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DETERMINING THE SUSTAINABILITY OF LAND-APPLYING BIOSOLIDS TO AGRICULTURAL LANDS USING ENVIRONMENTALLY-RELEVANT TERRESTRIAL BIOTA

by

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A Thesis
Presented To Ryerson University
In partial fulfilment of the requirements for the
degree of
Master of Applied Science
In the Program of
Environmental Applied Science and Management

Toronto, Ontario, Canada,
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Author's Declaration

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Abstract

Determining the Sustainability of Land-Applying Biosolids to Agricultural Lands Using Environmentally-Relevant Terrestrial Biota

Degree of Master of Applied Science, 2013
Karen Joan Puddephatt
Program of Environmental Applied Science and Management
Ryerson University

Biosolids, the treated solid by-product of a WWPT, have been land-applied for decades as a means of disposal of an inexpensive form of fertilizer. However, research has shown that many chemicals such as pharmaceuticals, herbicides, pesticides, plasticizers, detergents, or heavy metals pass through the WWTP, often unaltered, and potentially end up in the biosolids. Therefore, a need to determine if the land-application of biosolids has an impact on terrestrial biota exists. In this work, six different organisms were used including *Folsomia candida*, *Lumbricus terrestris*, *Zea mays*, *Glycine max*, *Phaseolus vulgaris*, and *Brassca rap*.

It was determined that government protocols were inadequate since they either prescribed organisms not environmentally-relevant or only looked at initial growth stages such as germination and emergence and not at effect, if any, on subsequent generations. Thus, new protocols were developed. Additionally, it was concluded that very little impact was seen on any of the terrestrial biota examined.

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List of Abbreviations

BNQ	Bureau de Normalization du Quebec
CCME	Canadian Council of Ministers of the Environment
CFIA	Canadian Food Inspection Agency
DMDW	Dechlorinated Municipal Drinking Water
EEC	European Economic Community
ESOC	Emerging Substances of Concern
F_0	Refers to parent plant and the seeds from parent plant
F_1	Refers to plant produced from seed grown from parent plants
ISO	International Organization for Standardization
MOE	Ministry of the Environment
NASM	Non-agricultural Source Material
OECD	Organization for Economic and Cooperative Development
OMAFRA	Ontario Ministry of Agriculture, Food, and Rural Affairs
PAR	Photosynthetically Available Radiation
PPCP	Pharmaceuticals and Personal Care Products
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
WEAO	Water Enviroment Asssociation of Ontario
WWTP	Wastewater Treatment Plant

Epigraph

"*Only by understanding the environment and how it works can we make the necessary decisions to protect it. Only by evaluating all our precious natural and human resources can we hope to build a sustainable future.*"

(UN Secretary-General Kofi Annan, 30 March 2005)

1. **Introduction**

1.1 Overview

Biosolids, and before that, their predecessor, night soils have been used for thousands of years as a beneficial, inexpensive source of fertilizer since they provide a source of slow-release nutrients to the soil that are essential for plant growth. Additionally, the present point in history marks a key stage in human population dynamics; it is the first time in recorded history when the majority of the population lives in an urban setting (Miller 2012). Thus, the question of what to do with the human waste produced each year remains an ever-pressing one.

Currently, biosolids are the treated continuously-produced solid by-product of a waste water treatment plant (WWTP) and there is a need for a sustainable practice for their disposal. To date, the city of Toronto produces 195,000 tonnes dry weight of biosolids a year and other major urban communities in the area generate another 110,000 dry tonnes per year which require disposal of (City of Toronto 2009). However, what does *sustainable* mean? V. Nazareth, an Environmental and Infrastructure Specialist for Water and Wastewater in Toronto, defines *sustainability* with respect to biosolids management as a program which considers the long-term view of protecting and preserving the environment for current and future generations and, as such, it encompasses all aspects of biosolids management from community well-being, economic prosperity, and environmental integrity (Nazareth 2007).

Due to the high organic matter and nutrient content of biosolids, there are many alternative choices for their disposal besides being landfilled or incinerated. Biosolids can be used for land reclamation in degraded areas such as on mines tailings, or for land-application such as the one that is of interest in this research as a source of inexpensive fertilizer for agricultural lands (CCME 2009). The natural biota present in the soil break down the organic matter which improves the soil structure, and in turn, leads to better crop yields and less soil erosion, both important factors to the farmer (Butt and Nuutinen 1998). However, since biosolids are the by-product of a WWTP, they may contain unwanted contaminants or

pathogens from industrial and residential wastes (Reilly 2001; Kinney *et al*. 2006; Kinney *et al*. 2008; Wu *et al.* 2008; CCME 2009; Clark *et al.* 2010). As a result, the public's perception of the worth of land-applying biosolids has become increasingly negative (LeBlanc 2007; Vyhnak 2008; City of Toronto 2009). It has been the public's opposition rather than any technical problems that has caused the discontinuing of a land-application program according to most regulatory agencies (USEPA 2000b). Consequently, this method of disposal has not been utilized to its full potential. To help answer the public's questions regarding biosolids, there is a need to determine if biosolids do in fact have any detrimental impact on the natural terrestrial biota and is thus the focus of this thesis.

When looking through the literature, it became obvious that most research focuses on chemical analysis of individual chemicals or classes of chemicals in the biosolids and not on the assessment of impact of the biosolids themselves to the actual terrestrial biota. Therefore, to fill in this gap, this thesis examined the impact, if any, of biosolids to terrestrial biota. Additionally, it is important for environmental relevance to examine not just one organism but to use a suite of organisms. Crouau *et al.* (2002) sum up the short-comings of using chemical analyses by stating that they are too expensive to use for all potential pollutants present, provide no information about bioavailability, and do not account for the significant number of possible antagonistic or synergistic reactions. These authors suggest that "chemical analysis must be complemented with ecotoxicological tests". Rogers from the USEPA also agrees, and adds that the slow turnaround time to get chemical results of regulated pollutants is additionally problematic (Rogers 1995).

Given the foregoing findings, there is a need to determine if the land-application of biosolids has a negative impact on the terrestrial environment and consequently if land-application is a sustainable practice.

1.2 Objectives

1) The overall objective is to determine if the land-application of biosolids to agricultural biomes is a sustainable practice. This objective is to be accomplished by means of a holistic approach to the investigation by examining selected environmentally-relevant, terrestrial organisms that are important to the Southern Ontario agricultural region (the area of study) and to determine if these organisms are negatively impacted by the land-application of biosolids. To achieve this objective, the following organisms were selected: *Folsomia candida* (springtails), *Lumbricus terrestris* (earthworm), *Zea mays* (corn), *Glycine max* (soya beans), *Plaseolus vulgaris* (common bean), *and Brassica rapa* (field mustard). These organisms were chosen not only because of their environmental relevance but because of the important role they each play in the environment.

   Although not discussed here in this work, preliminary bioassays were also carried out using *Daphnia magna* and *Hyalella azteca* to examine the potential impact of biosolids on the aquatic environment. For more information on these organisms and the bioassays see Appendix V.

2) A secondary objective of this research was to develop useable protocols. After examining and attempting to implement the existing government protocols, it was discovered that they were inadequate for the scope of this thesis. Therefore, useable protocols needed to be developed and tested with the individual indigenous terrestrial biota for use in a laboratory setup that simulated as closely as possible the natural environment.

1.3 History of Biosolids

Biosolids, as defined by the United States Environmental Protection Agency (USEPA), are "primarily organic solid product produced by waste water treatment processes that can be beneficially recycled" (USEPA 1994b). Biosolids are a *treated* by-product of a Waste Water Treatment Plant (WWTP) and are not raw sewage or sludge (CCME 2009). Once the sludge (the suspended or dissolved solids of the waste water treatment process) has been adequately processed and tested to meet government requirements for beneficial use it can then be termed "biosolids" (Kenney *et al.* 2006, 2008; NEBRA 2008; CCME 2009). The term "biosolids" was coined in 1991 by the Water Environment Federation (WEF), a group of sewage treatment plant operators and engineers and is used to denote a particular kind of sludge, one with beneficial uses (NEBRA 2008).

The use of biosolids is not a new concept. The recycling of human wastes or "night soil" has been a tradition in many parts of the world including China, many parts of Europe, South Korea, and many other areas for centuries (Crabtree 2000). The World Bank estimates that in China, to maintain their soil fertility, about one-third of their fertilizer requirements have been met by use of biosolids (Crabtree 2000). Along with manure, the land-application of human wastes as a means of disposal, as well as the recycling of nutrients, has been used by the Chinese for thousands of years, by the Europeans for more than a century and by the United States and Canada for over 50 years (Synagro 2002; O'Connor *et al.* 2005; Pepper *et al.* 2006; City of Toronto 2010a). Land-application of biosolids is still the most common method of disposal in the United States today (Wu et al. 2010). Additionally, Australia and New Zealand have been land-applying biosolids for over twenty years (Australian Water Association 2009). Closer to home, Ontario has been officially applying biosolids to agricultural land since 1979 when guidelines were first issued by the Ontario Ministry of the Environment (City of Toronto 2009; OMAFRA 2010a) without any documented health or environmental impacts when standards were followed (Environmental Commissioner of Ontario 2007). Biosolids are used as an inexpensive source of fertilizer to promote plant growth and to maintain soil structure due to their nutrient rich, organic content (Synagro

2002; O'Connor *et al.* 2005). And, land-application has been the most cost-effective and beneficial way to dispose of them (Pepper et. al 2006).

1.4 Benefits of Biosolids

In a natural setting, plants obtain their nutrients from their environment, grow and reproduce. These plants then die and decompose thereby returning the nutrients back to the soil. New plants take up these nutrients to use in their own growth and the cycle continues. Due to the introduction of agriculture and farming, this cycle is broken when the plants are harvested. As a result, the nutrients are not returned to the soil and farmers are now required to add fertilizers to replace what nature does automatically.

Biosolids can be used as a source of fertilizer or soil amendment since it provides the soil with a means of slow-release organic nutrients such as carbon and many macro-nutrients (elements required by plants in large quantities) such as nitrogen, phosphorus, potassium, calcium, magnesium and sulphur as well as micro-nutrients (elements required by plants in small quantities) including boron, cobalt, copper, iron, manganese, molybdenum, nickel, and zinc that are essential for plant growth (Brown 2006; Evanylo 2006; Carbonell *et al.* 2011). The addition of biosolids benefits crops also since very little, if any, leaching of these nutrients occur when application guidelines are adhered to (USEPA 2000c; Wang *et al*. 2009; Environmental Leverage Inc. 2010). Nutrients in the biosolids, present in the organic form, are less water-soluble and therefore more stable in the environment (Wang *et al*. 2009). Therefore, these nutrients remain in the cycle over time and are less likely to migrate through the soil to groundwater, as opposed to the inorganic forms found in the commercial fertilizers used by farmers and which usually only contain nitrogen, phosphorus and potassium (Brown 2006). These inorganic forms which are more water soluble, are quickly and easily washed out of the soil and into ground and surface waters (Wang *et al*. 2009).

Besides the macro- and micronutrient benefits that biosolids provide to the soil, they also add organic matter (Banks *et al*. 2006; Environmental Leverage Inc. 2010). The natural biota

present in the soil break down this organic matter. This in turn improves the soil structure by making the soil more porous which enhances the soil structure and improves moisture retention and permeability (Butt and Nuutinen 1998; Wang *et al*. 2008). This in turn improves the circulation of air and water in the soil which promotes better root systems leading to better crops yields (City of Toronto 2009). Additionally, this organic material helps to maintain good soil tilth (or 'health') which leads to a reduction in the potential for soil erosion (OMAFRA 2009). Land-application of biosolids to agricultural fields is an excellent means of recycling a continuously-produced useful by-product (Banks *et al*. 2006; Wang *et al.* 2009).

To the farmer, biosolids are more cost-effective compared to commercially-available fertilizers, especially since biosolids are provided free of charge as long as the user abides by the regulations established by the Ontario Ministry of Environment (MOE) and Ontario Ministry of Agriculture, Farming and Rural Affairs (OMAFRA) and other regulating bodies across Canada as to their use (Stewart 2005; Biosolids Management 2010). A typical application program could supplement the soil with 135 kg/ha of nitrogen, 250 kg/ha of total phosphorous, 250 kg/ha of total potash (potassium), 4000 kg/ha of organic matter, and other nutrients like copper, magnesium and zinc, (Environmental Leverage Inc. 2010), a cost saving to the farmer of $100-$250/acre (Stewart 2005; OMAFRA 2010a). In summary, to the agricultural sector, biosolids are a sustainable and cost-effective soil amendment that not only supports the economy (due to less waste being diverted to landfills) but reduces the dependence on the more costly fertilizers (Halifax Regional Municipality 2010; OMAFRA 2010a).

