Cerritos, CA) and the total weight plus the number of springtails weighed were recorded for each replicate. The weight per springtail was then calculated for each replicate and was averaged with the other replicate of each treatment to obtain the average weight per springtail for each treatment.

3.2.6.3 Statistical Analysis

Statistical differences were determined using the Student's t-test with a two tailed distribution and unequal variance (type 3). Quantal adult data were analyzed according to the Environment Canada statistical recommendations (Environment Canada, 2005b). Logit analysis was performed in R (www.r-project.org) and the concentration required to kill 50% and 25% (LC_{50} and LC_{25}) of the *Folsomia candida* adults were determined. Juvenile data were analyzed for the concentration required to inhibit juvenile production by 50% and 25% (IC_{50} and IC_{25}) in SYSTAT according to the Environment Canada biological test method (Environment Canada, 2007b). Weight, length, and width data were modeled in SYSTAT and an effective concentration to decrease each endpoint to 50% and 25% (EC_{50} and EC_{25}) was determined.

3.3 Results

The soils obtained from AB1 and BC1 for use in the toxicity tests were initially tested for PHC F2, F3 and F4. F4 is the least toxic of the fractions tested (ESG, 2003) and the F4 concentration did not exceed 120 mg/kg. Since the F4 concentration was unlikely to have an effect on *F. candida* it was excluded from the analysis. Both the F2 and F3 concentrations exceeded the criteria (150 mg F2/kg and 1,300 mg F3/kg) in most samples. Therefore, both F2 and F3 were included the data analysis.

3.3.1 Chronic Toxicity Tests on AB1 and BC1

Chronic toxicity tests were used to determine the LC₂₅ and the IC₂₅ for comparison to the CCME Tier 1 soil guidelines. Artificial soil was used as a control to determine the response of *Folsomia candida* under standard conditions and to determine if the physico-chemical characteristics of the reference soils influenced *F. candida* performance. The survival and reproduction results were plotted relative to the total F2 to F3 and F2 petroleum content.

3.3.1.1 Adult Survival

Adult survival for the AB1 tests was on average 7.4 ± 1.00 (n =10 springtails per test unit initially) in the artificial soil, while adult survival on the site control soil had an average of 5.0 ± 0.37 (Figure 3-1). The difference between the averages of the artificial soil and the site control soil are not statistically significant (p = 0.053).

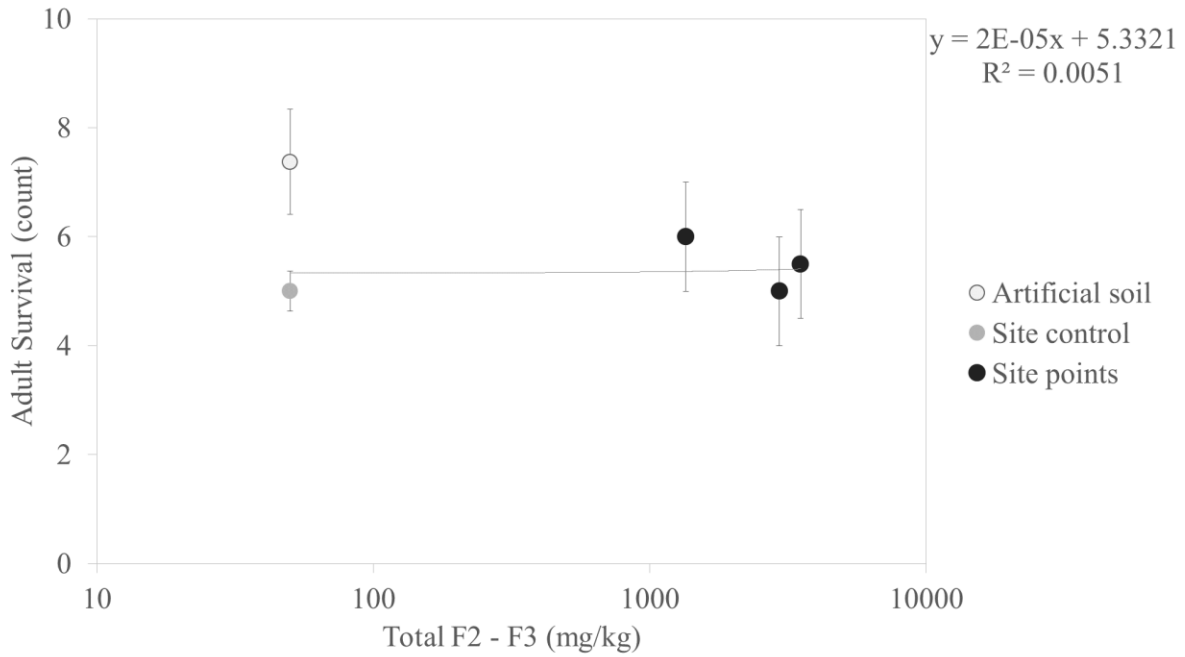


Figure 3-1: Adult survival counts from chronic toxicity tests with artificial soil, unimpacted control soil and impacted site soil from AB1.

Counts are plotted relative to the total F2-F3 concentrations; standard error bars are shown; no statistical differences were noted; artificial soil control n = 8, for all other points n = 6.

For the AB1 soils, adult survival at 1,350 mg F2 + F3/kg (low PHC) was 6.0 ± 0.97 of 10, at 2940 mg F2 + F3/kg (medium PHC) was 5.0 ± 1.10 of 10 and at 3,510 mg F2 + F3/kg (high PHC) was 5.0 ± 0.96 of 10. These adult survival averages were not statistically different from the site control (low PHC $p = 0.37$, medium PHC $p = 1.00$ and high PHC $p = 0.64$). The data were plotted with a linear trend as there were no statistical differences noted ($R^2 = 0.0051$; Figure 3-1), which indicates that there was no correlation between adult survival and petroleum concentration. An LC_{25} could not be determined because the total F2 to F3 PHC concentration is too low to extrapolate a line with any confidence and the trend line shows a slight increase in adult survival with increasing petroleum concentration. This indicates that the highest PHC concentration at AB1, 3,510 mg F2 + F3/kg, is non-toxic to *F. candida* adults.

The adult survival data obtained from AB1 soils were then plotted with F2 (Figure 3-2). The statistical differences between the numbers of adults obtained were not affected therefore, the data were also plotted with a linear trend line. Although, the R^2 doubled when the results were plotted with F2 (0.0051 versus 0.0104) there was no significant decrease in adult survival with increasing F2 concentration. This indicates that the F2 concentration of 610 mg/kg had no effect on *F. candida*.

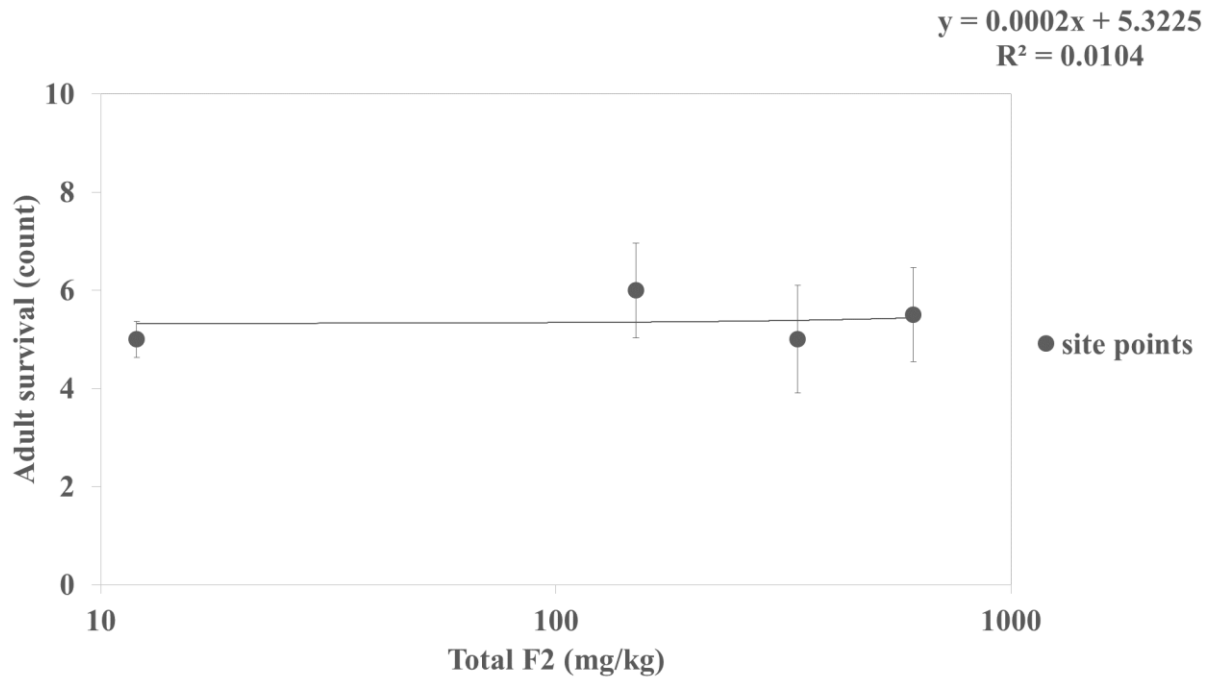


Figure 3-2: Adult survival counts from chronic toxicity tests on unimpacted control soil and impacted site soil from AB1 plotted with F2.

Adult survival for BC1 tests had an average of 7.8 ± 0.95 of 10 for the artificial soil and the site control had an average of 8.5 ± 0.85 of 10 adults. Adult survival in the artificial and site control soil were not statistically different ($p = 0.59$; Figure 3-3). The average adult survival at 660 mg F2 + F3/kg (low PHC) was 7.8 ± 1.05 of 10 which was not statistically different from the average of the site control ($p = 0.63$). However, the average adult survival at 6,810 mg F2 + F3/kg (medium PHC) was 0.8 ± 0.48 and, at 10,000 mg F2 + F3/kg (high PHC), it was 2.2 ± 0.83 ; which were statistically significantly different from the site control ($p < 0.0001$ for 6,700 mg F2 + F3/kg and $p = 0.0003$ for 10,000 mg F2 + F3/kg). Thus, BC1 tests showed a statistically significant decrease in adult survival between 660 mg F2 + F3/kg and 6,700 mg F2 + F3/kg ($p = 0.0005$) which indicates a correlation between adult survival and increasing petroleum concentration ($R^2 = 0.4634$; Figure 3-3). As there are at least two points that have partial effects (i.e., a value other than 0% or 100% of the site control value), the logit model could be used to determine the LC_{50} and LC_{25} . The logit model had a statistically significant fit to the data ($p < 0.0001$) and had an R^2 of 0.4634, indicating that 46% of the variability in the response data could be explained by the increase in petroleum content. The LC_{50} was determined to be 3,917 mg F2 + F3/kg (95% confidence interval 3,022 – 4,811) while the LC_{25} was 2,809 mg F2 + F3/kg (95% confidence interval 5,821 – 8,156; Figure 3-3). These lethality values indicate that the PHC content of the BC1 soils were toxic to *F. candida* adults.

The adult survival data obtained from BC1 soils were then plotted with F2 (Figure 3-4). The statistical differences between the numbers of adults obtained were not affected therefore, logistic analysis was also performed. The LC_{50} was determined to be 2,173 mg/kg and the LC_{25} was determined to be 1,518 mg/kg. These values indicates that the F2 concentration causes significant toxicity to *F. candida*.