1.5 How Biosolids are Produced

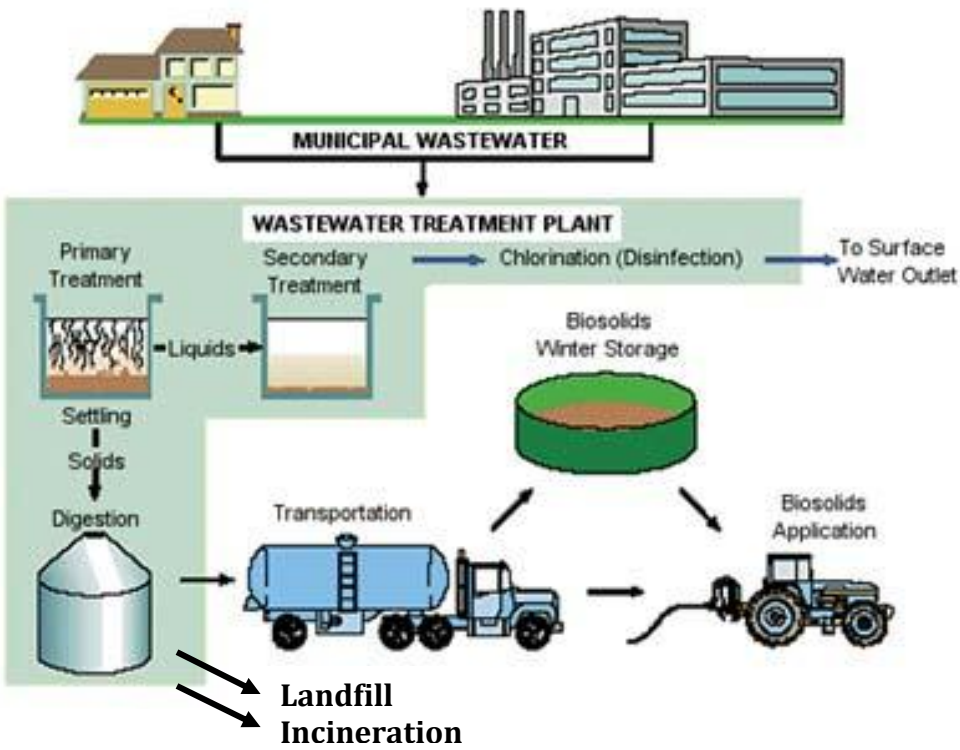

Figure 1: Illustration showing how biosolids are produced (Source: Modified from MOE 2010)

Figure 1 provides an overview of processes involved in producing biosolids. At a WWTP, the incoming wastewater is treated by several means including physical, mechanical, chemical, and biological strategies (City of Guelph nd). The primary treatment stage removes the heavier solids (such as trees branches, rocks and plastics) while the secondary treatment, a biological process, oxidizes organic pollutants (Sewage Treatment 2010). Additionally, nitrogen and phosphorus in the sewage is removed by the addition of chemicals such as aluminium sulphate ($Al_2(SO_4)_3 \cdot 12\ H_2O$) or calcium hydroxide ($Ca(OH)_2$) which precipitates or coagulates the nutrients (University of Waterloo nd). The solids are then allowed to settle. After the secondary treatment process, some of these solids are recycled back to the secondary treatment system as a source of bacteria to keep the system going, while the rest are sent to a digester where they remain until the organic solids are broken down to more

stable forms. In this stage, carbon dioxide (CO_2), ammonia (NH_3) and methane (CH_4) gases are produced (University of Waterloo nd). The sludge is then further treated to remove pathogens and to reduce volatile compounds (Pers. Comm. Michael Payne, OMAFRA; Sewage Treatment 2010). Depending on the municipality, different methods can be used and these will be discussed momentarily. The final treated product is now termed 'biosolids' (TorontoWater 2004; NEBRA 2008; Sewage Treatment 2010) and is then either sent for disposal (including incineration and landfilling), storage, or land-application. The various land-application methods will also be explained later, but first a closer look at pathogen and volatile compound reduction methods is warranted.

1.5.1 Aerobic Digestion

One of the more common methods of solids reduction for smaller wastewater facilities is liquid aerobic digestion (in the presence of oxygen). This method utilizes aerobic micro-organisms such as bacteria, fungi, protozoa, and rotifers (Seabloom and Buchanan 2005) to break down and stabilize the organic matter in the sludge, thus reducing the volume, the mass, and any pathogenic organisms originally present. The resulting products are water, carbon dioxide, and biosolids (Sewage Treatment 2010). As well as the organic materials that are present in the wastewater such as carbohydrates, fats, proteins, soaps, and urea, the micro-organisms also degrade the organically-bound nitrogen, sulphur, and phosphorus which results in the production of ammonia (NH_3), ammonium (NH_4), nitrate (NO_3), sulphate (SO_4), and phosphate (PO_4) (Seabloom and Buchanan 2005). Additionally, the aeration volatilizes organic substances such as hydrogen sulphide (H_2S), ammonia (NH_3), and other volatile organic compounds (Sewage Treatment 2010), thus eliminating the odour from the final product.

1.5.2 Anaerobic Digestion

Another popular process and a process that is important in the current research is liquid anaerobic digestion. The difference is this method utilizes anaerobic micro-organisms under anaerobic conditions (in the absence of oxygen) and without of aeration (Residua 2003;

DeBruyn and Hiborn 2007). Anaerobic digestion has been in use for over 150 years and the resulting product is much less offensive in terms of odour than the original raw sludge (Sewage Treatment 2010). Anaerobic digestion takes place in a series of steps in an enclosed vessel with the end goal again being the break down of the organic material present in sludge.

In the first step of anaerobic digestion, the sludge remains at 35°C for 12-20 days while the hydrolytic bacteria break down the insoluble organic material such as carbohydrates, fats, and proteins into their basic subunits of sugars, fatty acids and amino acids respectively (Residua 2003). These smaller subunits then go through the acidogenesis phase of anaerobic digestion where they are now available to the acidogenic bacteria and ultimately produce organic acids, hydrogen, carbon dioxide, and ammonia (Residua 2003). In the third step, the acetogenic bacteria present such as *Clostridium aceticum* and *Acterobacter woodii* convert organic acids to hydrogen, acetic acid, and carbon dioxide (Residua 2003; Marshall 2007). Finally in the fourth step, methanogens (which are obligate anaerobes) use the carbon dioxide as the ultimate oxidizing agent in their electron transport chain resulting in the reduction of CO_2 and H_2 and the production of methane.

Through anaerobic digestion, the volatile organic matter is converted to approximately 40% carbon dioxide and 60% methane (Residua 2003; Wang *et al.* 2008; Sewage Treatment 2010). Including the ammonia from the earlier step, these biogases can then be used for many beneficial uses such as in combustion process to run generators producing electricity, used as a fuel in furnaces for heat or cooking stoves, or the biogas can be cleaned and then used as a natural gas replacement (DeBruyn and Hiborn 2007). By utilizing the anaerobic digestion process instead of disposing of the solid waste in landfill, a municipality can in turn reduce their emissions of landfill gases (Residua 2003).

In the case of both aerobic and anaerobic digestion, the volume of the sludge is reduced (University of Waterloo nd) and pathogens are eliminated by 95% (Biosolids Management

2010). These two methods are the primary methods practiced and the least costly (Pers. Comm. Michael Payne, OMAFRA). In addition, both methods can be used in conjunction with other methods to also reduce the pathogens and/or further reduce the water content which will be discussed next (Pers. Comm. Michael Payne, OMAFRA).

1.5.3 Further Methods of Treating Biosolids

There are several methods to reduce the water content and pathogens of sludge thus making the product, biosolids, stable and less costly to transport. These methods include composting, dewatering, thermally dried (pelletization), alkaline stabilization, and the Lystek process. Except for dewatering, these methods of processing biosolids are more costly and usually only take place at the larger facilities (Pers. Comm. Michael Payne, OMAFRA). The biosolids used in this research were primarily stabilized using anaerobic digestion followed by subsequent mechanical dewatering (Region of Waterloo 2007; City of Guelph 2011) with one source being further treated using a pilot Lystek method (Region of Waterloo 2007) which incorporates alkaline stabilization in its process, and as such only these methods will be discussed further.

1.5.3.1 Dewatering

One such method to reduce water content and pathogens is dewatering. This is a mechanical process where chemicals such as ferric chloride as in the case as the biosolids used in this research, (Pers. Comm. Michael Payne, OMAFRA) are first added to release the water bound to the organic matter which is then removed by centrifugation, pressing, or belts (Sewage Treatment 2010). These mechanical methods remove 26% or more of the water and leave behind a ‘cake’ that can be land-applied (Pers. Comm. Michael Payne, OMAFRA). Due to the decrease in the amount of water, this form of biosolids is less expensive to transport to the fields than the liquid form (Pers. Comm. Michael Payne, OMAFRA; Evanylo 2006) and necessary if the biosolids are to be landfilled (Sewage Treatment 2010). At the Ashbridges Bay WWTP and the other three WWTP in Toronto, anaerobic digestion takes place in conjunction with dewatering which produces a biosolids cake that is 25-30% solids

(TorontoWater 2004; City of Toronto 2009). At this same facility, the methane gas produced is further utilized to heat and run the plant and the highly organic, nutrient-rich biosolids are sent to be either used as fertilizer on agricultural fields, incinerated, or is landfilled (TorontoWater 2004; Wang *et al.* 2008).

1.5.3.2 Alkaline Stabilization

Another method to treat the solids from the digester includes alkaline stabilization where lime (calcium hydroxide or calcium oxide) is added to raise the pH to as high as 12.4. This level of alkalinity kills any pathogenic bacteria present as well as destroys the odour by complexing with the odorous compounds such as H_2S and organic mercaptans (Pers. Comm. Michael Payne, OMAFRA; Sewage Treatment 2010). If water is present, the heat generated raises the temperature to 70°C which also helps kill bacteria. The low solubility of lime causes it to persist in the biosolids, thus preventing the re-growth of pathogens. Due to the high pH most metals are precipitated, thus reducing their solubility and mobility and phosphorous compounds are stabilized preventing eutrophication of surface waters (NLA 2010). Alkaline stabilization is considered a more cost-effective method, by as much as 60%, for treating solid wastes from a WWTP over composting, thermal drying or digesting methods (NLA 2010). The Lystek process uses a form of alkaline stabilization (Pers. Comm. Michael Payne, OMAFRA).

1.5.3.3 Lystek

A new method uses the Lystek technology that was developed in 2000 by Owen Ward at the University of Waterloo and has been in operation since 2003 as a pilot project at the WWTP in Guelph, Ontario (City of Guelph 2009). This process, illustrated in Figure 2, utilizes a municipality's dewatered biosolids and through a propriety process that involves chemical (alkalization with potassium hydroxide (KOH)) with elevated temperatures (steam at 65-70° C) and high-shear mixing, creates a liquid fertilizer that is purportedly pathogen-free (Lugoqski *et.al.* 2007).

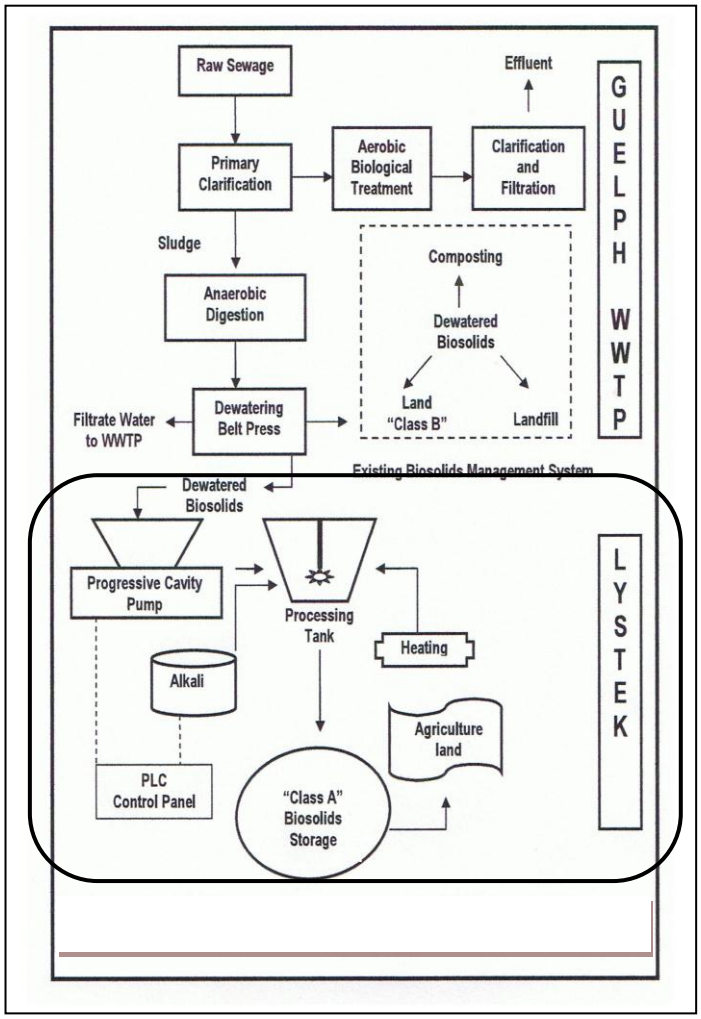

Figure 2: Process Flow Diagram of Guelph WWTP and Lystek System (Source: Singh *et al.* 2007)

The high-shear mixing contributes to the microbial and particulate breakdown while creating a homogenous, high solid content (20-28%) liquid that is not only stable for several years, but also has a low viscosity (reduced to 6,000 centipoises (cP) from 2 $x10^6$ cP) and can therefore be pumped and, as a result, is still able to be used in conventional machinery (City of Guelph 2009; Lystek 2009). These liquid biosolids can then be transported to farmers and safely land-applied. The economic advantage of the Lystek biosolids is not only the reduced operational costs associated with handling and storage of a more homogeneous product that facilitates better quality control but also when land-applied since it is much cheaper to transport a pumpable liquid than to haul dewatered biosolids (Singh *et al.* 2008).