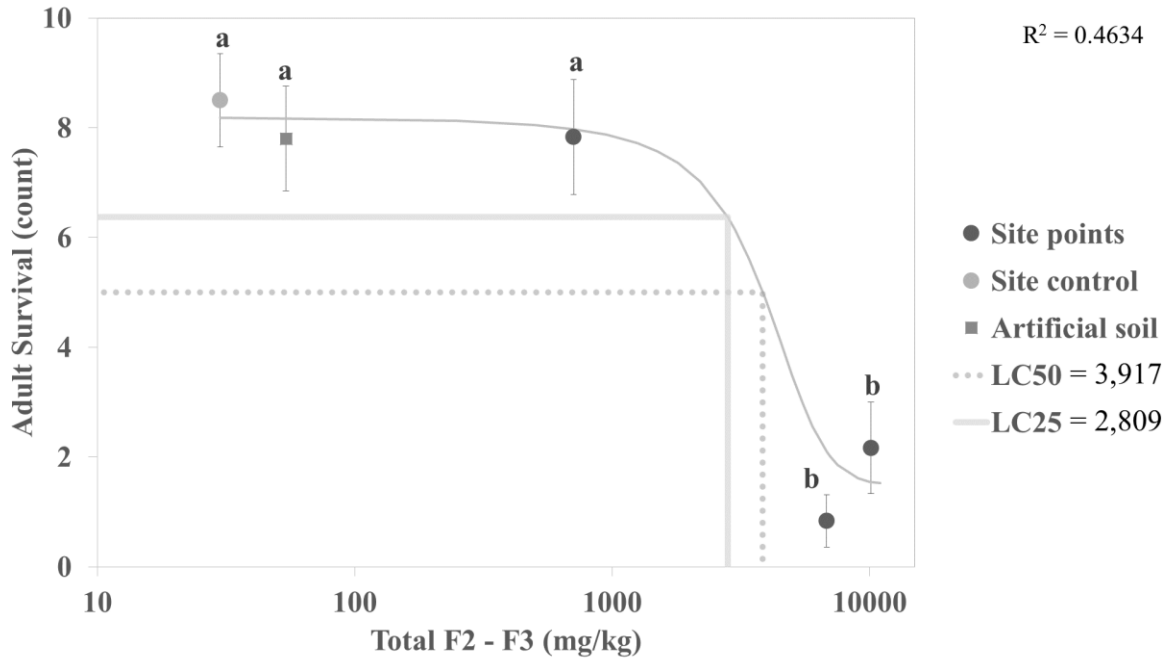


Figure 3-3: Average adult *Folsomia candida* survival from chronic toxicity tests with artificial soil, un-impacted control soil, and impacted site soil from BC1.

Adult survival averages were plotted relative to the total F2-F3 concentrations; letters that are the same represent no statistical differences; bars represent one standard error of the mean; artificial soil control n = 10, for all other points n = 6.

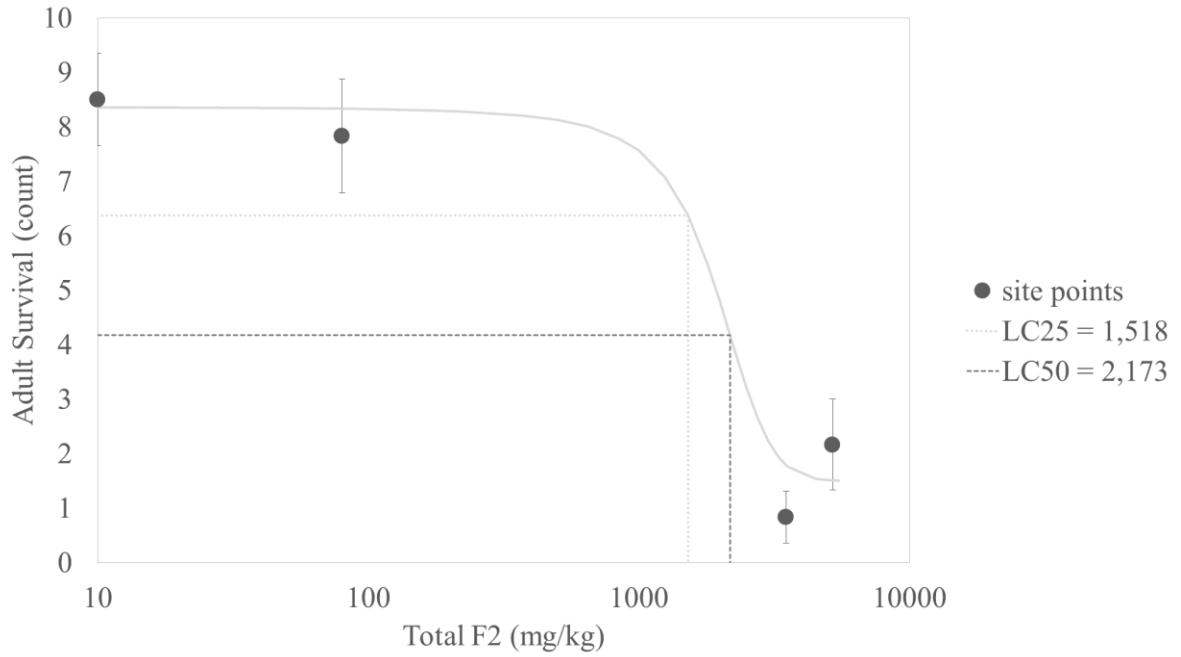


Figure 3-4: Average adult *Folsomia candida* survival from chronic toxicity tests on un-impacted control soil and impacted site soil from BC1 plotted with F2.

3.3.1.2 Juvenile Production

F. candida juvenile production was on average 672 ± 73 for the AB1 site control soil ($n = 6$) and 500 ± 87 juveniles for the artificial control soil ($n = 8$; Figure 3-5). The difference between the juvenile averages obtained from the site control and the artificial soil was not statistically significant ($p = 0.156$). The average number of juveniles obtained from the three weathered and phytoremediated PHC-impacted soils obtained from AB1 were 576 ± 121 for 1,350 mg F2 + F3/kg (low PHC, $n = 6$), 666 ± 81 for 2,940 mg F2 + F3/kg (medium PHC, $n = 6$) and $561 \text{ mg/kg} \pm 119$ for 3,510 mg F2 + F3/kg (high PHC, $n=6$). These values were not statistically significant from the average of the site control soil ($p = 0.514$, $p = 0.955$ and $p = 0.445$, respectively). No statistical differences were noted so the data were plotted with a linear trend ($R^2 = 0.19$; Figure 3-5). The results indicate that the highest concentration of the soil obtained from AB1 (3,510 mg F2 + F3/kg) had no effect on juvenile production of *F. candida*.

The adult survival data obtained from AB1 soils were then plotted with F2 (Figure 3-6). The statistical differences between the numbers of adults obtained were not affected therefore, the data were also plotted with a linear trend line. Although the R^2 increased by 1.5 times when the results were plotted with F2 (0.19 versus 0.3058), there was no significant decrease in adult survival with increasing F2 concentration. This indicates that the F2 concentration of 610 mg/kg had no effect on *F. candida*.

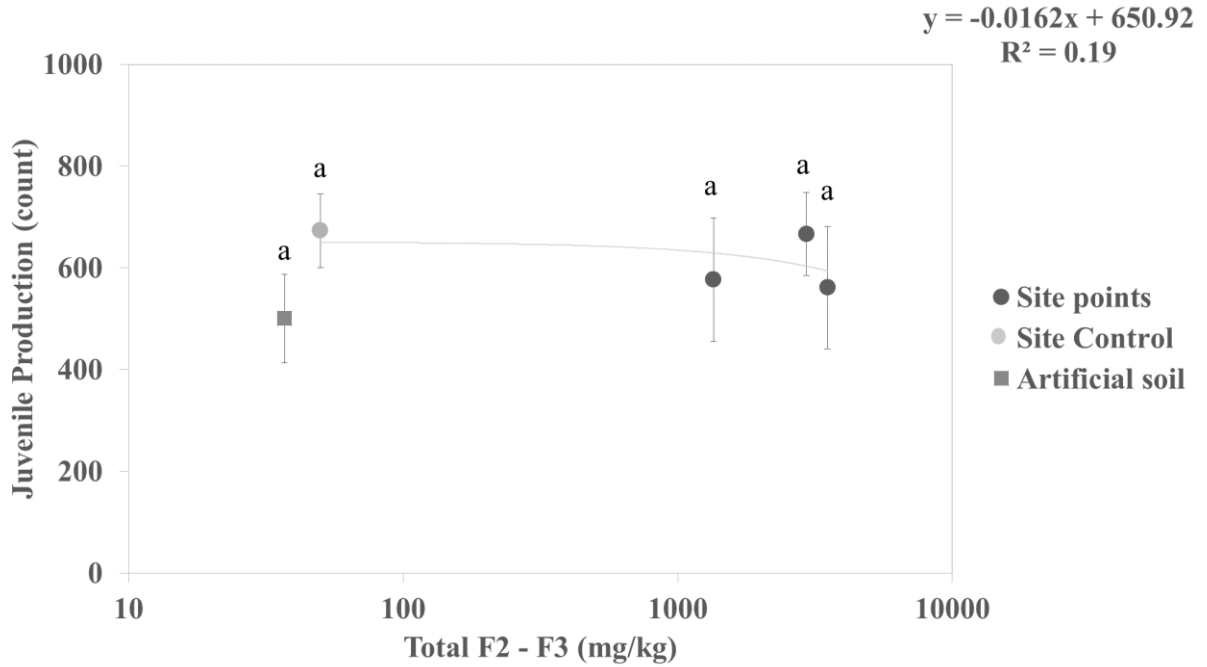


Figure 3-5: Average *Folsomia candida* Juvenile production for chronic toxicity tests on artificial soil, un-impacted control soil and impacted site soil from AB1.

Juvenile counts are plotted relative to the total F2-F3 concentrations; standard error bars are shown; no statistical differences were noted; artificial soil control n = 8, for all other points n = 6.

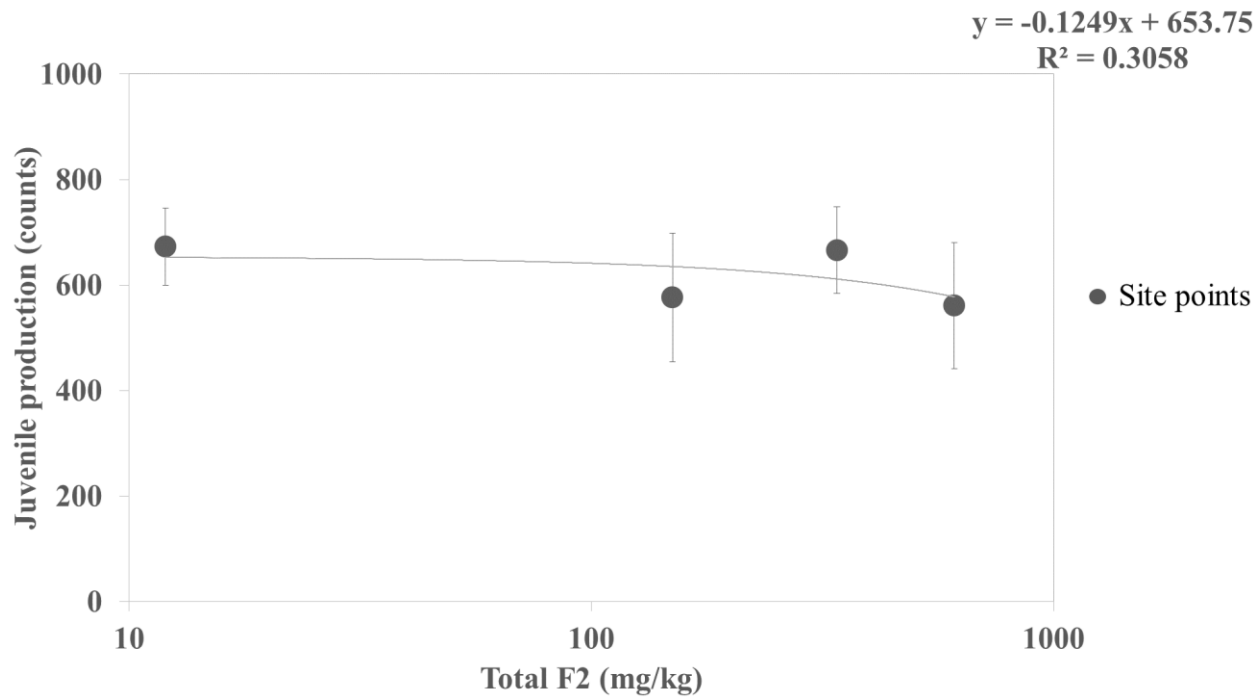


Figure 3-6: Average *Folsomia candida* Juvenile production for chronic toxicity tests on un-impacted control soil and impacted site soil from AB1 plotted with F2.

For the BC1 tests, juvenile production was 671 ± 87 for the artificial control soil and 450 ± 78 for the site control soil. The differences between the averages were not statistically significant ($p = 0.08$; Figure 3-7). The average juvenile production at 660 mg F2 + F3/kg (low PHC) was 397 ± 68 which was not statistically different from the site soil ($p = 0.624$). The average juvenile production in the medium PHC soil (6,700 mg F2 + F3/kg) was 24 ± 9 and in the high PHC soil was 10 ± 4 . Juvenile production was significantly lower in these soils relative to that in the site control soil ($p = 0.0027$ and $p = 0.0025$, respectively). The average juvenile production for 660 mg F2 + F3/kg was statistically significant from the juvenile production determined at 6,700 mg F2 + F3/kg ($p = 0.0027$) which indicates a correlation between juvenile production and increasing PHC concentration. The data were described best with a logistic regression model ($R^2 = 0.923$). The IC_{50} was determined to be 1,655 mg F2 + F3/kg (95% confidence interval 390 – 7,030) and the IC_{25} was 1,030 mg F2 + F3/kg (95% confidence interval 253 – 4592). An IC_{25} of 1,030 mg F2 + F3/kg at a site that contains 10,000 mg F2 + F3/kg indicates that the PHC found at BC1 significantly effects juvenile production.

The juvenile production counts obtained from BC1 soils were then plotted with F2 (Figure 3-8). The statistical differences between the numbers of adults obtained were not affected therefore, logistic analysis was also performed. The IC_{50} was determined to be 987 mg/kg while the IC_{25} was determined to be 310 mg/kg. These values indicate that the F2 concentration causes significant toxicity to *F. candida*.

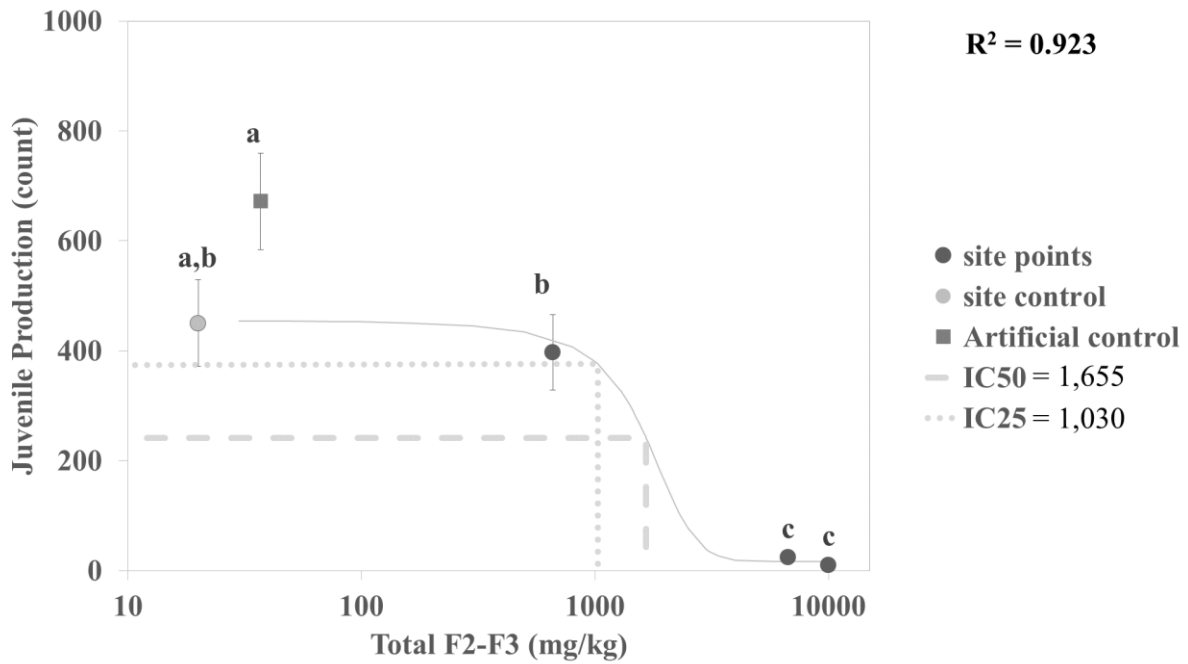


Figure 3-7: Average number of juveniles produced during chronic toxicity tests on artificial soil, un-impacted control soil and impacted site soil from BC1.

The average number of juveniles were plotted relative to the total F2-F3 concentrations; letters above standard error bars represent statistical differences; artificial soil control n = 10, for all other points n = 6; error bars are present on the two highest site points, they are covered by the point.

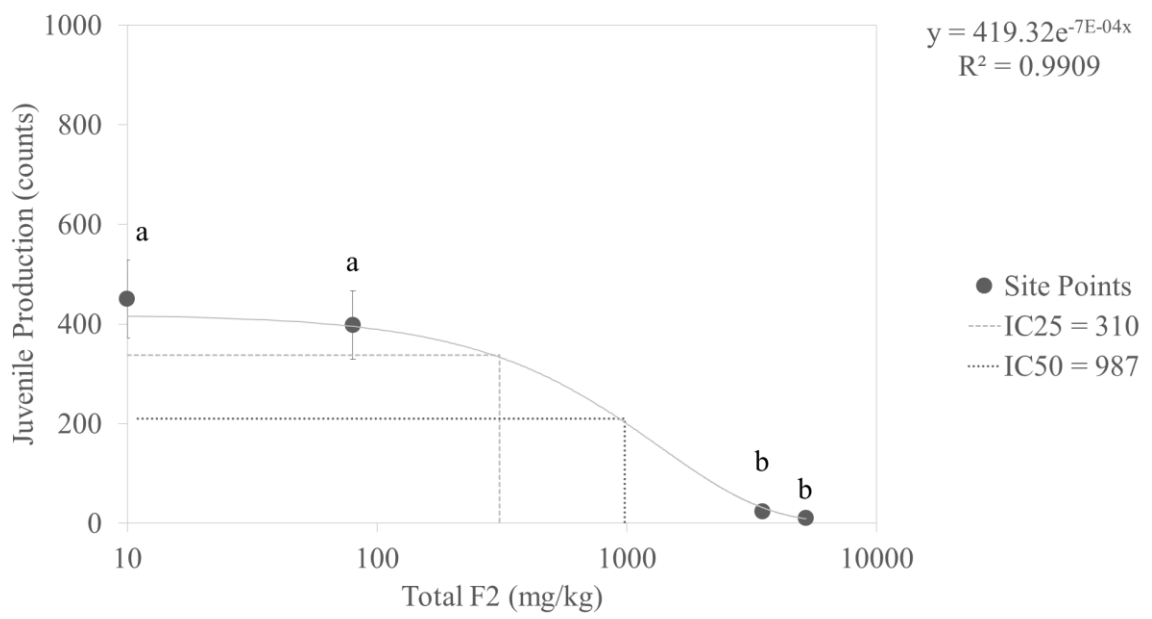


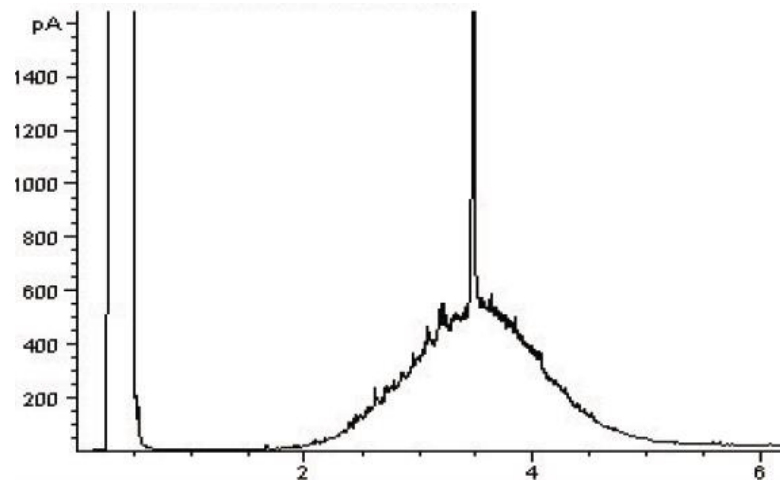
Figure 3-8: Average number of juveniles produced during chronic toxicity tests on un-impacted control soil and impacted site soil from BC1 plotted with F2.

3.3.2 AB1 and BC1 Chromatograms

Chromatograms were prepared by Maxxam to determine the concentration of each PHC fraction in each field site soil. The chromatograms were generated by injecting a sample of the PHC-impacted field soil, that had been extracted with a 1:1 of Acetone:Hexane and put through a silica gel column to remove biogenics, into a gas chromatograph with a flame ionization detector. The lower molecular weight hydrocarbons are lighter and more volatile and thus will reach the detector faster while an increase in temperature is required to volatilize the higher molecular weight hydrocarbons. Hydrocarbon standards between C₁₀ and C₁₆ for F2 and C_{>16} and C₃₄ for F3 were used to determine the retention time to compare to the sample chromatogram. The area under the UCM (Unresolved Complex Mixture) in each sample chromatogram between these retention times is proportional to the concentration of that fraction in the sample.