As can be seen in Figure 3, by reducing the volume, the result is that there is less to transport, as much as 40-80% less than the biosolids from conventional treatment processes (Burtt 2008). The Lystek processed biosolids are also less odourous than conventional biosolids, are pathogen-free (exceeding Class A biosolids as defined by the USEPA as pathogens inactivated *before* they are land-applied), can be stored for extended periods of time without alteration, and have increased methane production (by 50-100%) (Lugowski 2007; Lystek 2009). Even after storing the Lystek-treated biosolids for about three years at ambient temperatures (between 17-22 °C), there is no evidence of re-growth of pathogenic bacteria such as *Salmonella*, *Escherichia coli* or faecal coliforms (Singh *et al*. 2007). However, they have not been tested for impact to biota.

Conventional dewatered biosolids (20%) with a viscosity of > 2,000,000 cp (Lystek 2009)

Lystek biosolids (20%) with a viscosity of < 6,000 cp (Lystek 2009)

Figure 3: Comparison of conventional and Lystek produced biosolids (Source: Lystek 2009)

1.6 Public Perceptions and Reality

Although Canada has been land-applying biosolids for over 50 years, mainly for agricultural purposes (CIELAP 2008; City of Toronto 2010a), there has been an increase in public concern and growing opposition towards the use of biosolids for land-application purposes (Nazareth 2007; LeBlanc 2007; Vyhnak 2008; City of Toronto 2009). As well as the possible odour caused by biosolids, people are concerned with the potential contaminants entering groundwater or surface water (Nazareth 2007) as well as the transport of these contaminants to other locations (Schoof and Haukal 2005). When the public get their information from sources such as the *Toronto Star* article "Sludge Series: Soiled Land….Worries grow over 'stew' of chemicals spread on farmland" and "Biosolids a 'disaster waiting to happen" (Vyhnak 2008) or the more recent *Globe and Mail* article "Loosening of rules about spreading sewage sludge on Ontario farms ….people who live near farm fields fertilized with sludge have gotten ill….with symptoms of headaches, vomiting and fevers" (Maurino 2012), concerns grow. However, the last article failed to mention the people who do not live beside fields spread with 'sludge' but whom also have the same symptoms. The general public are not getting all the facts. Sludge is not land-applied, biosolids are, and only if they meet the

requirements set out in the regulations. Otherwise, they are disposed of by other means such as land-filling or incineration (O. Reg338/09). The industry is presently focusing their efforts on these concerns of the public (WEAO 2009). New sewer use by-laws at the municipal level are being implemented that prevent the dumping of contaminants in to the sewer systems the first place, as well as the implementation of the Household Hazardous Waste Programs to help increase public awareness and again reduce the amount of contaminants entering the system (WEAO 2009). Government agencies as well as others are working on outreach programs to help inform the public about the benefits and the overall background of biosolids so that they are more aware and better informed to make decisions (LeBlanc, 2007). However, these concerns need to be addressed because contaminants are present and is thus the focus of this research. These concerns will be discussed next.

1.7 Concerns with Biosolids

To date, there has been much research on the presence and fate and transport of contaminants in sewage sludge and biosolids. As has already been mentioned, wastewater treatment facilities were designed to treat incoming sewage from residential (90%) and industrial (10%) sources (OMAFRA 2010b) by dividing it into two fractions, solids and liquids, and processing the liquid waste so that the effluent can safely be returned to a surface water source nearby (Kinney *et al.* 2008). During this process there is the potential for unwanted hydrophobic compounds in this waste (which have relatively large octanol-water partitioning coefficients (K_{ow})) entering a WWTP to partition into the organic-rich solid phase (biosolids) and settled out. Additionally, but to a much lesser extent, hydrophilic compounds that are not removed with the effluent by coagulation and flocculation can also end up in the biosolids adsorbed to the particles and thus have the potential to be applied to the soil as well (Fent 1996; Kinney *et al*. 2006, 2008; Smyth *et al*. 2007; Wu *et al.* 2008; Sabourin *et al.* 2009; Wang *et al*. 2009). Many contaminants are neither completely metabolized nor degraded by the treatment processes at the WWTP and thus leave the facility as they entered (Kinney *et al*. 2006; Wu *et al*. 2008). As a result, they can persist or breakdown in the environment. Several major categories have been reported as substances of concern in biosolids (WEAO

2010). These include industrial chemicals (pesticides, plasticizers, and alkylbenzene sulfonates, etc.), alkyphenols, flame retardants, hormones, pharmaceuticals, personal care products, certain metals (arsenic, mercury, silver, and selenium, etc.), polyaromatic hydrocarbons, polychloinated dioxins and furans, and pathogens (CCME 2009; WEAO 2010; IJC 2011). However, their environmental fate and their significance is not known or well understood.

1.7.1 Fate and Transport of Biosolids

Once land-applied, contaminants present in the biosolids have the potential to enter the environment either as particulates into the air, run off directly into surface waters, or to migrate further into the soil and thus enter groundwater (Schoof and Haukal 2005). If the contaminants enter surface or groundwater through runoff or leaching they could potentially affect aquatic biota as well (Wu *et al*. 2008). If the contaminant enters the air as particulate matter for example through the farmer tilling the field, it could migrate via long range transport elsewhere (Schoof and Haukal 2005). If there is a significant rainfall event after land-application, there can be transfer of contaminants or pathogens down slope from the site (WEAO 2010). Figure 4 diagrammatically shows potential routes of environmental contamination considered by the USEPA when they were performing their risk assessment (the largest at the time) for the land-application of biosolids to agricultural lands and Table 1 follows with a brief description of these potential exposure pathways of contaminants to humans that were assessed.

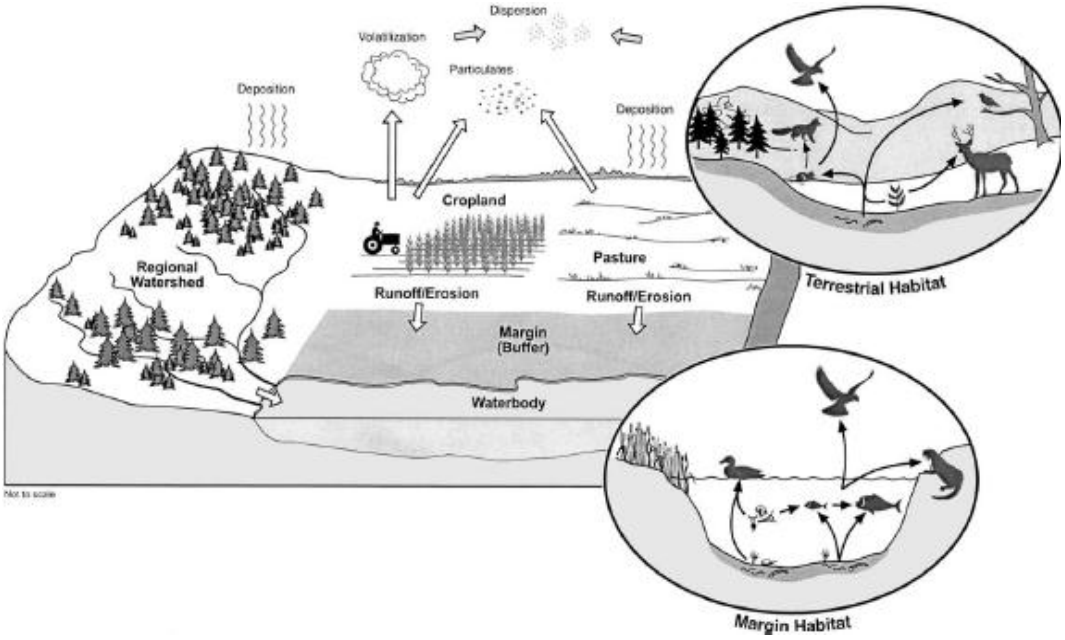

Figure 4: Sources of Environmental Contamination by Land-application of Biosolids (Source: Schoof and Haukal 2005)

Table 1: Human Exposure pathways evaluated in the Part 503 Rule Risk Assessment (Modified from Schoof and Haukal 2005)

Pathway	Potential Risk
biosolids ⇨ human	ingesting biosolids (e.g. child eats soil amended with biosolids)
biosolids ⇨ soil ⇨ plant ⇨ human	Ingesting of plants grown in biosolids amended soil
biosolids ⇨ soil ⇨ plant ⇨ animal ⇨ human	Ingesting animal product of animal raised on forge grown on biosolids amended soil
biosolids ⇨ soil ⇨ airborn dust ⇨ human	inhalation of particles (dust) when working fields
biosolids ⇨ soil ⇨ surface water ⇨ human	drinking surface water or ingesting fish from water polluted by contaminants from biosolids
biosolids ⇨ soil ⇨ groundwater ⇨ human	drinking well water containing pollutants leached from soil amended with biosolids

1.7.1.1 Organic contaminants

The presence of organic contaminants in biosolids are of concern because of their ability to adversely affect human health and the ecosystem due to their potential toxicity (such as carcinogenicity or endocrine disruption) and since biosolids which are enriched with organic material could provide a ubiquitous non-point source of organic contaminants to the environment (CIELAP 2008). Schowanek *et al.* (2004) describes a need for a more systematic approach to derive quality standards for organic contaminants in biosolids that are to be applied to agricultural lands in response to the outdated European Union Sludge Directive 86/278/EEC. Closer to home, Table 2 indicates that the regulations for organic contaminates in biosolids in Canada is very limiting (CCME 2010). In the United States, organic contaminants are not required to be tested for in biosolids at all (Harrison *et al*. 2006).

Table 2: Standards for Organic Contaminants in Biosolids in Canada (Modified from CCME 2010).

Jurisdictions	Contaminant			
	Dioxins& Furans (ngTEQ/kg)	**PCB**	**PAH**	**Other Organic Chemicals**
NL	currently uses USEPA Part 503 Rule and BNQ Standard 0413-400/2009 on Soil amendments, if applicable			
NS (CLASS A)	17	Levels of contaminants not specified		
NS (CLASS B)	50	Levels of contaminants not specified		
PEI	Levels of contaminants not specified			
NB	Levels of contaminants not specified			
QC (CLASS C1)	17	rarely detected- not deemed of concern	rarely detected- not deemed of concern	
QC(CLASS C2)	50			
ON	Levels of contaminants not specified			
MB	Levels of contaminants not specified			
SK	Levels of contaminants not specified			
AB	Levels of contaminants not specified			
BC	risk is managed by site specific review by medical health officer			
YUKON	not available			
NWT	Levels of contaminants not specified			
NU				
CFIA	27 (Interm)			
BNQ 0413-400/2009 Biosolids	27			

Kinney *et al.* (2006) determined which of a specified eighty-seven organic contaminants were present in biosolids and found a minimum of thirty in any one biosolids of the nine different sources of biosolids tested. Harrison *et al.* (2009) suggests that the Environmental Risk Assessment initiated in 1988 by the USEPA for their Part 503 Regulations on biosolids are outdated and emphasises the needs to revisit the regulations (Harrison *et al*. 2009). Very recently, standards have been proposed for inclusion into Part 503 of the USEPA biosolids regulations to limit the concentration of dioxin and dioxin-like compounds in biosolids (CCWA 2007). While some provinces in Canada have Standards for Organic Contaminants in Biosolids (under the Canadian Food Inspection Agency) such as PCB, PAH, dioxins and furans, Ontario is not one of them (CCME 2010). (Table 2). Part 503 of the USEPA is presently undergoing a review in the form of the Targeted National Sewage Sludge Survey (TNSSS) and it is expected that new guidelines on their use and the level of contaminant will be coming in the near future from this study (National Biosolids Partnership Webcast 2012).

1.7.1.1.1 Legacy Organic Contaminants

Few biosolids impact studies exist that examine legacy contaminants. Legacy contaminants are not well defined, but pertain to those chemicals that are persistent in the environment, present on-going challenges in clean-up strategies, and have a history of regulation. This group of chemicals consists of more than 23,000 chemicals including but not limited to polychlorinated biphenyls (PCBs), polynuclear aromatic hydrocarbons (PAHs), chlorinated dioxins and furans, pesticides, flame retardants linear alkybenzene sulphonates (LAS), and alkylphenol surfactants (AP) (Harrison *et al.* 2006; WEAO 2010). The older 2001 report by WEAO found that Ontario regulations were lacking in acceptable concentration data of dioxins and furans allowed in biosolids destined for agricultural application. From the work done by Harrison and her group (2006), they found 516 organic compounds in biosolids and identified groups of chemicals that are more abundant, such as pesticides, PAHs and PCBs, and emphasised the need for caution when applying these soil amendments. This group summarized that 83% of these 516 compounds were not even on the EPA list of priority pollutants and 80% are not target compounds of the EPA (Harrison *et al.* 2006). Table 3 is a

representation of these legacy organic contaminants along with the range of concentration in mg/kg (or ppm) dry weight that have been found in biosolids and thus have the potential to be land-applied (Harrison *et al.* 2006). However, Harrison *et al.* did not indicate the environmental relevance of these numbers since impact studies were not conducted.

Table 3: Concentration of organic chemicals reported in biosolids (Modified from Harrison *et al*. 2006). ND = non detect.