The chromatograms obtained for AB1 and BC1 soil (Figure 3-9 a and b and Figure 3-10 a and b, respectively) indicate that the F2 section of the UCM (approximately 1 to 5 minutes) is largely composed of high molecular weight F2 hydrocarbons (C₁₃ to C₁₆), while the F3 section of the UCM (approximately 5 to 7 minutes) is largely composed of low molecular weight F3 hydrocarbons (C_{>16} to C₂₁). The AB1 chromatogram also indicates that the distribution of F2 and F3 is approximately 25% and 75%, respectively. While, the BC1 chromatogram indicates that the distribution of F2 to F3 is approximately 50% and 50%, respectively. These distributions suggest that the PHC in the AB1 soil is mostly F3.

a)



b)

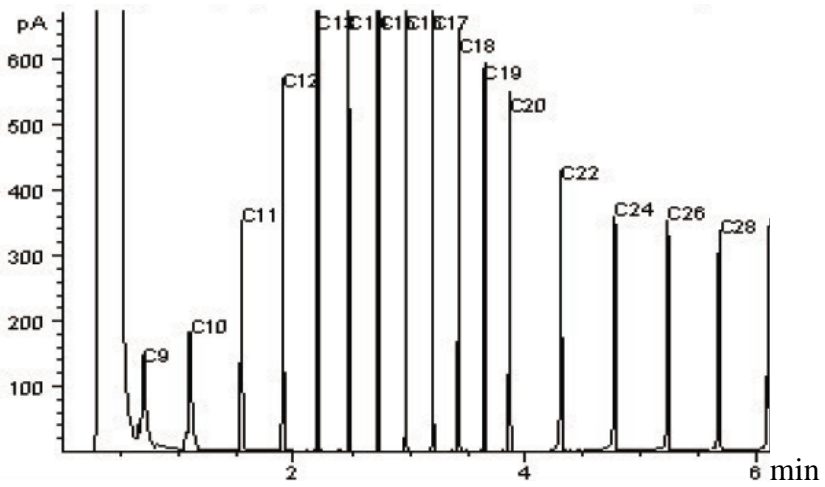
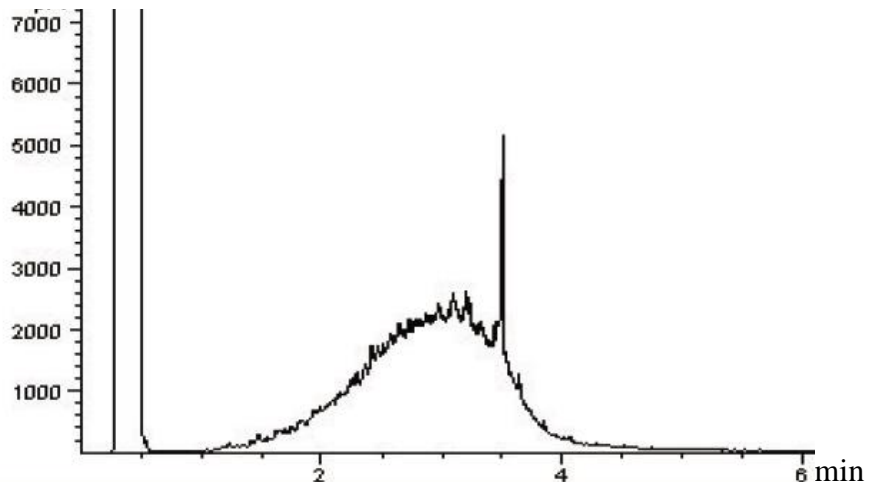


Figure 3-9: a) AB1 Chromatogram b) Typical elution times of different carbon chain lengths.

a)



b)

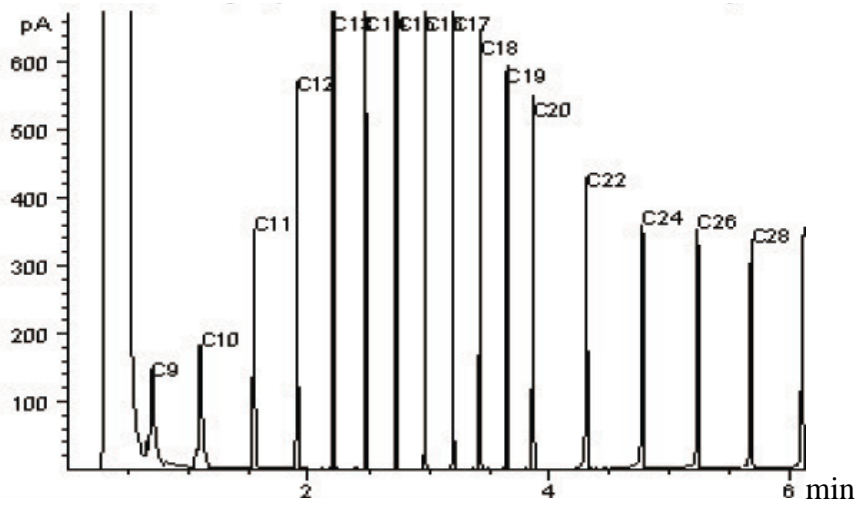


Figure 3-10: a) BC1 Chromatogram b) Typical elution times of different carbon chain lengths.

3.3.3 *F. candida* Endpoints of Weight, Length and Width to Assess the Toxicity of AB1 and BC1 Soils

Weight, length and width measurements for *F. candida* were assessed for their suitability as sublethal endpoints to include in the chronic toxicity test protocol with springtails. Weight, length and width measurements were also assessed for their sensitivity to PHC by comparison to IC₂₅ data obtained from juvenile production endpoints for each site. Each measurement was plotted against the total F2 + F3 petroleum concentration (mg/kg). The artificial soil was used to obtain control levels for these endpoints under standard conditions and to determine if the soil properties influenced these endpoints.

3.3.3.1 Effects on *Folsomia candida* Weight

The mean weight of *F. candida* exposed to the AB1 control soil and to the artificial control soil was 0.0313 ± 0.0071 mg and 0.0345 ± 0.0034 mg, respectively; these values were not statistically different ($p = 0.657$; Figure 3-11). The weight of 0.0285 ± 0.0098 mg obtained at 3,510 mg F2 + F3/kg was not statistically different from the control soil ($p = 0.83$) and there was no trend between weight and increasing petroleum concentration ($R^2 = 0.0217$). This indicated that the highest concentration of 3,510 mg F2 + F3/kg was determined to have no effect on *F. candida* weight.

The average weights of *F. candida* obtained after exposure to the artificial soil and the BC1 control soil were 0.0266 ± 0.003 mg and 0.0215 ± 0.004 mg, respectively. This difference in weight was not statistically significant ($p = 0.331$; Figure 3-12). The average *F. candida* weight of 0.0186 ± 0.005 mg at 660 mg F2 + F3/kg (low PHC) and 0.0106 ± 0.001 mg at 6,700 mg F2 + F3/kg (medium PHC) were not statistically different from the site control soil ($p = 0.652$ and $p = 0.102$, respectively; Figure 3-12). The weight of adult *F. candida* was significantly decreased ($p = 0.048$) at 10,000 mg F2 + F3/kg BC1 soil and the linear trend ($R^2 = 0.395$) suggests a significant decrease in weight with increasing petroleum concentration. The EC₅₀ was estimated to be 6950 mg/kg total F2 to F3 while the EC₂₅ was estimated to be 421 mg/kg total F2 to F3 (Figure 3-12).

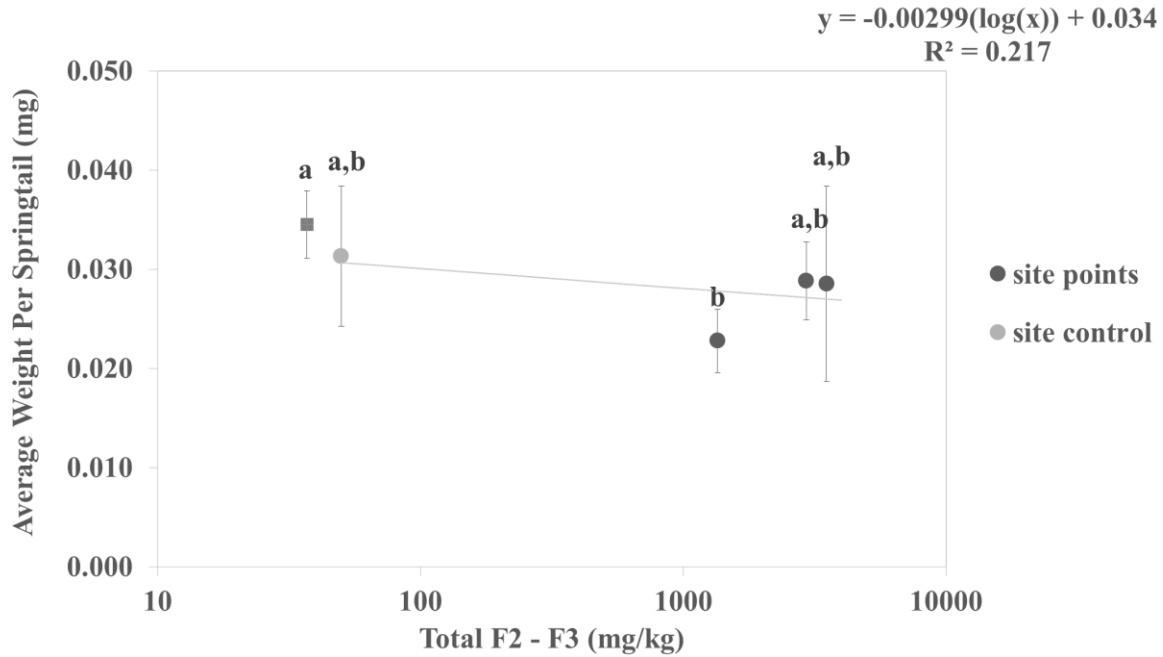


Figure 3-11: Average *Folsomia candida* adult weight from chronic toxicity tests on artificial soil, in-impacted control soil and impacted site soil from AB1.

Average *Folsomia candida* adults plotted relative to the total F2-F3 concentrations; letters above the standard error bars indicate statistical differences; artificial soil control n = 8, site control and medium PHC n = 5, high and low PHC n = 6 .

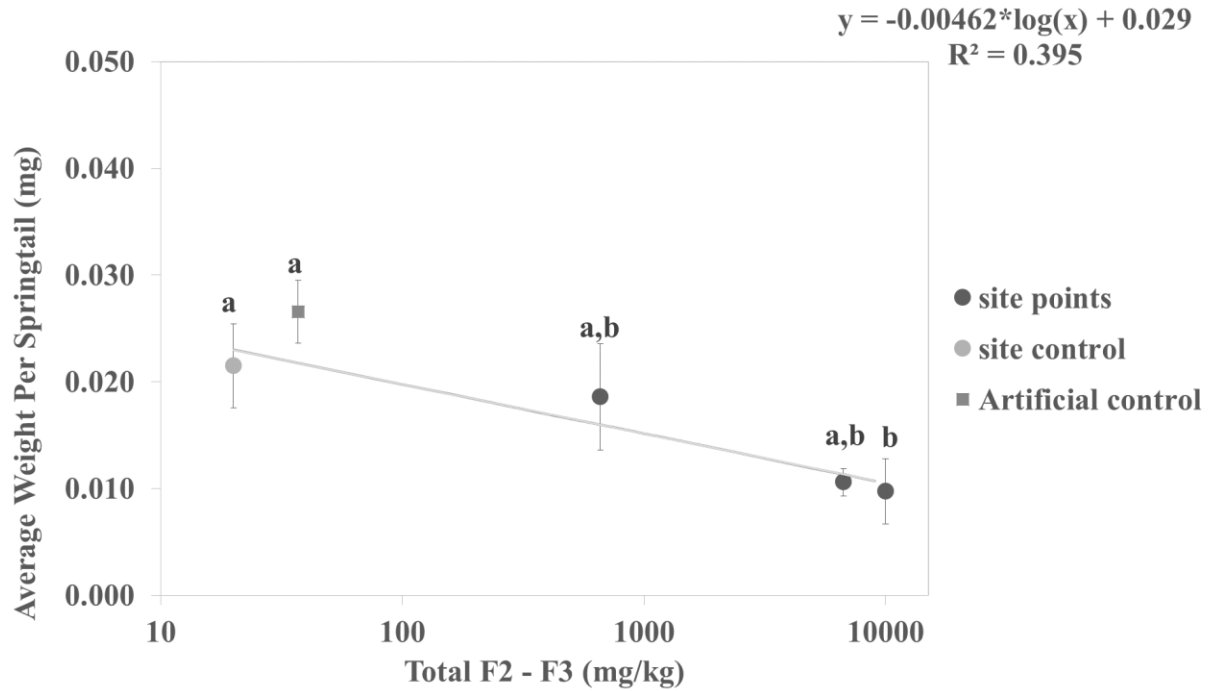


Figure 3-12: Average *Folsomia candida* adult weight for chronic toxicity tests with artificial soil, un-impacted control soil and impacted site soil from BC1.

Average adult weight was plotted relative to the total F2-F3 concentrations; letters above the standard error bars indicate statistical differences; artificial soil control n = 10, site control and low PHC n = 6, medium PHC n = 3, high PHC n = 5.

3.3.3.2 Toxicity Effects *Folsomia candida* Length

The *F. candida* used in soils from AB1 had an average length of 1.79 ± 0.06 mm for the site control which was not statistically significant from the 1.89 ± 0.06 mm average length obtained for the artificial soil ($p = 0.144$; Figure 3-13). The average length of *F. candida* after exposure to 1,350 mg F2 + F3/kg (low PHC) was 1.76 ± 0.03 mm, to 2,940 mg F2 + F3/kg (medium PHC) was 1.76 ± 0.06 mm and to 3,510 mg F2 + F3/kg (high PHC) was 1.72 ± 0.05 mm. These lengths were not statistically different from the lengths of *F. candida* that were exposed to the site control soil ($p = 0.633$, $p = 0.71$ and $p = 0.36$, respectively). No EC_{25} or EC_{50} could not be determined. This indicated that the highest concentration of 3,510 mg F2 + F3/kg was determined to have no effect on *F. candida* length.

The adult length of springtails exposed to the BC1 control soil (1.58 ± 0.056 mm) did not differ significantly ($p = 0.080$) from that (1.71 ± 0.036 mm) of the artificial control soil (Figure 3-14). The length of adults in soils with F2 + F3 concentrations of 660 mg F2 + F3/kg (low PHC) and at 6,700 mg F2 + F3/kg (medium PHC) were 1.68 ± 0.051 mm and 1.41 ± 0.102 , respectively and neither were statistically different from the site control soil ($p = 0.191$ and $p = 0.242$, respectively). The length of adult *F. candida* at 10,000 mg F2 + F3/kg (high PHC) was statistically shorter (1.28 ± 0.078 mm) than the Collembola that were placed in the site control soil ($p = 0.010$; Figure 3-14). The significant decrease in the adult length of surviving *F. candida* with increasing PHC concentration was best described with the Gompertz regression model ($R^2 = 0.927$). The EC_{25} was estimated to be approximately 13,750 mg F2 + F3/kg while the EC_{50} was estimated to be approximately 47,150 mg/kg.

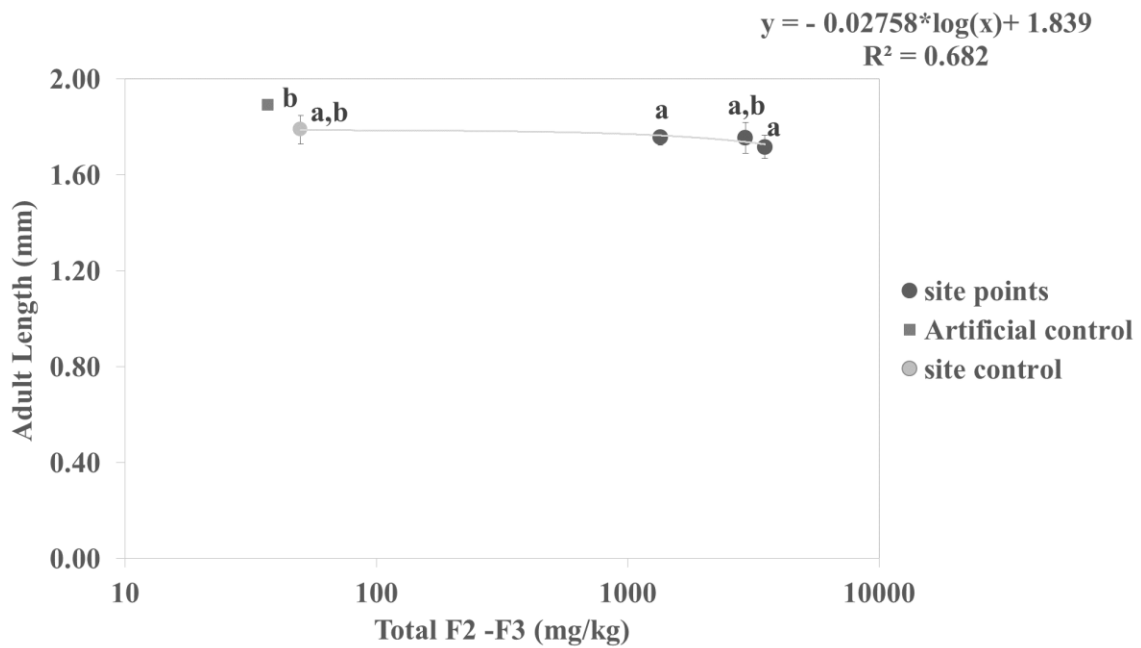


Figure 3-13: Average *Folsomia candida* adult length from chronic toxicity tests on artificial soil, un-impacted control soil and impacted site soil from AB1.

Adult length was plotted relative to the total F2-F3 concentrations; the letters over the standard error bars represent statistical differences; artificial soil control n = 8, medium PHC n = 5, for all other points n = 6.

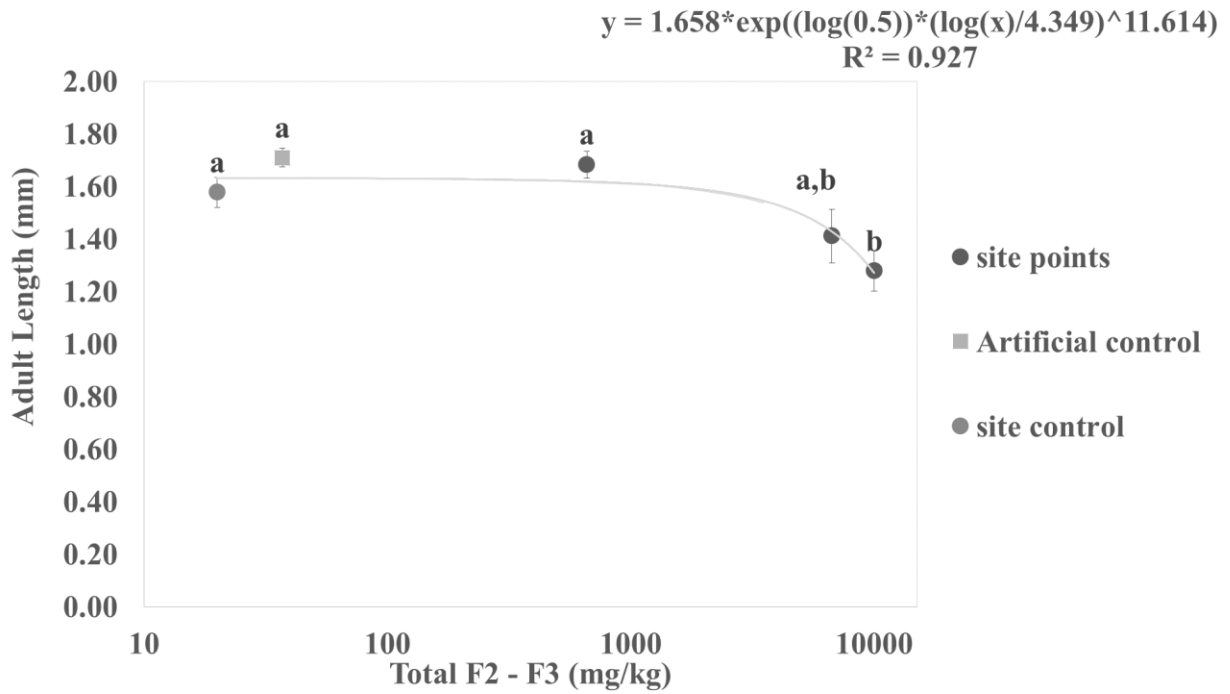


Figure 3-14: Average adult *Folsomia candida* length from chronic toxicity tests on artificial soil, un-impacted control soil and impacted site soil from BC1.

Average adult *Folsomia candida* length was plotted relative to the total F2-F3 concentrations; letters above the standard error bars indicate statistical differences; artificial soil control n = 8, site control and low PHC n = 6, medium PHC n = 3, high PHC n = 7.

3.3.3.3 Toxicity Effects on *Folsomia candida* Width

F. candida exposed to the AB1 reference control soil had an average width of 0.53 ± 0.02 mm for the artificial soil which was not statistically different from the 0.50 ± 0.01 mm average width obtained from the site control soil ($p = 0.198$; Figure 3-15). The average width was determined to be 0.51 ± 0.01 mm at 1,350 mg F2 + F3/kg (low PHC), 0.48 ± 0.01 mm at 2,940 mg F2 + F3/kg (medium PHC) and 0.51 ± 0.01 mm 3,510 mg F2 + F3/kg (high PHC). None of these points were statistically different from the site control soil ($p = 0.467$, $p = 0.349$ and $p = 0.330$, respectively). Data was plotted with a linear trend ($R^2 = 0.098$) which indicates only a 1% correlation between adult width and increasing petroleum concentration (Figure 3-15). These results indicate that 3,510 mg F2 + F3/kg did not affect *F. candida* width (Figure 3-15).

The adult width of the springtails exposed to the site control soil of BC1 was 0.45 ± 0.013 mm and was 0.53 ± 0.008 mm in the artificial control soil. These widths were statistically different ($p = 0.0012$; Figure 3-16) which indicates that there was likely a soil effect of BC1 on width of *F. candida*. The width of adults at 6,700 mg F2 + F3/kg (medium PHC) was 0.47 ± 0.073 which was not statistically different from the width obtained in the site control soil ($p = 0.825$); however, the width of adults at 660 mg F2 + F3/kg (low PHC) was 0.54 ± 0.033 mm which had a statistically higher average weight than the site control. This indicates the possibility of a hormetic effect (an increase in width before a drop), but the data fit best with the Gompertz model ($R^2 = 0.438$), which does not incorporate a hormetic effect into the model. The width at 10,000 mg F2 + F3/kg (high PHC) was 0.40 ± 0.022 mg which was not statistically different from the site control soil ($p = 0.165$) but indicates a trend toward decreasing width with increasing PHC concentration (Figure 3-16). The EC_{25} was estimated to be approximately 17,425 mg/kg total F2 to F3 (Figure 3-16).