Legacy Contaminants	Category	Range mg/kg dry wt
dieldrin	pesticide	ND-64.7
toxaphene	pesticide	51
bisphenol-A	phenols	0.00010-32.100
phthalates	phthalate acid ester/plasticizers	ND-58.300
dioxins and furans (polychlorinated dibenzo)	polychlorinated biphenyls, naphthalenes, dioxins and furans	ND-1.7
PCB congeners	polychlorinated biphenyls, naphthalenes, dioxins and furans	ND-765
anthracene	polynuclear aromatic hydrocarbons acenaphthene	ND-44
benzopyrene congeners	polynuclear aromatic hydrocarbons acenaphthene	ND-24.7
naphthalene	polynuclear aromatic hydrocarbons biphenyl	ND-6610
total PAH	polynuclear aromatic hydrocarbons biphenyl	ND-199
coprostanol	sterols, stanols and estrogens	216.9
alkylbenzene sulfonates	surfactants	<1-30,200

Kinney and his team (2006) examined nine different biosolids products destined for land-application and found median concentrations of PAHs (by-product of fuel burning) present. Phenanthrene was found at levels of 342 µg/kg, anthracene at 139 µg/kg, fluoranthene at 1090 µg/kg, and pyrene at levels of 1110 µg/kg. Little work has been done on terrestrial organisms, but these contaminants are known to have toxic effect on the aquatic environment. In Kenny *et al.* (2006) study, only their presence was determined, not their impact. Clark *et al.* (2010) investigated the distribution of polybrominated diphenyl ethers (PBDEs) in an Australian wastewater treatment plant and found that over 99% of the PBDEs entering the WWTP were removed, and this was mainly from sedimentation (96%).

The study by Bright and Healey (2003) looked at organic chemicals such as volatile organics, PCBs dioxins/furans, chlorinated pesticides, and petroleum hydrocarbons in biosolids produced at the Greater Vancouver WWTP and found they were not detected at environmentally-relevant concentrations in the 36 samples analysed. They also found that when these biosolids were mixed with uncontaminated soil at agronomic rates (8 tonnes/ha), there was adequate protection against environmental risk from these organic chemicals with the exception of the petroleum hydrocarbons or the microbial metabolites due to reducing concentrations below levels at which risk might occur. However, they do not assess impact; they only looked at risk assessment and present British Columbia soil concentrations benchmarks.

Fent (1996) examined the raw effluent entering a Zurich WWTP and found the concentrations of monobutylin (MBT), dibutylin (DBT), and tributylin (TBT) (used for various industrial and agricultural applications such as polyvinyl chloride stabilizers, pesticides, and preservatives) to be in the range of 140-560, 130-1030, and 60-220 ng/L respectively and after being processed, found concentrations of MBT, DBT, and TBT in the range of 0.3-0.8, 0.5-1.0 and 0.3-1.0 mg/kg dry weight respectively in the biosolids. While these organotin compounds are known to have adverse effects on the aquatic biota (gastropods), their ecotoxicological implication to the terrestrial biota is not well understood, but bioaccumulation up the food web is a possibility (Fent 1996). Fifty mg/kg TBT has been shown to enhance nitrate-nitrogen production in the soil and at concentrations of 100-250 mg/kg were shown to stimulate ammonification (Fent 1996). These levels are at much higher concentrations than were found in the biosolids.

1.7.1.1.2 Emerging Substances of Concern

This relatively new class of contaminants termed emerging substances of concern (ESOC) (CCME 2009) are environmental contaminants that are either synthetic or naturally-occurring. ESOCs consist of the many chemicals used daily by society that are either unregulated, or inadequately regulated, and has been recently detected in the environment in trace levels usually due to new, or improved, analytical methods in chemistry, hydrology and engineering to do so (Battaglin *et al.* 2007). ESOCs are organic compounds such as pharmaceuticals and personal care products (PPCP) that society consumes or washes down the drain daily which have the potential to pass through the WWTP and end up in the biosolids and thus be land-applied (Wu *et al.* 2008; CCME 2009). Since there is inadequate toxicological data on these substances, it is often difficult to determine their risk to human beings or the environment (Battgling *et al*. 2007). ESOCs have been appearing in wastewater but to date there is a noticeable lack of a database recording their presence in Canadian biosolids (CCME 2009). The detection of these compounds in the biosolids (in ng/L or ng/g total solids (dry weight) (ppb) levels) does not necessarily mean there is a risk to humans or the environment but the concern is due to their persistence in the soil and the potential bioaccumulation in the food chain with repeated application (CCME 2009). ESOCs can be categorized into several groups including brominated flame-retardants, alkylphenols and their ethoxylates, industrial contaminants (including plasticizers, and surfactants), current-use pesticides, synthetic musks, and other organic wastewater constituents such as pharmaceuticals, personal care products, hormones, sterols, and disinfectants (CCME 2009;WEAO 2010; IJC 2011).

In humans and other biota, fifty to ninety percent of the active ingredient of a pharmaceutical or prescription drug that enter the body is not absorbed but instead is excreted (Jjemba 2002; CIELAP 2008). PPCP such as ciprofloxacin (antibiotic), tetracycline (antibiotic), carbamazepine (anticonvulsant), triclosan (antibacterial and antifungal agent) or galazolide (fragrance in some perfumes) are ending up in the environment (Kinney *et al*. 2006; Wu *et al*. 2008; CCME 2009). At the date of their research in 2006, Kinny *et al.* focused mainly on

the susceptibility of the aquatic environment due to wastewater discharge, and less was understood about the presence and fate of the organic wastewater contaminants (OWC) in biosolids. The information summarized in Table 4 provides the median concentration (in µg/kg) of OWC that were found in different biosolids products (cake, kiln dried, composted, or dewatered) from nine sources. With larger K_{OW} values, these contaminants would be removed in the primary sedimentation stage and would partition preferentially into the organic rich biosolids (Kinney *et al.* 2006; Clarke *et al*. 2010). Again, this research does not indicate the environmental relevance of these numbers since impact studies were not carried out, just that these contaminants were found in biosolids.

Table 4: Carbon Normalized Concentrations, Organic Carbon (µg/kg), of Organic Wastewater Contaminants Detected in all Nine Biosolids (Modified from Kinney *et al*. 2006)

Organic Wastewater Contaminants	Use	Log K_{ow}	Median of all Biosolids (µg/kg)
carbamazapine	antiepileptic	2.45	68
diphenhydramine	antihistamine	3.27	340
fluoxetine	antidepressant	4.05	370
d-limonene	fragrance	4.57	630
tonalide (AHTN)	fragrance	5.70	11,600
galaxolide (HHCB)	fragrance	5.90	3,900
indole	fragrance	2.14	19,600
4-tert-octylphenol	detergent metabolite	5.28	4,030
para-nonylphenol-total	detergent metabolite	5.92	261,000
nonylphenol, dithoxy-total	detergent metabolite	4.21	7,010
bisphenol A	fire retardant	3.32	4,690
3-beta-coprostanol	steroid	8.82	126,000
cholesterol	steroid	8.74	209,000
beta-sitosterol	steroid	9.65	131,000
stigmastanol	steroid		174,00
phenol	disinfectant	1.50	2,180
triclosan	disinfectant	4.53	10,200
diethylhexyl phthalate	plasticizer	7.88	10,500
para-cresol	preservative	1.97	4,400
skatol	fecal indicator	2.60	2,510

The WEAO Report (2001, updated 2010) examined different contaminants and their fate in biosolids. They found, for example, surfactants such as linear alkylbenzene sulphonate (LAS), which are widely found in commercial products and detergents, were being degraded in aerobic conditions and are more biodegradable than the branched version they replaced. Although LASs have a half-life of 7-9 days in the soil, their presence was found in biosolids in higher concentrations (in the mg/kg total solids dry weight levels) than other contaminants and had the potential, once land-applied, to dissolve bio-membranes of the natural soil biota and invertebrates as well as increase the mobilization of hydrophobic contaminants which could also be present in the soil (WEAE 2010). Additionally, alkylphenol ethoxylates (APEs) are the most common non-ionic surfactant used worldwide in paints, detergents, and pesticides, to name a few sources. APEs were found to be degraded by soil microorganisms but are of concern because of their weak endocrine disrupting potential and the fact they are being found in biosolids in the range of 500-1000 mg/kg (dry weight). Also, concentrations of these hydrophobic contaminants have been found in higher amounts in anaerobically digested biosolids than in other forms of treated biosolids (WEAO 2010; IJC 2011). With a half-life of 10-25 days, these contaminants are not persistent in the soil nor do they easily migrate through the soil, mineralize, or get taken up by plants. Thus, they are not presently felt to be a problem (WEAO 2010).

Carballa *et al.* (2007) worked on the fate of PPCPs during anaerobic digestion of sewage sludge. They found better removal efficiencies with antibiotics, musks and natural oestrogen, but other PPCPs such as tranquilizers, anti-inflammatory, and X-Ray contrast mediums only had a 20-60% removal while the anti-epileptic drug, carbamazepine showed no elimination at all. Therefore, what was not removed at the treatment facility could be sent to fields and be land-applied. The summary report by CCME (2009) indicated there was a lack of data on the removal efficiency of the different methods for treating biosolids and there was not sufficient data on the reduction of ESOCs. Therefore, what was being land-applied was not known. The work by Jelic *et al*. (2011) showed that although the WWTP met their regulatory requirements for wastewater treatment, (91/271/EEC) they were only somewhat successful in

the removal efficiency of pharmaceutical compounds. They looked at the fate of forty-three pharmaceutical compounds from three different WWTP, sampling over a two-year time span and found that twenty-one of the pharmaceuticals accumulated in the sludge of all three plants in concentrations up to 100 µg/kg. For example, naproxen's recommended adult dosage is 5.50x 10^3 µg every 6 to 8 hours. Since these compounds were found in the sludge (the pre-treated product) and chronic toxicity of such mixtures was unknown, the risk posed to the environment was not fully assessed. They concluded that subsequent work needed to take place at the WWTP on the biosolids before they could be released, to prevent the introduction of these pharmaceutical compounds into the environment. Wu *et al*. (2008) looked at the persistence of pharmaceuticals in biosolids during storage when exposed to sunlight and aeration and found no elimination of carbamazepine, ciprofloxacin, or triclosan. They did find that clindamycin, clarithromycin, erythromycin, doxycycline, and tetracycline were eliminated under these conditions. The work by Daughon and Ternes (1999) examining the aquatic system stated that the primary source of PPCP to the terrestrial environment is *probably* from the disposal of biosolids from WWTPs onto the land and thus entering the waterway by this means. They indicate that while many of these individual contaminants are found in the environment in the ppb range, they do not exist in isolation but instead are present along with the many other contaminants and often share the same mode of action and thus could lead to a significant effect on the indigenous biota due to additive exposure (Daughon and Ternes 1999). But again, this work does not look at their impact, just how PPCP could get to the terrestrial environment.

The work by Lapen *et al.* in 2008 spiked biosolids with various PPCP then simulated a rain event to observe the tile runoff for the presence or absence of these spiked compounds. This was not an environmentally-relevant study since the contaminants would not normally enter biosolids at this stage and consequently may not even be in the biosolids in the form, or concentrations, as spiked in this research. Therefore, the later work by Sabourine *et al*. that was continued in 2009 using dewatered municipal biosolids is a more relevant body of work to examine the fate of PPCPs in biosolids since this work used the PPCP that were originally

present in the biosolids and did not unnaturally spike them. Once again, impact on the terrestrial biota was not investigated. Sabourine *et al.*'s work showed that < 1% of certain PPCPs in the biosolids were found in the runoff (those with log K_{ow} greater than 3.18) such as triclocarban, ibuprofen, and naproxen but others such as acetaminophen, carbamazepine, or caffeine (compounds with log K_{ow} of 2.45 or less) had greater than 1% migration thus indicating that the log K_{ow} could be used as a potential indicator for migration of various PPCPs present in biosolids.

All these researchers were looking for the presence of the contaminant, either in the sludge, biosolids, or the soil but not if it had any impact on the natural biota to which it was being applied though the land-application of biosolids. This is an area that needs to be examined. To reiterate, contaminant research examines fate and transport. Few studies examine impact.

1.7.1.2 Heavy Metals

Once land-applied, any inorganic ions including heavy metals which can be found in most municipal biosolids could leach into the soil if the soil or biosolids are acidic (Biosolids Management 2010). If near-neutral or alkaline conditions are maintained as prescribed in the regulations, these metals will not be readily leached from the biosolids or through the soil (O. Reg. 338/09; Biosolids Management 2010; Beecher 2012). Metal movement through the soil and their availability to plants can increase under certain conditions such as soil type, soil pH, and water availability. The research by Sloan *et al.* (1998) examined a plot of agricultural land that had cumulative biosolids loadings and examined them for the recovery of Cd, Cr, Cu, Ni, Pb, and Zn after sixteen years. They found the concentrations of these six metals to be significantly greater ($p=0.01$) at a depth of 0.30m compared to a control plot; were only slightly greater ($p=0.05$) at a depth of 0.45m and the same as the control below 0.45m. Ninety-five percent of Cd, Cu, Pb, and Zn were retained in the top 0.3m of soil. Sloan *et al.* also found that greater than 90% of the heavy metals could still be found in the top 0.15m in the first six years after application of biosolids to the land and the movement deeper into the soil profile could be due to leaching or tillage. They concluded that the land-

application of biosolids can, over time, significantly increase the concentration of heavy metals in agricultural soils (Sloan *et al*. 1998).