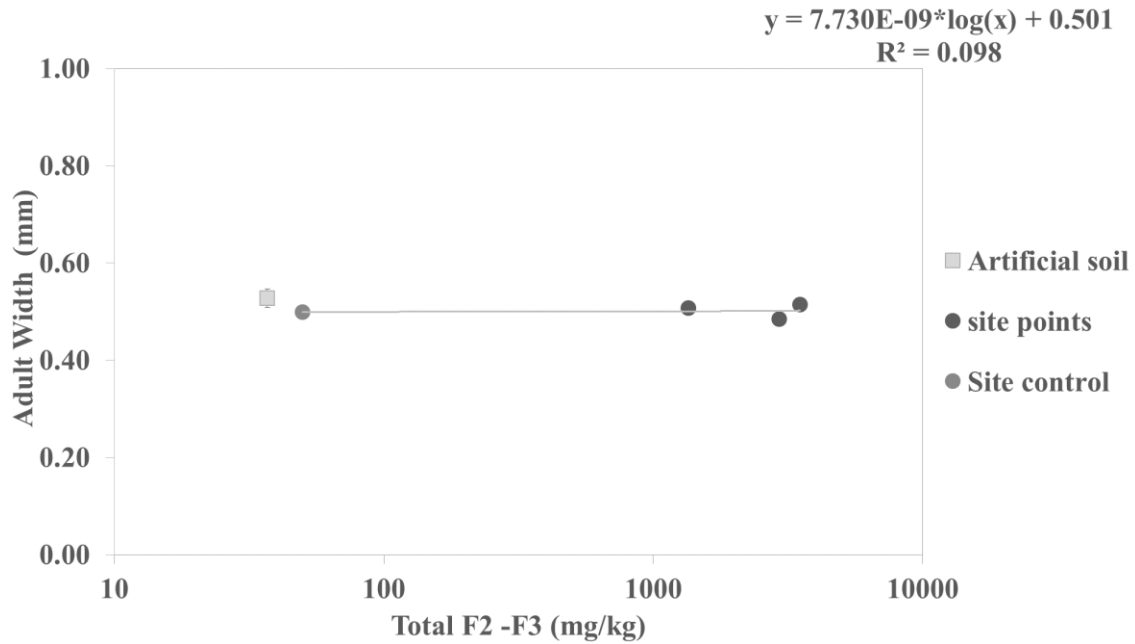


Figure 3-15: Average *Folsomia candida* adult width for chronic toxicity tests on artificial soil, un-impacted control soil and impacted site soil from AB1.

Average widths were plotted relative to the total F2-F3 concentrations; standard error bars are shown; no statistical differences were noted; artificial soil control n = 8, medium PHC n = 5, for all other points n = 6.

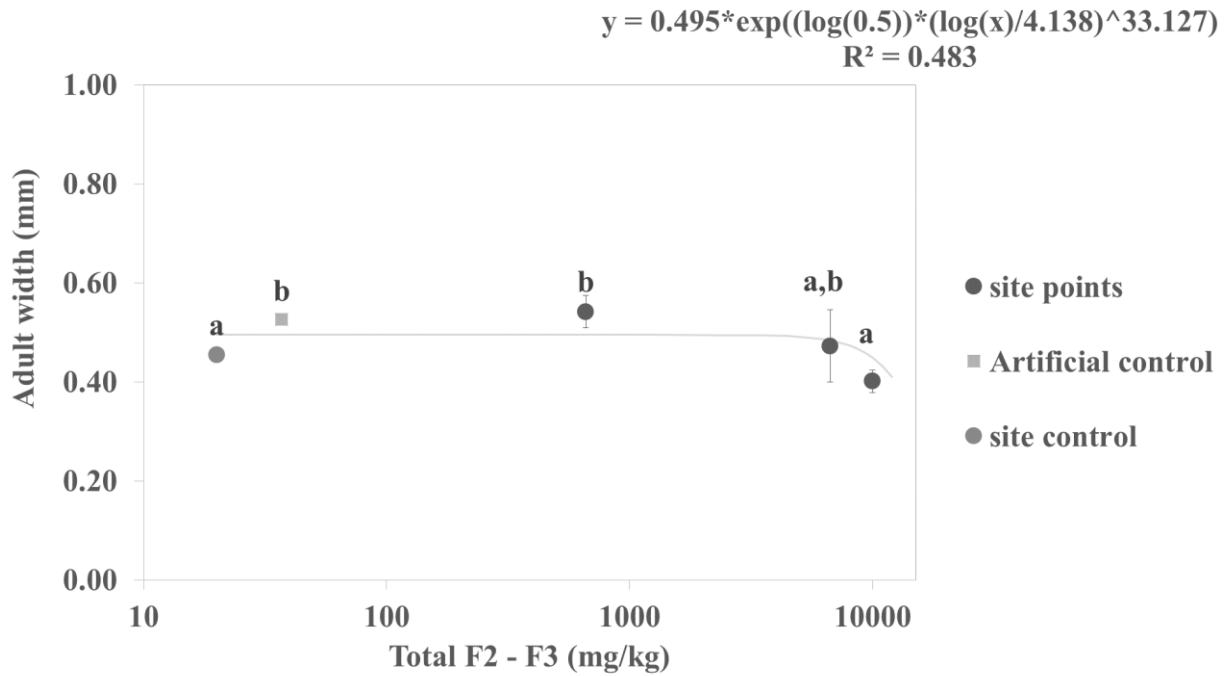


Figure 3-16: Average *Folsomia candida* adult width for chronic toxicity tests on artificial soil, un-impacted control soil and impacted site soil from BC1.

Average adult width plotted relative to the total F2-F3 concentrations, letters above the standard error bars indicate statistical differences; artificial soil control n = 9, site control and low PHC n = 6, medium PHC n = 3, high PHC n = 7.

3.3.4 *Folsomia candida* Avoidance Tests on AB1 and BC1 Soils

Avoidance tests were conducted to determine their suitability as an assessment tool for the toxicity of PHC-impacted sites. The site control soil was used as the control soil to determine if an avoidance response to the impacted soils was noted. Avoidance test responses were converted into net responses and graphed relative to total F2 to F3 petroleum content. Positive net response values represent a preference for the site control soil while negative net response values indicate a preference for the PHC-impacted soil.

The *F. candida* avoidance test responses for AB1 showed that when the site control soil was placed on both sides of the divider the net response was -11.5 ± 10.4 which was not statistically different from zero ($t = 0.123$; Figure 3-17). The two AB1 site soils highest in PHC, 2,940 mg F2 + F3/kg and 3,510 mg F2 + F3/kg, had a net response of 2.1 ± 9.6 and 6.0 ± 16.7 , respectively. These values were not statistically different from the response obtained from the site control ($p = 0.319$ and $p = 0.373$) nor were they statistically different from zero ($t = 0.218$ and $t = 0.359$) which indicates that *F. candida* does not prefer the control soil over the impacted soil. The low PHC site soil, 1,350 mg F2 + F3/kg, had a net response value of 44.0 ± 11.7 which was significantly different from the control soil ($p = 0.002$) and statistically different from zero ($t = 3.78$). This significant difference indicates that *F. candida* prefer the site control soil over the low PHC site soil. The results of the avoidance test indicate that there was no trend between soil preference and PHC concentrations which suggests that the preference for the control soil noted at 1,350 mg F2 + F3/kg was likely due to the soil properties and not the PHC (Figure 3-17).

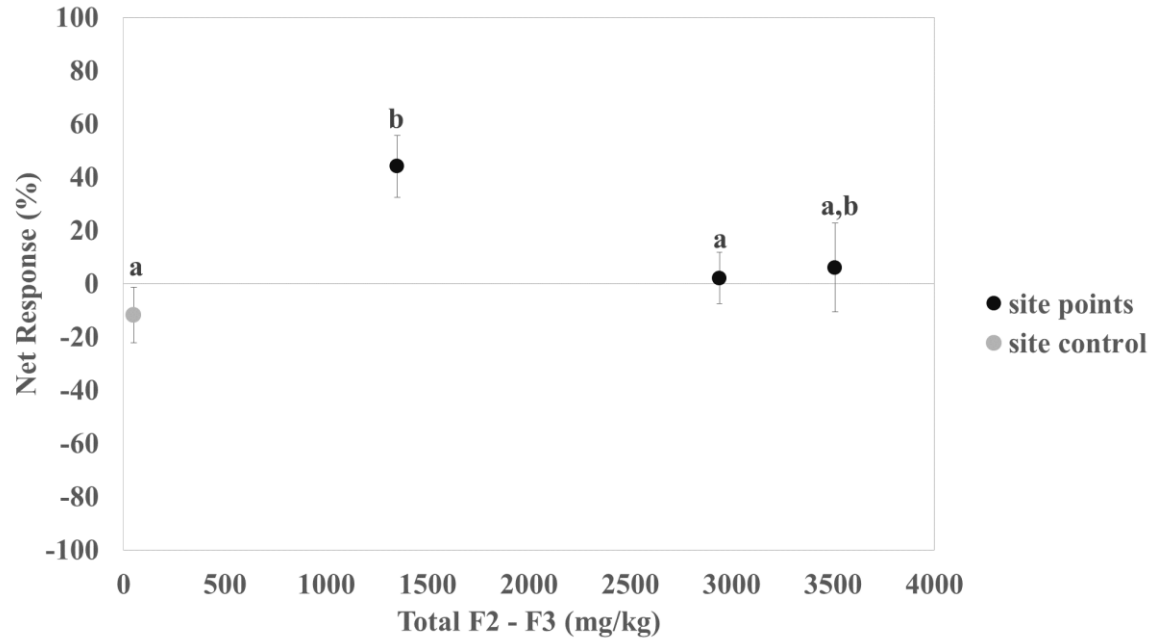


Figure 3-17: Avoidance test results AB1

Each test soil was tested against the site control soil. Positive values indicate preference for site control soil; letters above standard error bars indicate statistical differences; site control soil n = 10, all other points n = 8.

The *F. candida* avoidance test for BC1 showed that when the site control soil was placed on both sides of the divider the net response was 5.3 ± 12.1 which was not statistically different from zero ($t = 1.39$; Figure 3-18). The low PHC site soil (660 mg/kg total F2 to F3) had a net response of -16.8 ± 17.5 which was also not statistically different from zero ($t = 2.72$). The net response of the medium and high site soils (6,700 mg F2 + F3/kg and 10,000 mg F2 + F3/kg) was 77.4 ± 8.7 and 59.4 ± 10.5 , respectively. These values were statistically different from the control soil ($p = 0.0002$ and $p = 0.0038$) and were statistically different from zero ($t = 25.16$ and $t = 16.0$). These results indicate that there was a trend between increasing petroleum concentration and increasing preference for site control soil (Figure 3-18).

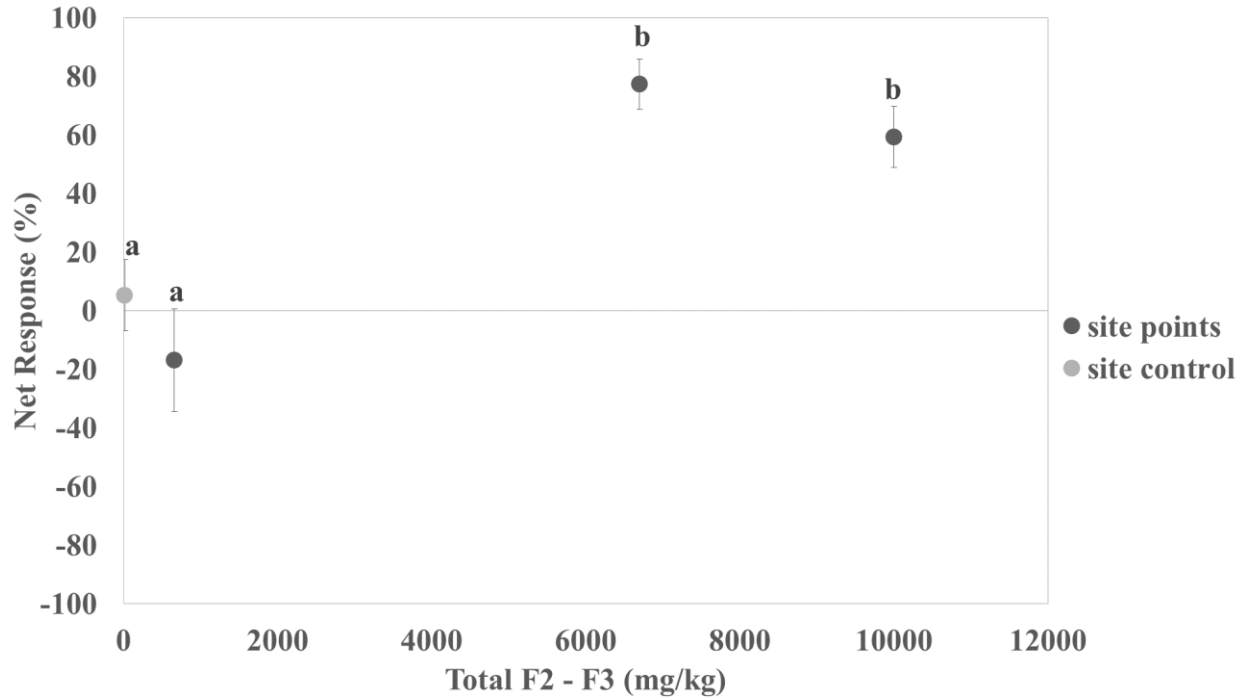


Figure 3-18: Avoidance test results BC1

Each test soil was tested against the site control soil. Positive values indicate preference for site control soil; letters above standard error bars indicate statistical differences; site control soil n = 10, all other points n = 8.