Kulbat *et al*. (2003) observed that heavy metals such as Ag. Cd, Cr, Cu, Ni, Pb and Zn were greatly decreased in the biological treatment stage of a WWTP over the mechanical treatment stages. Wang *et al.* (2006), looking at similar metals, show that metal uptake by sludge is appreciably affected by pH, stating that Ni(II) and Co(II) are the least adsorbable, while Pb(II) and Cu(II) are the most easily adsorbable. Although the Toronto's Sewer Use By-Law, one of the strictest in North America, has successfully reduced the amount of metals in Toronto biosolids, they still remain in small quantities (Biosolids Management 2010). Due to the fact that the content of the biosolids depends largely on what the industrial, commercial, and residential activities are in the surrounding community, the amount of metals in the biosolids may vary and must be monitored (OMAFRA 2010a). Table 5 is a summary of the metal concentrations that were found in the biosolids of eleven Canadian WWTP (CCME 2009). To Table 5 for comparison, the amount of metals detected in biosolids after processing by the Lystek method as well as the allowable levels under Ontario regulations have been added as these values were determined based on what was needed by the plants while not being toxic to either plants or humans. (See section 1.8.3.1). While we can see that the metals concentration in both the Lystek and regular biosolids are well below Ontario regulated limits, continuous exposure to indigenous organisms upon repeated application has not been assessed.

Table 5: Metal Concentration Data in 11 Canadian Treated Sludge and Biosolids Samples (Modified from Singh *et al.* 2007; Lystek 2009; CCME 2009)

Metal	# Detected (out of 11 samples)	Concentration (mg/kg Total Solids dry weight		
		Maximum Detected Conc'n	Typical Lystek Conc'n	Maximum Conc'n Allowable
Arsenic	7	6.7	1.83	170
Cadmium	2	1.2	1.15	34
Chromium	10	120	124.96	2800
Cobalt	7	4.2	6.48	340
Copper	11	890	833.65	1700
Lead	9	55.5	36.18	1100
Mercury	11	3.2	1.08	11
Molybdenum	8	8.6	15.46	94
Nickel	9	21.1	28.53	420
Selenium	6	3.2	4.50	34
Zinc	11	647	1289	4200

Antonious *et al*. (2010) looked at the unique properties of biosolids from different sources to determine if heavy metals were being incorporated into plants grown in soil amended with biosolids. Their particular plant of interests was *Cucurbita pepo* (summer squash) and they found that when looking at the fruit, the human consumable portion of this plant, that of the heavy metals analyzed (Cd, Cr, Cu, Mo Ni, Pb, and Zn) there were none that were above permissible levels of U.S. guidelines for edible fruit. For example, maximum levels of Cd (0.03 µg/g), Ni (2.5µg/g), and Pb (0.01µg/g) were found based on dry weight of fruit. This work does not assess the impact to these squash i.e. their growth rates or reproduction capabilities, just the presence of these heavy metals.

1.7.1.3 Pathogens

Pathogens are also a concern since they are not completely destroyed (only reduced by 90 to 99%) (MOE 2009) at the WWTP and thus could cause harm to humans or animals when land-applied. Reilly (2001) found that the WWTP in Ottawa had no reduction in *Giardia* cyst levels and only a 50% reduction in Cryptosporidium oocyst. Gerba *et al.* (2002) did a literature review of the resistance of emerging pathogens and found that hepatitis A and adenoviruses are most likely to survive the WWTP process and prolonged exposure in the environment since they are thermally-resistant viruses, but microsporida and Cyclopora were

unlikely to survive an anaerobic digestion and could not survive under low moisture conditions in the environment. Rogers and Smith (2007) indicate in their work that the indigenous soil microorganisms may have a competitive edge and aid in the decay of pathogenic bacteria that could be land-applied with the biosolids. Canada has been land-applying biosolids for over 50 years and if the regulations are adhered to, there is little scientific evidence of harm from pathogens and to date there has not been any scientifically documented evidence of problems from pathogens in biosolids since they are strictly regulated and monitored (Environment Commissioner of Ontario 2007; Biosolids Management 2010). However, concerns have been voiced over the safety of crops grown on fields that use biosolids since the possibility of the transmission of pathogens such as bacteria, Helminth worms, protozoa, or viruses exists (Nazareth 2007). Table 6 from the WEAO updated report (2010) show the survival time of the different classes of pathogens present either on soil or plants. Most are destroyed in a few months (thus the need for the waiting period between the land-application of biosolids and land use) but observed that Helminths can survive in the soil for up to seven years (WEAO 2010).

Table 6: Pathogen survival time on soil and plants (WEAO 2010)

Pathogen	**Soil**		**Plants**	
	Absolute Maximum	**Common Maximum**	**Absolute Maximum**	**Common Maximum**
Bacteria	1 year	2 months	6 months	1 month
Viruses	6 months	3 months	2 months	1 month
Protozoa	10 days	2 days	5 day	2 days
Helminths	7 years	2 years	5 months	1 month

Gottschall *et al*. (2009) examined two methods of land-application of biosolids, direct injection and surface spreading, and found that the direct injection method had significantly higher *E. coli* and *Clostridium perfringens* in the tile water than the surface spreading method, but there was a 94% reduction in *E. coli* and 60% for *C. perfringens* of the initial loading at the 100 day post application study period. Zaleski *et al.* (2005) state that even though there are concerns of the risk from pathogens to the general public, as well as the

workers dealing with biosolids, there has never been an established link between the land-application of biosolids and an illness. Pepper *et al.* (2006) state that bacteria not only have the ability to replicate quickly, but also, to adapt genetically and therefore new pathogens will emerge. Thus there is a need to understand the occurrence of pathogens in the biosolids destined for land-application as well as the effectiveness of the treatment process at the WWPT to ensure the safety of biosolids.

Additionally, along with the pathogens present in the biosolids are the multitude of antibiotics that we consume and excrete and which could lead to antibiotic-resistant bacteria in the soil, potentially leading to pathogen resistance of the natural soil microbial population or be assimilated by the plants or other living organisms (Wu *et al*. 2008). Table 7 summarizes the data of a fifteen month study by Brook *et al.* (2007) with different sources of soil (without biosolids and with continuous applications of biosolids) and biosolids that would be land-applied looking for four different antibiotic resistant bacteria. They used heterotrophic plant count (HPC) (a standard microbial method to identify the presence heterotrophs or organisms that utilize organic compounds) to determine the percentage of antibiotic-resistant bacterial and concluded that there was no significant difference ($p>0.05$) between the antibiotic-resistance of the soil bacteria in either the soil with or without biosolids applied to it.

Table 7: Fraction of Antibiotic-Resistant Bacteria in Biosolids and Soil with and without Biosolids Application (Modified from WEAO 2010)

Matrix	Antibiotic Resistance (% of Total HPC Concentration)			
	Ampicillin	Cephalothin	Ciprofloxacin	Tetracycline
Field with no biosolids applied	8.1	10.1	3.1	2.4
Field with continuous biosolids applied	7.9	11.0	9.2	2.8
Biosolids (acceptable for land-application)	3.6	63.6	0.1	0.4

1.7.2 Impact of Biosolids on Terrestrial Organisms

While there is significantly less research looking at the impact of biosolids on terrestrial organisms, the few studies suggest that little impact occurs and that the public misgivings may be misplaced. However, chemical analysis suggest the abundance of contaminants, therefore impact studies must continue. The 2004 study conducted by Jaques Whitford Limited for Toronto Public Health performed a risk assessment on human, wildlife, and plants potentially exposed to biosolids in the pellet form in Toronto Parks. They found no risk to human health (including workers dealing daily with the making and distribution of the pellets as well as visitors to parks), nor to plant or soil organisms. When examining the potential impact on wildlife and pets, they again found no risk expected except for chromium III in robins. When carrying out the assumptions for risk assessment, they tend to overestimate the levels of exposure and therefore provide a very conservative potential risk factor (Jaques Whitford 2004). Consequently, in the case of the robins, the value does not mean an impact to the robins, but rather the risk cannot be ruled out. For example, if the robins do not migrate as normal away from the Toronto area where the pellets are applied during a portion of the year, their exposure to chromium III could put them at risk.

Banks *et al*. (2006) looked at several different organisms. Their work investigated the ecotoxicity of metals found in soils amended with municipal biosolids using earthworm (*Eisenia fetida)* and nematode mortality (14-Day and 24-hr respectively) and earthworm reproduction (7-weeks), seedling germination and root elongation of *Lactica sativa* (lettuce), *Panicum milliaceum* (millet) and *Rapharus sativus* (radish), and microbial respiration as indicators. The results they obtained for the different organisms saw no pattern emerging between the different locations analyzed and concluded that current regulations were adequate for the protection of the ecosystem. The most common negative effect with the different sources of biosolids tested was a reduction in biomass of the earthworms and poor germination with the lettuce seeds but these results came from sites that were contaminated pre USEPA 503 guidelines (i.e. before 1993), and were used because they had high levels of metals, low pH, and increased salinity. With the other amended sites, where there was higher

microbial respiration (they concluded due to the labile carbon), the plant bioassays provided shorter roots but not detrimentally so, and no restriction on survival, growth or reproduction was found with the nematodes or worms (Banks *et al*. 2006).

Snyder *et al*. (2011) looked at the effect of triclocarban (TTC) (antibacterial and antifungal agent in disinfectants and soaps) on *Eisenia fetida*, *Paspulum notatum* (Bahia grass) and soil microorganisms through chemical analysis of the organism's tissue. Due to the popularity of this antibacterial agent, found in many personal care products since the 1950s, TTC can now be found in biosolids at relatively high concentrations (0.19-441 mg/kg) (Snyder *et al.* 2011). The low solubility (0.045 mg/L) and log K_{ow} of 3.5 of TTC make it a substance of concern to the environment because it tends to stay at the site of application and thus increases the exposure time to the indigenous organisms present. Therefore, it has the potential to increase in concentration with repeated biosolids applications (Snyder *et al.* 2011). Snyder *et al.* found the bioavailability of TTC in spiked biosolids to *E. fetida* had an LC_{50} of 40 mg TTC/kg using the amended fine sand method, which corresponds to an application rate of 22 Mg/ha for 100 years to reach the equivalent of 40 mg TTC/kg amended fine sand assessment. Therefore, no impact was seen and the author suggested further work using *Lumbricus terrestris* or *L. rubellus* due to their burrowing behaviours and feeding habits and to examine sublethal effects such as substrate avoidance and juvenile hatching. This research also found no effect on respiration (measured as CO_2 evolution) of the soil microbial community. The bioaccumulation of TTC in the *Paspulum notatum* was 0.0004-0.0007g soil/g tissue, or in other words, insignificant. This 2011 research was one of the first to examine their biota by looking for writhing, stiffening, or inactivity as behavioural responses to their contaminant. They did not look at weight loss or reproduction but did also look for swelling or ulceration under the different treatment conditions (Snyder *et al*. 2011).

The work by Holt (2007) examined the effects of biosolids on nitrogen-fixing bacteria and found no effect on the microbial community from a one-time application. However, it was suggested that further study would be needed to determine if there was a cumulative effect

due to repeated land-application of biosolids. Rogers and Smith (2007) examined the effect on the indigenous soil microbial community due to the changes in soil matrix and potential addition of pathogens from biosolids to agricultural lands. Since these organisms play a crucial role in the soil food web and their role in nutrient cycling affects plant performance, it is important to understand if this ecosystem was disturbed. They found the dynamics and number of the bacterial communities changed with the application of biosolids to agricultural lands. This change in microbial structure could potentially cause the number of protozoa present to increase due to the increase in bacterial populations which might also allow viruses to become prevalent. This in turn, could suppress the indigenous bacteria.

Gebert (2010) examined the run-off and leachate of a laboratory-scale land-application of biosolids to determine the impact, if any, on the aquatic organisms *Daphnia magna* and *Hyalella azteca*. After examining respiration rates of both organisms exposed to the elutriate, there was a difference, but not detrimentally so, observed between the sources of biosolids used, thus indicating that biosolids need to be assessed on a plant by plant basis.

1.7.3 Excess Nutrients

Lastly, although not as big a problem as the other concerns, excess nutrients must be examined. Excess phosphorous is not toxic to plants but instead leads to problem if it leaches through the soil, particularly coarse soils, to ground water (Pierzynski and Gehl 2005), or enters slow-moving surface water where it could lead to the development of excess algae and weed growth and potentially to eutrophication (Payne *et al*. 2001). Hence, the amount of phosphorous in biosolids being land-applied is regulated. Nitrogen present in the biosolids can migrate through the soil and lead to elevated levels of nitrate nitrogen in the groundwater, or could accumulate in the edible foliage of plants which in turn could lead to a health risk for both humans and wildlife (Henry 2007; Biosolids Management 2010). Excess nitrate could cause birth defects, methaemoglobinaemia (haemoglobin cannot carry enough oxygen) and cancers in humans (Ontario Drinking Water Quality Standards). Additionally, nitrous oxide is one of the most potent greenhouse gases (Davidson *et al*. 2012). Ontario

Regulation 169/03 (Ontario Drinking Water Quality Standards) regulates nitrate (as nitrogen) in drinking water at 10 mg/L. Excess nitrogen in plants can also cause weakened stems in grain crops or reduced quality of fruits (Henry 2007). Additionally, ammonia volatilizes when biosolids are land-applied but not incorporated into the soil or when they are processed, producing unwanted odour and greenhouse gas emissions (Pierzynski and Gehl 2005).

1.7.4 Transporting Biosolids

A concern that arises indirectly occurs while transporting biosolids to the end user. This could prove costly depending on the distance that needs to be travelled. However that is not the main issue. Given that the biosolids are transported over major highways, there is the potential for traffic accidents which could lead to accidental spills causing adverse effects. Terratec Environmental Ltd. "Canada's premier biosolids management company" (their words) who do the majority of the hauling of biosolids across Ontario was fined $300,000 for three such spills between 2006 and 2008 (MOE Court Bulletin 2008). In these particular cases, a portion of the load was spilled on the road and, and luckily, no one was hurt. The potential is there for a major environmental spill. The regulated application of 22 tonnes/ha over a *five*-year time frame could easily be violated. If a spill occurred over a bridge, the contamination to the watercourse below would be impossible to prevent.

1.7.5 Concerns with Biosolids Summary

Again in the research previously examined, very few determine impact. Instead, their work indicate a potential chemical(s) or biological hazard to humans or the environment with the land-application of biosolids. They look at specific contaminant or class of contaminant or organism(s). Therefore there is a need for a holistic approach to determine if the land-application of biosolids has a negative impact on the terrestrial biota and consequently if land-application is a sustainable practice.

Now that concerns of biosolids have been considered, it is important to also address the regulations pertaining to biosolids and the methods available for their disposal.

1.8 Canadian Regulations Pertaining to Biosolids

Canada, as in the United States, uses an acceptable risk approach to their biosolids management, unlike the Europeans who use a more preventative approach (Schoof and Houkal 2005; Page 2009). Unlike in the United States where the biosolids regulations are at a federal level (Penn State 2000), in Canada, the regulations and guidelines pertaining to the use and disposal of biosolids are at the provincial and territorial level. The exception is in the case of compost or pellets. Here, the biosolids are sold as a commercial fertilizer: thus, they are regulated under the Federal Government's authority (Fertilizers Regulations, C.R.C., c 666; R.S., 1985, c F-10). Additionally, since Canada does not have a national biosolids guideline like the Code of Federal Regulations (CFR) 40 Part 503 of the United States, they also do not have unified, defined classes or grades of biosolids (CCME 2010). Biosolids vary by definition and between provinces and territories. In Ontario, under the new regulations that took effect January 1, 2011, there are three Categories based on biosolids quality (CCME 2010). These new regulation now classify biosolids as a nutrient instead of a waste and fall more under the Ministry of Agriculture's purview instead of the Ministry of the Environment as was previously the case (Maurino 2012). Under the old classification, Ontario's biosolids were considered Class B by USEPA standards; pathogens having been reduced significantly (by 90-99%) and they are pathogen-free after being land-application, and do not exceed the metals or nutrient levels as prescribed in the regulations O. Reg. 338/09 (MOE 2009).

Table 8 lists the relevant legislation or guidelines for each of the provinces in Canada that have them (CWWA 2003, 2007). The complexities are numerous. Many provinces, through governmental consultation with scientists and agrologists, have adapted existing regulations such as Part 503 of the USEPA which was promulgated in 1993, while others use provisions found in the Canadian Environmental Protection Act (CEPA) 1999 or other regulatory permits and guidelines (Nazareth 2007). In Ontario, an Inter-ministerial Committee was commissioned in 1971 consisting of members from OMAF (Ontario Ministry of Agriculture and Food), MOE (Ministry of Environment), and MOH (Ministry of Health) to develop

guidelines for the management of biosolids (Pers. Comm. Michael Payne, OMAFRA) which were first published in 1973. These initial guidelines did not restrict heavy metal additions to the soil. Those restrictions came in 1979 with revisions to the guidelines that included limits for the eleven regulated metals that are seen today (Pers. Comm. Michael Payne, OMAFRA).

When the USEPA was initially carrying out their risk assessment for biosolids for their Part 503 Regulations, twelve organic chemicals were initially included along with the metals but were eventually left out of the regulations since many were already banned and concentrations that were found in biosolids would have been below the regulatory concentrations (USEPA 1994c).

*Note land-application of biosolids is not permitted in Newfoundland. Their disposal method is by landfill with permit (CCME 2010).

Table 8: Canadian Legislation and Guidlines for Usage and Disposal of Biosolids

Province	Legislation
British Columbia	Environmental Management Act and Health Act - Organic Matter Recycling Regulation, 18/2002; amendments 321/2004
	The Compost Facility Requirements Guideline: How to Comply With Part 5 of the Organic Matter Recycling Regulation, 2004
Alberta	Environmental Management Act and Health Act - Organic Matter Recycling Regulation, 18/2002; amendments 321/2004
	Guidelines for the Application of Municipal Wastewater Sludge to Agricultural Lands, 2001
Saskatchewan	Land-application of Municipal Sewage Sludge Guidelines, 2004, EPB 296
Manitoba	The Environment Act, E125, 1998
Ontario	Nutrient Management Act, S.O. 2002, Chapter 4
	O. Reg. 267/03 General Regulation under the Nutrient Management Act, 2002
	O. Reg. 338/09 September 2009 amending O. Reg. 267/03 General Regulation under the Nutrient Management Act, 2002
	Reg 347 of R.R.O. 1990 (General – Waste Management)
	O. Reg 336/09 Made under the Environmental Protection Act amending Reg 347 of R.R.O. 1990 (General – Waste Management)
	Publication 811: Soil Management and Fertilizer Use, Chapter 2, point 17. Adjustments to Fertilizer Recommendations (Legumes and Manure) 2002.
	Interim Guidelines for the Production and Use of Aerobic Compost in Ontario, 2004
	Guidelines for the Utilization of Biosolids and Other Waste, 1996
	Guide for Applying for a Certificate of Approval to Spread Sewage and Other Biosolids on Agricultural Lands (Organic Soil Conditioning) [Sewage Biosolids and Other Wastes], 1997
Quebec	Guidelines for the beneficial use of fertilizing residuals, 2004 and addendum - Feb 2006
Newfoundland	*
Nova Scotia	Guidelines For Land-application and Storage of Biosolids in Nova Scotia, 2004, revised March 2010
New Brunswick	Guidelines for Issuing Certificates of Approval for the Utilization of Wastes as Soil Additives
	Guidelines for the Site Selection, Operation and Approval of Composting Facilities in New Brunswick
Prince Edward Island	Environmental Protection Act – Chapter E-9 Waste Resource Management Regulations. Updated 2009
	Sewage Disposal Systems Regulations
Yukon	Environment Act
Northwest Territories	Environmental Protection Act
	Environment and Natural Resources – Guideline for Industrial Waste Discharges
Nunavut	Water Licence
Canada	Fertilizer Act R.S. 1985,c F-10
	Fertilizer Regulations, C.R.C., c 666

1.8.1 At the Provincial Level in Ontario

Table 8 also shows that Ontario has many regulations and guidelines pertaining to biosolids. The ones that are pertinent to this research are mainly the Guidelines for the Utilization of Biosolids and Other Waste of 1996, Ontario Regulation 267/03 (amended with O. Reg. 338/09 effective January 1, 2011) which has the Nutrient Management Act, 2002 as its enabling statute and is enforced by Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) and O. Reg. 347 of the Environmental Protection Act, R.R.O. 1990, General – Waste Management, (as amended O. Reg. 157/98) which is enforced by the Ontario Ministry of the Environment (MOE) and was also recently amended to Ontario Regulation 336/09 again with the same effective dates.

1.8.2 At the Municipality Level

The municipalities in most provinces are responsible for the operation of the WWTP in their jurisdiction (CCME 2010). Therefore, under afore mentioned regulations, they are responsible to ensure that the biosolids being land-applied are tested and meet the pertinent provincial government requirements, thereby ensuring that the biosolids maintain their beneficial use to the soil and crops. City of Toronto additionally performs yearly WWTP performance evaluations where polychlorinated biphenyls, and dioxins and furans are also assessed (Jaques Whitford 2004). For the chemical and biological analysis of the biosolids used in this research, see Appendix III.

1.8.3 What is Regulated in the Biosolids in Ontario

As in the American regulations, Ontario regulations use quantitative standards listing the maximum allowable limits, except Ontario monitors eleven metals, including cobalt and molybdenum that the Americans regulations do not. In addition to metals, pathogens levels and the final nutrient value of the biosolids are also monitored.

1.8.3.1 Metal Guidelines

For biosolids to be land-applied on fields, they need to have a beneficial use to the soil and/or the crops and must not be detrimental to them. Some metals in small quantities are essential to plants and animals, but in larger amounts can be harmful. Thus, they are regulated. When the regulated limits for metals were originally developed in the mid 1970s for Ontario, they were based on: i) background soil concentrations, ii) toxicity data at the time to plants and grazing animals, and iii) reasonable concentrations that could be expected to be found in biosolids thus providing application rates that would prevent accumulation above levels that were deemed of concern. The USEPA values for their Part 503 were developed much later (starting in 1988) and included human health risk in their assessment, which was the largest environmental risk assessment undertaken at the time (Pers. Comm. Michael Payne, OMAFRA). Therefore, there are some differences in the allowable levels of the metals that are regulated. USEPA biosolids regulations examined the direct ingestion of biosolids by different animals as well as plant uptake when exposed to biosolids. The major focus of their research was the uptake of metals (Brown 2006). Using this data and human dietary exposure studies, they were able to established limits (Brown 2006). Table 9 lists the eleven regulated metals of various governments' with their limits. For Ontario, these were established by the MOE (O. Reg. 347/09, Section 14.2 (e)). Ontario Biosolids Guidelines allows for higher levels than the Canadian Fertilizer Standards (Kingston Biosolids 2003; O. Reg. 347/09). Under Ontario Guidelines, the land must first be assessed prior to land-application of biosolids to ensure that the metal concentrations in the soil are below acceptable levels and there are stringent restrictions as to where the biosolids can be applied. This is not the case for fertilizers, which fall under the federal regulation, and can be purchased by the general public. These products could be applied almost anywhere, thus the lower allowable concentrations.

Table 9: Metals Regulated under Various Government Legislation (Modified from (USEPA 1993; Kingston Biosolids 2003; O. Reg. 347/09)

Metal	Ontario Biosolids Guidelines (mg/kg)	USEPA 503.13 Rule (mg/kg)	Canadian Fertilizer Standards (mg/kg)
Arsenic	170	75	75
Cadmium	34	85	20
Chromium	2800	3000	N/A
Cobalt	340	N/A	150
Copper	1700	4300	N/A
Lead	1100	840	500
Mercury	11	57	5
Molybdenum	94	75*	20
Nickel	420	420	180
Selenium	34	100	14
Zinc	4200	7500	1850

*Molybdenum was removed from the amendment to the 503 rule in 1994 pending EPA further consideration (USEPA 1994a)

Although field crops removed < 0.4kg of metals per year from the soil (MOE and OMAFRA 1996; Beechar 2012), Table 10 provides the reasoning behind why these metals were regulated (MOE and OMAFRA 1996; Brown 2006; Biosolids Management 2010). Metals tend not to leach significantly in the soil.

Table 10: Reasons for Eleven Metals Regulated in Ontario Biosolids (Modified from Brown 2006; Biosolids Management 2010)

Metal	to Animals	to Plants	Notes
Arsenic	toxic in [low]	toxic in [low]	but plant uptake is minimal, therefore allowed in small quantities in the biosolids
Cadmium	toxic	toxic	not essential to either therefore set to [low] to prevent build-up in biomass
Chromium	essential	not easily absorbed	therefore regulations allow for [higher] in biosolids to be present
Cobalt	required but toxic in [higher]	not needed	utilized in small quantities by nitrogen fixing soil bacteria therefore allowed in biosolids
Copper	essential but toxic in [high]	essential but toxic in [high]	some plants deficient, therefore allowed in [higher] in biosolids than found naturally in soil
Lead	toxic	not needed insoluble in soil	insoluble in soil, less readily absorbed by plants than other metals, therefore allowed in [higher] than found naturally in soil
Mercury	toxic	not needed toxic	not readily absorbed by plants, therefore, since found in [low] in biosolids, regulations allow for more to be present
Molybdenum	non-toxic in [small]	essential	To date the amount of molybdenum found in biosolids has been low and thus of no concern
Nickel	essential	toxic	therefore only [low] allowed in biosolids
Selenium	essential	toxic above [low]	deficient in some feed crops, therefore regulated at [low]
Zinc	essential but toxic at [high]	essential but toxic at [high]	some plants are deficient in zinc, therefore regulations allow for [higher] than found in the soil naturally

To give a perspective, Table 11 lists the amount of these eleven regulated metals found in the biosolids processed at Ashbridges Bay WWTP in 2008. Also in this Table are the metal concentrations expressed as a percentage of MOE levels. These biosolids were below approximately 10% of MOE guidelines, copper being the only exception at 58 % but still well below regulated limits.

Table 11: Summary of Analysis of Toronto's ABTP Biosolids, 2008 (Biosolids Management 2010)

Regulated metals	Average (mg/kg of solids)	Percentage of MOE Limit
Arsenic	6.26	4%
Cadmium	0.99	3%
Chromium	151.5	5%
Cobalt	4.2	1%
Copper	994	58%
Lead	61.2	6%
Mercury	1.07	10%
Molybdenum	11.54	12%
Nickel	29.1	7%
Selenium	2.3	7%
Zinc	755	18%

1.8.3.2 Pathogens

Pathogens are a specific causative agent of disease or illness such as a bacterium, parasite, or virus and are abundant in untreated wastewater and as such are regulated (O. Reg. 338/09). The type and amount of pathogens found in wastewater is dependent on: i) population density (larger populations would have more sources, therefore higher concentrations of pathogens), ii) sanitary habits (poor habits lead to greater number of pathogens), and iii) time of year (warmer weather promotes more growth) (Biosolids Management 2010). Bacteria are the most commonly found pathogen in untreated wastewater. Biosolids from Toronto WWTPs that are to be land-applied are tested twice monthly and have always been under the regulated limit (Biosolids Management 2010). Table 12 provides the limits for pathogen content regulated under O. Reg. 338/09 and are similar to that in Part 503 of the USEPA and includes non-agricultural source material (NASM). NASM are biosolids that do not come from a farm source such as manure, runoff from a barnyard, or wastewater for an agricultural operation (O. Reg. 338/09).