3.4 Discussion

Acute and chronic toxicity tests with two field sites were used to determine the EC₂₅, IC₂₅ and LC₂₅ for comparison with the CCME criteria. The F3 concentrations had no effect on *Folsomia candida* at the concentrations tested. This indicates that the F3 criteria can be increased from 1,300 mg/kg when used as remediation guidelines for weathered PHC-impacted field soil. The F2 concentrations tested were determined to be toxic to *F. candida*. However, the F2 criteria can likely be raised to 250 mg/kg without having an effect on *F. candida*.

3.4.1 Weathering of PHC and Impact on CCME Guidelines

Volatilization, degradation, and sorption are processes involved in weathering of PHCs (Alexander, 1995; Gallego *et al.*, 2010; Maletic *et al.*, 2011; Osuji *et al.*, 2006). PHCs from C₆ to C₁₄ will largely be lost to the air through volatilization (Pichtel and Liskanen, 2001). PHCs from C₆ to C₃₂ can be degraded, for the most part, by microorganisms (Beilen and Funhoff, 2007). PHC compounds greater than C₃₂ can interact with soil humus through van de Waals forces or diffusion into pores sequestering them in the soil (Brusseau, 1997; Maletic *et al.*, 2011; Yong and Rao, 1991). All of these weathering processes are able to make PHC less biologically available and therefore less toxic (Maletic *et al.*, 2011). Maletic *et al.* (2011) showed that after a site impacted with diesel and crude oil that had been weathered for 9 years, the site soil concentration of 35,000 mg/kg total petroleum hydrocarbons was not toxic when compared to freshly spiked soil at 35,000 mg/kg. AB1 and BC1 have been weathered for six and ten years, respectively, thereby theoretically removing most, if not all, of the toxicity at these sites.

Toxicity was not observed at AB1 which corresponds with the six years of weathering and the 2 years of phytoremediation, indicating that the low total F2 and F3 concentration of 3,510 mg/kg was not toxic. The total F2 to F3 concentration of 10,000 mg/kg at BC1 was expected to show no toxicity after the 10 years of weathering and 3 years of phytoremediation. However, BC1 soils showed toxicity with increasing PHC concentration. Toxicity occurs between the low (660 mg F2 + F3/kg) and medium (6,700 mg

F2 + F3/kg) site points. The LC₂₅ was determined to be 1,030 mg/kg, therefore, the soil properties of these two points were further investigated to determine if there was another possible cause to the increased toxicity (Table 3-1).

The soil properties of pH, percent organic matter, percent sand, percent silt, percent clay, cation exchange capacity and EC_e were measured for each soil (Table 2-1). The percent differences in values of each soil property listed in Table 2-1 between the low and medium PHC site points ranged from 1% to 39% with the exception of F3 and F2 which had percent differences of 81% and 98 %, respectively. The EC_e had a 39% difference with values increasing from 0.620 at low PHC (660 mg F2 + F3/kg) to 1.022 at medium PHC (6,700 mg F2 + F3/kg). An EC of 1.03 dS/m was found to decrease juvenile production, but had no effect on adult survival of *Folsomia candida* (Maletic *et al.*, 2011). This indicates that the soil properties are not the likely cause of toxicity at BC1 and PHC is the most likely cause.

As the soil properties were not likely the cause of the toxicity noted at BC1, the F2 and F3 concentrations were investigated as the likely cause for the toxicity. Toxicity was determined to occur at a concentration of 1,030 mg F2 + F3/kg which is between the low and medium site points (Table 2-1). The F3 concentrations at these site points was 580 and 3,200 mg/kg, respectively. The 3,200 mg/kg F3 is only 300 mg/kg higher than the F3 concentration that was determined to cause no effect in the soil of AB1 (2,900 mg/kg) indicating that F3 was not the likely cause of the toxicity at BC1. These findings suggest that F2 is the likely cause of toxicity at BC1.

The 3,510 mg F2 + F3/kg value obtained from AB1 and was determined to have no effect, contained only 11% F2, so this value was compared to the CCME guideline value for F3 (1,300 mg/kg). The F3 concentration in the soil from AB1 was determined to be 2,900 mg/kg. This suggests that the guideline value can be raised from 1,300 mg/kg to 2,900 mg/kg without having an effect on *F. candida*.

As stated previously, the toxicity of BC1 was attributable to the F2 concentration at the site and the most sensitive endpoint was juvenile production. An LC₂₅ of 2,809 mg/kg and IC₂₅ of 1,030 mg/kg were derived for weathered PHC at BC1. The LC₂₅ and IC₂₅ values

derived by Angell *et al.* (2012) for F2 were much lower for *F. candida* when artificially weathered soil was used (LC₂₅ of 337 mg/kg and IC₂₅ of 533 mg/kg). This suggests that artificial weathering may not be representative of natural weathering processes.

The F2 value that can be suggested for CCME criteria is 1,030 mg/kg; however, the large 95% confidence interval of 253 – 4,592 indicates that more replicates and more concentrations should be used to more confidently determine the concentration to which the guideline values can be raised. The CCME and Angell *et al.* (2012) also incorporated multiple species with varying sensitivities in their determination of a guideline value. As this research relied solely on results from a single invertebrate species, which may not be the most sensitive or most representative species, the IC₂₅ may not be protective of most species. Therefore, the recommended guideline value for F2 is 250 mg/kg, which falls at the low end of the 95% confidence interval. This value is verified by plotting the juvenile production in soils at BC1 with F2. The IC₂₅ obtained was 310 mg/kg, indicating that 250 mg/kg would not likely have an effect on *F. candida* juvenile production. The recommended guideline value for F3 is 2,900 mg/kg, the F3 concentration in AB1 soil that was determined to have no effect on *F. candida*.

3.4.2 Endpoint Sensitivity in Chronic Toxicity Tests

Two endpoints were investigated during toxicity tests: adult survival and juvenile production. BC1 had an LC₂₅ of 2,809 mg/kg for adult survival and 1,030 mg/kg for juvenile production which indicated that juvenile production was a more sensitive endpoint than adult survival. This result corresponds to results in literature which show that for all invertebrate species juvenile production is a more sensitive endpoint than adult mortality (Angell *et al.*, 2012; Cermak *et al.*, 2010).

3.4.3 Sublethal Endpoints

Sublethal endpoints such as juvenile production and length assessments are preferred in chronic toxicity tests because toxicity is observed at lower concentrations than those that cause mortality (Environment Canada, 2005a; Environment Canada, 2007b; Folker-Hansen

et al., 1996; Petersen *et al.*, 1997). The effects of heavy metal and pesticide soil in soil have been studied using growth endpoints (weight and length) for several Collembola species; similar studies have yet to be applied to PHC-impacted soils (Crommentuijn *et al.*, 1993; Folker-Hansen *et al.*, 1996; Petersen *et al.*, 1997). Sublethal endpoints (length, width and weight) were investigated to determine if they would be a valuable addition to the chronic toxicity test protocol for PHC-impacted soils.

As stated earlier, juvenile production is a more sensitive endpoint than adult survival which indicates that that the EC₂₅ of any new endpoints for use with *Folsomia candida* should be compared with the IC₂₅ obtained from juvenile production. At BC1 the IC₂₅ was determined to be 1,030 mg/kg. Length was determined to have an EC₂₅ of approximately 13,750 mg/kg which indicates that production of juveniles is 13 times more sensitive than length. This suggests that length is not a suitable endpoint to add to chronic toxicity tests with *F. candida*. Width was determined to have an EC₂₅ of 17,425 mg/kg. Not only is this endpoint even less sensitive than length, the lack of statistical significance between the points suggest that there may be no effect on *F. candida*. This indicates that width should not be considered as an endpoint for chronic toxicity tests with *F. candida* involving PHC. Dry weight was determined to have an EC₂₅ of 421 mg/kg. This value is twice as sensitive as the IC₂₅ obtained for juvenile production which indicates that weight would be a valuable addition to chronic toxicity tests of PHC using *F. candida*. Crommentuijn *et al.* (1993) has also indicated that weight was a more sensitive endpoint for *F. candida* than juvenile production when testing cadmium.

3.4.4 Avoidance Tests

Avoidance-response tests have shorter durations than chronic toxicity tests (Environment Canada, 2007a). The ability to obtain rapid results from a sensitive endpoint has led to their investigation for use as a preliminary screening tool for soils impacted with metals and pesticides (Heupal, 2002; Liu *et al.*, 2010). Avoidance tests with *Folsomia candida* have been recommended for use as endpoints in toxicity tests (Natal-da-Luz *et al.*,

2008) and were used here to determine their suitability for use with weathered PHC-impacted soils.

Avoidance test results for AB1 and BC1 show similarities to the results obtained for the chronic toxicity test endpoints of adult survival. AB1 avoidance test showed no preference for control or PHC-impacted soil of any concentration which correlates well with results obtained for chronic toxicity test with AB1. However, the low PHC site point indicated a preference for the control soil which was statistically different from zero. This response was not likely due to the presence of petroleum because the two site points highest in petroleum showed no statistical differences from zero. This indicates that the preference for the control soil may likely be due to soil properties found at the low PHC point. Investigations of the soil properties (Table 3-1) indicated that the pH and clay content were highest at the low site point. The pH and clay content were 15% and 31%, respectively, higher than the site control soil. As previously stated, *F. candida* prefer soils with a low bulk density and therefore a lower clay content (Brady and Weil, 2010; Domene *et al.*, 2011; Natal-da-Luz *et al.*, 2008). Studies have shown that soil pH values of 7.74 and 6.83 have no effect on *F. candida* survival or reproduction (Kaneda and Kaneko, 2002; Sorenson and Holmstrup, 2005). The results with BC1 soil indicated that Collembola avoidance increased with increasing petroleum concentration. Toxic effects were predicted to occur when chronic toxicity tests with BC1 were conducted. This was indeed the result obtained which indicates that avoidance tests would be a good preliminary screening tool to test the toxicity of petroleum contaminated sites.

3.5 Conclusions

The main purpose of this research was to determine if the CCME guidelines are too conservative to apply to sites with weathered PHCs in soil. This objective was tested using chronic toxicity tests with *Folsomia candida*. Results from these tests indicate that concentrations of 250 mg/kg F2 and 2,900 mg/kg F3 did not adversely affect *F. candida* adult survival and reproduction. These results support the premise that the Tier 1 CWS for PHCs in soil are overly protective when PHC residuals are well weathered. Six years of

weathering and phytoremediation measures likely resulted in recalcitrant PHC residuals in soil that were not bioavailable to organisms such as springtails (Alexander, 1995; Maletic *et al.*, 2011; Michel and Hayes, 1999).

The emphasis on sublethal endpoints in chronic toxicity testing (Environment Canada, 2005a; Environment Canada, 2007b; Folker-Hansen *et al.*, 1996; Petersen *et al.*, 1997) lead to the investigation of length, width and weight as endpoints to add to the Environment Canada springtail chronic toxicity test protocol. Weight was determined to be a more sensitive endpoint than reproduction, length and width for *Folsomia candida*. Thus it can be concluded that the addition of weight to the chronic toxicity test protocol would be helpful in the determination of a guideline value as it provides more sensitive information than both mortality and reproduction.

Avoidance tests with *Folsomia candida* have been recommended for use as an endpoint in toxicity tests (Natal-da-Luz *et al.*, 2008) and were investigated in this study for use as an initial site assessment screening tool to predict chronic toxicity. Results showed that avoidance tests were able to predict the outcome of the chronic toxicity tests. The ability of the avoidance tests to predict the outcome of the chronic toxicity tests leads to the conclusion that they would make a good preliminary screen for determining the toxicity of PHC-impacted soils.

3.6 Recommendations and Further Research

The use of field soils in toxicity tests is beneficial because they provide values applicable to known environmental contamination; however, it is difficult to work with field soils because of the limited concentrations, the limited amount of soil that can be shipped to laboratories for use in testing, the availability of an appropriate control soil and the availability of information of previous soil manipulations and contamination sources. It is recommended that for research on field soils a minimum of four replicates per treatment should be used for chronic toxicity tests to decrease response variation and aid in statistical analysis. It is also recommended that a concentration curve be attempted at all field sites with a minimum of 10

concentrations. This would aid in the determination of more accurate LC₂₅, IC₂₅ and EC₂₅.

It is recommended that weight be added as a measurement endpoint to the test protocol. Further research should be undertaken to determine if this endpoint can be used for other Collembola species such as *Orthonychiurus folsomi* and *Folsomia fimetaria*. Until this research has been completed, it is recommended that the weight endpoint only be used for chronic toxicity tests involving *Folsomia candida*.

The use of an avoidance test for a preliminary toxicity determination of PHC-impacted field sites should be used to determine if chronic toxicity tests are necessary for the site. Avoidance tests are quick and simple to perform in comparison with chronic toxicity tests. It is recommended that at least three points (a high, medium, and low PHC concentration) and an appropriate site control be incorporated to determine general results for the entire site. Using the avoidance test would save time, effort, resources and money if chronic tests were not deemed necessary to perform for a PHC-impacted site. The avoidance test would also indicate the extent of cleanup necessary and would allow for faster implementation of remediation strategies.

The CCME determines the guideline values through a ranked response distribution of the LC₂₅, EC₂₅ and IC₂₅ (CCME, 2008b). This distribution looks at all of the endpoints obtained for a single soil and petroleum type in comparison to the control and ranks them from the most to least sensitive response (CCME, 2008b). The use of a ranked response distribution was the method by which the current F2 and F3 guidelines were determined (CCME, 2008b). For F2, three studies with soils spiked with Federated crude oil were used (CCME, 2008b). Numerous endpoints for several plant species were included along with both survival and reproduction of *Eisenia* and *O. folsomi* (CCME, 2008b). The 25th percentile of the ranked responses of both plants and invertebrates determined the current guideline value of 150 mg/kg F2 (CCME, 2008b). Using multiple species and multiple endpoints for BC1 would be advantageous to determining an appropriate guideline for weathered sites with high initial F2.

The research outlined in this study as well as that of Angell *et al.* (2012) support the development of soil quality criteria for sites with weathered PHCs separately from sites impacted with fresh product. This study also supports the need for chronic toxicity testing of more field soils with a wide variety petroleum contamination to determine a guideline value that can be applied to weathered sites.

Chapter 4 Summary and Conclusions

The Canadian Council of Ministers of the Environment (CCME) has developed guidelines for petroleum hydrocarbon (PHC) impacted field soils that are intended to protect ecological receptors such as springtails, earthworms and plants (CCME, 2001b). These guidelines were intended to be highly conservative and, as such, they were based on the “worst case” scenario of a fresh petroleum spill. This would ensure that all PHC-impacted field sites that had the potential to affect ecological receptors were identified (CCME, 2001b; CCME, 2008b). Weathering of PHC in soil, caused by volatilization, sorption to soil particles and microbial degradation, occurs over a period of time and has been shown to decrease the toxicity of PHC-impacted field soils (Alexander, 1995; Gallego et al., 2010; Maletic et al., 2011; Osuji et al., 2006). When applied as remedial benchmarks, these CCME guidelines may be too conservative to be used as realistic targets as they do not account for sites where weathering has been occurring for a number of years. This research investigated the applicability of CCME criteria to weathered PHC-impacted field soils. It is hypothesized that the toxicity of weathered PHC-impacted field soils will occur at higher concentrations than the CCME guidelines. The four objectives used to test this hypothesis were to: (1) use chronic toxicity tests with *Folsomia candida* to calculate a LC₂₅ and an IC₂₅ of weathered PHC-impacted field soil at three different field sites, (2) compare the heat and floatation extraction methods to determine their efficiency at removing *F. candida* adults and juveniles from soil, (3) determine if the sub-lethal endpoints (weight, length and width) will be a suitable addition to the chronic toxicity test protocol and (4) investigate the feasibility of using *F. candida* avoidance-response tests as a preliminary test method to assess the toxicity of weathered PHC-impacted sites.

F. candida are small parthenogenic, soil dwelling arthropods that have a hydrophobic cuticle (Ghiradella and Radigan, 1974; Hopkin, 1997; Hopkin, 2007; Marshall and Kevan, 1962). *F. candida* are commonly used in toxicity testing because of the aforementioned physical features and their ease of culturing (Fountain and Hopkin, 2005). Chronic toxicity tests were performed following the Environment Canada biological tests methods using

weathered PHC-impacted field soil from three different field sites (ON1, AB1 and BC1) to determine an LC₂₅ or IC₂₅ of the test species *F. candida*.

The highest PHC concentration obtained for the ON1 field soil (635 mg/kg F2 and 12,000 mg/kg F3) and the AB1 field soil (610 mg/kg F2 and 2,900 mg/kg F3) did not affect *F. candida* survival and reproduction. However, there was significant adverse effects to *F. candida* survival and reproduction when organisms were exposed to PHC-impacted soil obtained from the BC1 site. For this soil, toxicity occurred between PHC concentrations of 80 to 3500 mg F2/kg and 580 to 3200 mg F2/kg. A LC₂₅ of 2,809 mg F2 + F3/kg was calculated for adult survival and an IC₂₅ of 1,030 mg F2 + F3/kg was calculated for juvenile production. Two possible causes for the toxicity noted at BC1 were investigated: the soil properties and the concentrations of each petroleum fraction. The soil physical properties at the BC1 field site, as well as the AB1 and ON1 field sites, were determined not to be a significant cause of toxicity. The petroleum concentration of the soil obtained from BC1 was determined to have a significant effect on *Folsomia candida* adult survival and juvenile production and when individual fractions were considered, it was suspected that the toxicity at BC1 was likely caused by the F2 concentration.

The Environment Canada protocol recommends *F. candida* be removed from the soil at the end of the exposure period using either the floatation or the heat extraction methods (Environment Canada, 2007b). The floatation method involves the addition of water to the soil to allow the hydrophobic *Folsomia candida* to float to the surface (Environment Canada, 2007a). The heat extraction method uses heat to drive the organisms, away from the heat source, out of the soil and into a vessel where they can be quantified (Edwards and Fletcher, 1971). The total number of adults recovered using the heat extraction was compared to the total number recovered using the floatation method. In all cases the number of adults obtained using the floatation method was higher than the total number of adults obtained using the heat extraction method. However, only two of these results had statistically significant increases in the number of adults obtained from the floatation method compared to the number of adults obtained from the heat extraction method. This suggests that the

floatation method is the best method to use to extract *F. candida*, but it also indicates that either method can be used with no significant effect on the conclusions made.

Chronic toxicity tests using organisms other than *F. candida* have focused on measuring sub-lethal endpoints such as weight and length (Folker-Hansen et al., 1996; Pedersen et al., 1997). This is because decreases in weight and length are observed at lower concentrations than those that cause mortality (Folker-Hansen et al., 1996; Pedersen et al., 1997). Although *F. candida* weight has been used for toxicity testing with cadmium (Crommentuijn et al., 1993), the only sub-lethal endpoint currently included in the Environment Canada protocol is juvenile production. The third objective was to determine if the sub-lethal endpoints (weight, length and/or width) will be a suitable additions to the chronic toxicity test protocol. The endpoints of weight, length and width were added to chronic toxicity test for AB1 and BC1. During the chronic toxicity test the highest concentration tested for AB1 (610 mg F2/kg and 2,900 mg F3/kg) was determined to have no effect on the weight, length or width of the adults. The concentrations obtained from BC1 did show a significant decrease on *F. candida* weight, length and width. The EC₂₅ was calculated to be 421 mg F2 + F3/kg for weight while the EC₂₅ was calculated to be 13,750 mg F2 + F3/kg and 17,425 mg F2 + F3/kg for length and width, respectively. The EC₂₅ of 421 mg F2 + F3/kg obtained for the weight of adults is lower than the IC₂₅ of 1,030 mg F2 + F3/kg obtained for juvenile production. This indicates that weight is a more sensitive endpoint than adult survival and suggests that it would be a valuable addition to chronic toxicity tests of PHC-impacted field soils using *F. candida*.

Acute toxicity tests use shorter exposure times and measure either lethal effects, such as survival, or sub-lethal effects, such as avoidance behaviour (Environment Canada, 2007a). Avoidance-response tests have been used extensively with earthworms and have been shown to have the same trends as the results obtained in the longer chronic reproduction tests (Environment Canada, 2007a). Avoidance-response tests employed a method adapted from Liu *et al.* (2010) and Environment Canada (2007a) which involved dividing a cylindrical container in two, placing a control soil in one half and a test soil in the other, removing the divider and adding 20 *Folsomia candida* to the midline. The avoidance-response test, using

soil obtained from AB1, showed no trend between soil avoidance and increasing PHC concentration. However, the avoidance-response test, using soil obtained from BC1, indicated that *F. candida* avoidance increased with increasing petroleum concentration. As noted above, chronic toxicity tests results with soils from AB1 showed no effect on survival or reproduction with increasing petroleum concentration while soils obtained from BC1 showed a decrease in adult survival and reproduction with increasing petroleum concentration. These results show that avoidance-response tests were able to predict the outcome of the chronic toxicity tests and led to the conclusion that they would make good preliminary screening tools for determining the toxicity of PHC-impacted field soils.

Overall, the above results indicate that the CCME guidelines are too conservative to apply to weathered PHC-impacted field soil. Results also indicate that F2 and F3 concentrations of 250 mg/kg and 2,900 mg/kg, respectively, would not adversely affect *F. candida* adult survival, juvenile production or adult weight. These values support the premise that the Tier 1 guidelines are overly conservative when PHC residuals are weathered. It must be noted that the increase in guidelines recommended here (150 mg/kg to 250 mg/kg for F2 and 1,300 mg/kg to 2,900 mg/kg for F3) are based on toxicity tests with *F. candida* and other organisms should be used to support these values along with testing of more samples of weathered PHC-impacted field soil.

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