Table 12: Schedule 6 Table 2 – CP1 Non-Agricultural Source Material (NASM) that is Sewage Biosolids (O. Reg. 338/09)

Column 1	Column 2	Column 3
Pathogen	**Level in aqueous material (containing less than 1% total solids, wet weight)**	**Level in non-aqueous material (containing 1% or more total solids, wet weight)**
E. coli	1,000 CFU per 100 ml	1,000 CFU per gram of total solids, dry weight
Salmonella	3 CFU or MPN per 100 ml	3 CFU or MPN per 4 grams of total solids, dry weight
Viable Helminth ova	No detectable level in 100 ml	No detectable level in 4 grams of total solids, dry weight
Total culturable enteric virus	No detectable level in 100 ml	No detectable level in 4 grams of total solids, dry weight

Note in Table 12 CP1 means that the pathogen in Column 1 does not exceed the levels as set out in Column 2 or 3.

During the secondary treatment stage at a WWTP, the majority of pathogens are removed via anaerobic digestion. This process can remove 95% of the bacteria and viruses present in the wastewater and parasites by over 60% (Biosolids Management 2010). The pilot Lystek process operating at Guelph, Ontario can produce biosolids that are better than Class A designation under the US biosolids classification system (a 99% reduction in pathogens) (Scrozzo, 2006). Other Ontario biosolids are considered Class B under Part 503 of the USEPA in terms of the pathogen content since the pathogens have been reduced by 95% at the WWTP and are pathogen-free *after* they are land-applied. This is due to the fact that many bacteria cannot survive outside their normal incubation temperature and are destroyed by the conditions of the natural environment (USEPA 2000c).

1.8.3.3 Nutrients

The nutrient value in the form of phosphorus and total nitrogen/ammonia are regulated in the biosolids to maintain their beneficial use to the land and crops. The allowable amount of phosphorus in biosolids is 60 mg extractable/L in the top fifteen cm of soil and nitrogen

cannot exceed 135 kg of nitrogen/ha over a five year period (MOE and OMAFRA 1996). Levels of 50-60 ppm of phosphorus are needed for successful growth of some crops (OMAFRA 2008; Section 52 and 98 of O. Reg. 338/09) while nitrogen is present in biosolids in both organic and inorganic forms (ammonium and nitrate nitrogen). The inorganic forms are immediately available to the crops while the organic nitrogen must be first broken down to the inorganic compounds, providing a 'time release' form of nitrogen (Pers. Comm. Michael Payne, OMAFRA: City of Toronto 2009). The ammonium nitrogen that is delivered to the soil is available to plants and gets converted to nitrate, which, in turn is either taken up by plants or migrates further into the soil (Biosolids Management 2010).

1.9 Disposal Methods of Biosolids

Municipalities play a key role in regulating biosolids. They are responsible for the operation of the WWTP in their jurisdiction and as such are required to ensure that not only the plant functions as specified and the criteria as laid out in the regulations are followed (O. Reg. 347/09 (MOE); Table 3 s.98 Part XI; O. Reg.267/03 (OMAFRA)), but the products they produce and dispatch also meet the requirements set out in these regulations. Besides the energy recycling from the process, there are several options available to the municipality for the disposal, recovery or recycling of the solid waste produced at their facility. Theses include landfilling, incineration, land-application, land reclamation, or for commercial use (compost or pellets). The end use is determined by the quality of the biosolids (CCME 2009). If the biosolids produced do not meet government guidelines for land-application in terms of metals, pathogens, or nutrients, that batch cannot be land-applied and must be disposed by another means such as incineration or sent to a landfill. Table 13 below lists the various possible options for biosolids used in Canada showing, by percentage, how the biosolids are disposed of. Although composting, pelletization and site remediation are all methods of land-application, their use is regulated differently, as mentioned earlier, therefore they are listed individually in Table 13. Also provided are 2009 statistics of how the different methods are used to dispose of the 660,000 dry tonnes of biosolids produced in Canada per year (CIELAP

2009; Biosolids Management 2010; Environmental Leverage Inc 2010; Region of Waterloo 2011).

Table 13: Disposal methods of Biosolids across Canada in 2008

Method	Ontario	British Columbia	Quebec
Land-application	40%	90%	27%
Landfill	40*%		31%
Incineration	20%		42%
Pelletization	16%		
Site Remediation	4%		
Composting		5% of the 90%	

* Note, in Ontario the percentages do not add up because as in the case of landfill, this could include biosolids treated by other methods also. For instance, in the case of Peel Region their incinerated biosolids are landfilled (Region of Waterloo 2011) and biosolids that are pelletized could be land-applied or used for site remediation. In Canada, on average, approximately fifty percent of all biosolids are applied to the land in one form or another, with most provinces being more than eighty percent (Region of Waterloo 2011; CWWA 2012).

1.9.1 Disposal Methods of Biosolids Used in Toronto

Since biosolids are produced on a continual basis, there must be a sustainable practice for their disposal. In the past, Toronto has used landfill, incineration, and land-application as means of disposal (Biosolids Management 2010) (Table 9). According to the report prepared by KMK Consultants, prior to 2002, the biosolids produced at the four wastewater treatment plants in Toronto were either incinerated at Highland Creek Wastewater Treatment Plant (HCTP) (22%) or transported to Ashbridges Bay Wastewater Treatment Plant (ABTP) for further treatment (as is the case for Humber Treatment Plant (HTP) and North Toronto Treatment Plant (NTTP)) (78%) (City of Toronto 2004; Biosolids Management 2010). Until August 2006, dewatered biosolids cakes were sent to Carleton Farms, Michigan, USA for

disposal in landfill sites (City of Toronto 2009). In 2006, Michigan closed its borders and stopped accepting Ontario biosolids.

Combining the biosolids from all four facilities, Table 14 indicates by what methods Toronto disposed of its biosolids for the years 2008 and 2010. Land filling went down while land-application went up over this time frame (Biosolids Management 2010). Since Table 14 is strictly Toronto data, the method of land-application here refers to biosolids being disposed of on agricultural lands, while pellets (from pelletization) and composting would be disposed on gardens and parklands as regulated under the Federal Fertilizer Act.

Table 14: Disposal Methods of Biosolids in Toronto in 2008 and 2010

Method	2008	2010
Landfill	41%	27%
Incineration	23%	22%
Land-application	11%	17%
Pelletization	16%	30%
Site Remediation	4%	3%
Composting	0%	0%

To date, the City of Toronto processes over 1.4 million m^3 of water daily which generates 195,000 dry tonnes of biosolids every year (Biosolids Management 2010) which it competes with other major urban communities in the area which generate a further 110,000 dry tonnes per year for disposal locations (City of Toronto 2009).

The focus of this thesis is the land-application of biosolids to agricultural lands in southern Ontario. Therefore only this method will be further discussed.

1.9.2 Land-Applying Biosolids

Where biosolids can be applied is very detailed in Ontario regulations (Guidelines for the Utilization of Biosolids, 1996, s6; Part VI, s.43-52.1 of O. Reg. 338/09) and stipulates buffer requirements that biosolids must be a minimum distance away from residences, from wells (both municipal and private), and from watercourses. The use of the land also plays an

important role in determining if biosolids can be applied (OMAFRA 2010a). For example, biosolids cannot be applied to a crop that are for direct human consumption, i.e. that is not processed, such as tomatoes or lettuce, but can be applied to corn, soya beans, or wheat since they are not considered "field-to-table" crops because it is unlikely for direct contact of the edible portion of the crop and biosolids (Pers. Comm. Michael Payne, OMAFRA; Brown 2006). There are also different waiting periods stipulated between application of the biosolids and land use (MOE and OMAFRA 1996). This time lag before the farmer can use the land is to minimize health risk to livestock and humans by ensuring biosolids are incorporated effectively into the soil and by providing the natural processes, for instance soil micro-organisms and climatic conditions (such as sunlight and temperature), to completely destroy any remaining pathogens. By Ontario classification, biosolids are pathogen-free *after* they are land-applied (MOE and OMAFRA 1996; USEPA 2000c; Brown 2006; O. Reg. 338/09; CCME 2009).

A farmer must receive a Certificate of Approval (CofA) from the MOE and have their soil tested to ensure that the location is suitable to receive the biosolids (MOE and OMAFRA 1996). These tests look for concentrations of phosphorus, available potassium, nitrogen, and eleven regulated metals already present in the soil, as well as pH and soil composition. All results must meet provincial standards as set out in the guidelines (MOE and OMAFRA 1996). If the site fails to meet any of the criteria, then biosolids cannot be land-applied.

Soil pH is determined due to the fact that metals are fairly stable in alkaline soil and therefore would not easily migrate through the soil to plants or the groundwater (Biosolids Management 2010). All of the regulated metals, except molybdenum, are more mobile in acidic soils. For this reason, the regulations prohibit the addition of biosolids to soil with a pH of less than 6 (O. Reg. 338/09 98.0.17(3)). Furthermore, in acidic conditions, the organic and inorganic compounds found in the biosolids to which the metals can sorb are more readily dissolved, thus making the metals motile (Biosolids Management 2010).

Since the climate of this area of study exists where the ground does freeze, Ontario restricted the land-application of biosolids between December 1 to March 31 of the following year during which time they must be stored, (Part VI, s.52.2 to 52.4 O. Reg. 338/09). During the remainder of the year (April 1 through November 30), biosolids are restricted from being applied during heavy rains or pending rains to prevent the potential of runoff not only into surface waters but also to the groundwater through tiles and natural percolation (USEPA 1993; O. Reg 267/03).

The term 'land-application of biosolids' covers such uses as agricultural fields, golf courses, and parks, or as a soil amendments for site remediation locations such as mining pits, construction sites and forests. In the next section, only the methods pertaining to agricultural land will be examined.

1.9.2.1 Methods of Land-applying Biosolids

In Ontario, the method of application (either direct injection or applied to the surface) depends on several factors such as if the biosolids are liquid or dewatered, the equipment that is available, as well as the physical landscape (Evanylo 2006; Pers. Comm. Michael Payne, OMAFRA). For agricultural purposes, if the biosolids are to be surface-applied, existing equipment that the farmer already has for spreading manure, such as can be seen in the picture on the left in Figure 5, can be used, and if necessary incorporated into the land using disks or plows as seen on the right in Figure 5. This method would be appropriate for dewatered biosolids and was the method that was simulated in this research when incorporating the biosolids into the soil of the crop troughs.

Figure 5: Farmer using manure spreader to distribute biosolids onto the land (Source: Centre for Urban Horticulture 2003)

If biosolids are in the liquid form (or fluid as in the case of Lystek-produced biosolids) they can be land-applied by 'spraying' or applied directly to the surface using the appropriate machinery. This method of application depends on the water content of the biosolids as well as the land use and could be used, for example, on a hay field. With direct application to the surface, photolysis plays an important role in further degrading the compounds found in the biosolids.

Yet another method for land-applying biosolids is the injection method (MOE and OMAFRA 1996; O. Reg. 338/09). This method is best suited for low solid content (or liquid) biosolids as well as agricultural fields that use a no-till system and require a low application rate (Evanylo 2006). To achieve the desired consistency, water can be added at the site at the time of application. Here, specially designed machines (Figure 6) are used to inject the liquid biosolids below the surface of the ground and to cover it over immediately. The advantage of this method is not only the reduction of odours but it limits the loss of ammonia volatization since the biosolids are immediately incorporated into the ground. The disadvantage with this method is the potential damage to the sod due to the deep furrows left by incorporating the biosolids into the top 15 cm of soil (Evanylo 2006).

Figure 6: Injection Equipment for Biosolids Application (Source: USEPA 2000b (left), merrellbors.com (right))

1.10 Biota to Evaluate Potential Impact of Biosolids

To determine if the land-application of biosolids is an environmentally-sustainable practice, the indigenous terrestrial biota need to be assessed for potential impact since, as seen in the previous discussion, little research has been conducted on the impact to these organisms by the land-application of biosolids. The biota that were chosen to evaluate impact were: *Folsomia candida* (springtails) and *Lumbricus terrestris* (earthworms) and *Zea mays* (corn), *Glycine max* (soya beans), *Phaseolus vulgaris* (common bean), and *Brassica rapa* (field mustard).[1]

1.11 Folsomia candida

Table 15 shows the taxonomy for *Folsomia candida* (Willem, 99403), TSN 1902. Willem is the taxon author who in 1902 developed this taxonomic key for *Folsomia candida* (ITIS 2008). The TSN or Taxonomic Serial Number is specific for each species and is similar to a CAS number for chemicals.

[1] Photographs, unless otherwise specified, are those of the author

Table 15: Taxonomic Key of *Folsomia candida* (ITIS Report nd)

Kingdom:	Animalia
Phylum:	Arthropoda
Subphylum:	Hexapoda
Class:	Entognatha*
Order:	Collembola, (springtails)
Family:	Isotomidae
Genus:	*Folsomia*
Species	*candida* (Willem)

*Note: some sources categorize *Folsomia candida* in the Class Insecta instead of Entognatha (wingless) (Environment Canada 2007c; Storey 2009).

1.11.1 Ecology of Folsomia candida

Folsomia candida, colloquially termed springtails, are macro-arthropods and one of the most abundant macroscopic organisms on earth with estimations of 100,000 springtails per meter squared of topsoil and leaf litter (Hopkins 1997; Wiles and Krogh 1998; Fountain and Hopkin 2005; Scott-Fordmand and Krogh 2005; Krogh 2008), but even with such large numbers, springtails only represent 1% of the animal biomass due to their small size (Krogh 2008). The 6000 species worldwide (Hopkin, 1997; Houseman 2007) are ubiquitous to the various soil types found in Canada (Hopkin, 1997; Environment Canada 2007c). *F. candida* are widely disseminated in the soil ecosystem, dwelling in the upper soil layer, living in and around the surface of soil of such environments as mines, caves, agricultural systems, leaf litter, along the edges of streams, and locations with a high organic content (Fountain and Hopkin 2005; Aldaya 2006; Environment Canada 2007c). Many of which, are locations where biosolids could be applied. *F. candida* plays a key role in the soil foodweb for three reasons. They act as the primary detritivores and as such are one of the main biological modes responsible for the creation of soil (Campiche *et al*. 2007; Environment Canada 2007c). They also are prey for many endogeic (in the soil) and epigeic (on top of the soil) invertebrates such as beetles, centipedes, mites and spiders (Krogh 2008). Additionally, they are primarily responsible for keeping microbial populations under control. Through their feeding behaviours, springtails enrich the soil by breaking down algae, lichens, fungi, bacteria, insect faeces, decaying vegetable matter and other organic material thus releasing

the nutrients back into the soil (Hopkin 1997). As such, these organisms are an integral part of the soil environment and their presence is a good indicator of soil health (Campiche *et al.* 2007). Since collembolans can inhabit acidic soils, they play a role in its decomposition also since other decomposers such as earthworms and diplopoda (e.g. millipedes) are typically absent in this soil type (Krogh 2008).

1.11.2 Anatomy of Folsomia candida

Springtails are tiny (4-day olds are 0.5 mm; adults are 1.5-3.0 mm in length), unpigmented, segmented, wingless white insects (or entognatha) (Wiles and Krogh 1998; Fountain and Hopkin, 2005) (Figure 7, left). Besides relying on their six appendages for locomotion, *F. candida* possess a forked structure (furcula) attached to the underside of the abdomen which is used to flip them into the air (Figure 7, right). This feature is how they have obtained the moniker of *Springtails* (Hopkin 1997; Environment Canada 2007c). The release of the furcula leads to rapid jumping movements (as much as 8 to 10 cm in a single motion) (Fountain and Hopkin, 2005; Houseman 2007). This movement is used as a means of escape when they feel threatened (Wiles and Krogh 1998) since the organisms do not have control of where they land.

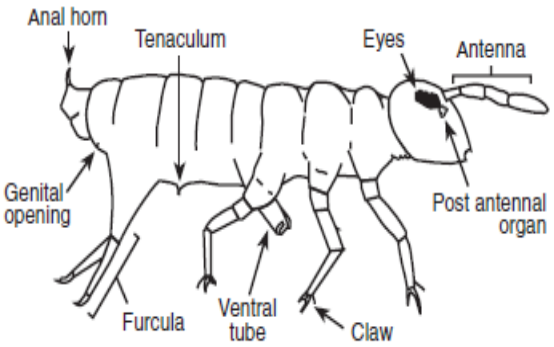

Figure 7: Adult and Juvenile *Folsomia candida* on a leaf. Actual size of adult here is 2mm (left). (Source: *Folsomina candida* 261 FOcan nd). Anatomy of *F. candida* showing furcula (right). (Source: Houseman 2007)

Folsomia candida are blind but have internal photoreceptors and are thus sensitive to light. These organisms breathe through their thin body covering since they do not have a trachea system. Therefore, springtails can easily become dehydrated and must seek out a moist, humid habitat. They possess a physiological adaptation to prevent desiccation by absorbing water vapour (Fountain and Hopkin, 2005; Houseman 2007). Their thin exoskeleton is not only highly permeable to water and air, but also chemicals and other components in the soil (Krogh 2008). *F. candida* do not go through a metamorphosis but grow by continuously moulting throughout their life-cycle (Campiche *et al*. 2007). Their behaviour and reproductive capacity are endpoints assessed in the current study.

1.11.3 Reproduction of Folsomia candida

Springtails reproduce by parthenogenesis, an asexual means of reproduction where growth and development of the offspring takes place without the fertilization of the eggs. The offspring produced by this method are almost always female with the XY chromosome system determining gender (Krough and Petersen 1995). The end result is the production of clones. The females are sexually mature at sixth or adult instar, which occurs in twenty-one to twenty-four days. The average lifespan of a female springtail at 15°C is 240 days, but at 24°C it is only 111 days. At 15° C, the average number of eggs a female lays in her lifetime is 1100, compared to 900 at 21° and only 100 at 27° C. As can be seen, temperature plays an important role (Fountain and Hopkin 2005). The eggs (about thirty-fifty) are laid in communal heaps, with one female adding to the previously laid batch. These small white spheres (about 80-110 µm in diameter) take seven to ten days to hatch at 20°C. If a chamber is crowded (> 1 animal/cm^2) this could lead to a reduction in the number of eggs (Fountain and Hopkin 2005). A reduced egg population can also be caused by light, stress, pheromones, or their container being contaminated by waste products. Although *F. candida* are blind, there are more eggs produced when they are kept in constant darkness than if they were in a light:dark cycle (Fountain and Hopkin 2005).

1.11.4 Ecotoxicology Studies using Folsomia candida

F. candida are considered representative of the terrestrial invertebrate group and are extensively dispersed in the soil ecosystem. Therefore, it is considered an environmental relevant test organism (Aldaya 2006). Additionally, they are known to be able to 'taste' chemicals and thus avoid them (Fountain and Hopkin 2005). Therefore, their presence is a good indicator of soil health (Houseman 2007; *Folsomia candida* 261 FOcan nd). In ecotoxicological studies, these organisms have been successfully used to indicate soil pollution levels since there is a point along a pollution gradient where the species dies out (Crouau *et al.* 1999). *F. candida* were first used in 1956 assess impact to DDT (dichlorodiphenyltrichloroethane) and many other pesticides (Wiles and Krogh 1998) leading to the development of the ISO 1999 protocol No 11267 Soil Quality – Effects of soil pollutants on collembola (*Folsomia candida*): Method For Determination of Effects on Reproduction, Geneve. *F. candida* in now a standardized ecotoxicological test organism, one of only three animal organisms used for ecotoxicological testing in Europe (Crouau *et al*. 2002). Crouau *et al*. (2002) have used this organism to evaluate the toxicity of waste since they feel that chemical analysis is often unsuitable, expensive, and extensive knowledge of the waste being tested is needed while the synergistic and antagonistic interactions are not revealed. When using these organisms, they noted that pH and organic matter content of the waste did have an effect on the reproduction of *F. candida* and needed to be taken into consideration. Wilke *et al.* (2008) examined the ecotoxicity of various hazardous wastes using different biological organisms including *Folsomia, Lemna,* and earthworms *(E. feitida)* as part of the requirement in Europe to do so. Since *Folsomia* have been used successfully in environmental risk assessment of chemicals and soil quality testing, these researchers concluded that the collembolan reproduction test would be a first choice candidate for future assessments of wastes based on response observed. Aldaya *et al.* (2006) compared the longer duration (forty-day) reproduction bioassay with a shorter 100-minute avoidance bioassay using varying concentrations of PAHs. They concluded that with heavily polluted samples, the longer duration bioassays were at a disadvantage since the contaminant could either kill the organism outright or deprive the organisms of food. Their results also showed a good

correlation between the 100-minute avoidance bioassay and the 40-Day reproduction bioassay.

1.11.5 Relevance to Project

Folsomia candida have been extensively studied as an ecotoxicological organism and used as a standard soil test organism for environmental studies (some of which have been discussed above) for over 40 years since they possess many ideal attributes for ecotoxicological research (Scott-Fordmand and Krogh 2005), including their sensitivity to soil contaminants, ease of sampling in the field, ease of maintenance under laboratory conditions, as well as their natural distribution in the upper soil surfaces of agricultural ecosystem where biosolids would be land-applied (Wiles and Krogh 1998; Crouau *et al*. 1999; Fountain and Hopkin 2005; Aldaya 2006; Campiche *et al*. 2007). Therefore, *F. candida* is a relevant organism to use in this study. Determining if biosolids has an impact on these organisms would be crucial due to the important role they play in the ecosystem.

1.12 Lumbricus terrestris

Table 16 below is the taxonomy for *Lumbricus terrestris* that was used to assess for impact of the land-application of biosolids.

Table 16: Taxonomic Key of *Lumbricus terrestris* (NBII *Lumbricus terrestris* 2009; Environment Canada 2007a; The Taxonomicon - *Lumbricus terrestris*)

Kingdom:	Animalia
Phylum:	Annelida
Class:	Clitellata
Subclass:	Oligochaeta
Order:	Haplotaxida
Family:	Lumbricidae
Genus:	*Lumbricus*
Species:	*terrestris* Linne

1.12.1 Ecology of Lumbricus terrestris

Lumbricus terrestris are commonly referred to as earthworms, dew worms, or night crawlers. Although *L terrestris* was not originally native to Canada, and was brought here by European

immigrants 400 years ago, it has now been incorporated into the terrestrial landscape of all parts of Canada except the Yukon and Northwest Territories (Environment Canada 2007a; Lowe 2008). Aristotle regarded them as the *"intestines of the earth"* since they play a key role in soil fertility and structure (Colorado State 2011). Their ability to increase soil fertility has ensured their importance to this part of the world. *L terrestris*, being able to tolerate a fairly wide range of soil pH (4-8), make up sixty to eighty percent of the soil biomass in some locations (Environment Canada 2007a; Kinney *et al.* 2008). They are now found to inhabit a variety of moist soil types such as meadows, grasslands, pastures, and fields commonly occupied with wheat-corn-soya bean rotations (Environment Canada 2007a; Kinney *et al.* 2008) and prefer a temperatures range between 4 and 21°C (Colorado State 2011). Like the collembolan, earthworms are not only vital to the terrestrial food web as a significant dietary component to birds, reptiles, fish, and mammals, but are important soil invertebrates in the cycling of nutrients and energy and have been linked directly to soil health (Yeardley *et al.* 1996; Banks *et al*. 2006; Kinney *et al*. 2008; CEPA 2009).

L. terrestris are anecic (burrow deep within the soil and come to the surface for food), selectively feeding on material found at the soil surface and can consume their weight in fallen leaves and other organic materials daily (Environment Canada 2007a; Ayers 2010; Frund *et al.* 2010). These vertical dwellers, which can usually be found in the top 60 to 180 cm of the soil, pull the plant material into their burrows where the soft parts are eaten along with soil particles (Colorado State 2011). Through their digestive processes, earthworms grind large particles of organic material into smaller ones; thus, the soil and plant debris are bound together into crumbs as they pass out of earthworms, incorporating minerals and organic matter (Yeardley *et al.* 1996; Butt and Girgoropoulou 2009; Ayers 2010). Soil microorganisms consume these smaller particles and complete the conversion of once-living plant material into rich, organic soil. In so doing, earthworms literally eat their way through the earth. The maze of tunnels created allows water and air to penetrate down into the soil, which improves the soil structure and aeration (Butt and Nuutinen 1998; Butt and Girgoropoulou 2009; Frund *et al.* 2010). Therefore, it can be seen that these organisms play

an essential role in soil fertility as well as the creation of soil. Any change in this rate of detritus decomposition and soil fertility could, in turn, affect soil biomass which could lead to changes in the entire food web (Yeardley *et al.* 1996; Butt and Girgoropoulou 2009).

Earthworms are negatively-phototactic and very sensitive to light and only come to the surface at night when the surface temperatures are lower and humidity higher, to feed and copulate (Environnent Canada 2007a; Lowe 2008; Ayers 2010). Therefore, these worms are more visible than other worm species (Lowe 2008). During the day, *L. terrestris* lie in their burrows. On warm, damp nights, earthworms will stretch out along the surface of the soil in search of food, using their flattened tails to anchor themselves for a quick retreat back into their burrows to avoid predation (Butt and Nuutinen 1998). They do not tolerate a soggy soil but earthworms need an environment with a certain moisture content to keep their skin damp (Lowe 2008; Colorado State 2011).

Ayers (2010) mentions that an active worm population can boost plant production. Many farmers use the number of earthworms present in their fields as an indication of the health of their soil. The United States Department of Agriculture in 2010 stated that within an acre of land, there could be up to 500,000 earthworms which can move up to five tons of soil in a year! As such, it is important to determine whether land-application of biosolids impact the life of these extremely important terrestrial dwellers. In addition to improving soil structure and fertility, research has found soil rich with earthworms contain fewer parasitic nematodes (Ayers 2010). Earthworm activities directly stimulate beneficial organisms that trap, eat, and out-compete plant-eating nematodes (Ayers 2010).

1.12.2 Anatomy of Lumbricus terrestris

These invertebrates possess a cylindrical body with the posterior being dorsoventrally compressed and acting as an anchor in their burrows (Figure 8). An adult *Lumbricus* has an average body length of 90-300 mm with an average diameter of 6-10 mm, and a life expectancy ranging from six to fifteen years (Environment Canada 2007a). Their tanylobic

prostomium, a completely divided proboscis-like flap, contains chemoreceptors and project from their first segment anteriorly (Environment Canada 2007a). This enables an earthworm to grasp object which permits surface browsing (Evans 1948; Sims and Gerard 1985). The clitellum, which is only present in sexually mature adults, is used to secrete material for the formation of cocoons which holds the young until they hatch. Setae, tiny bristle-like hairs located along the length of the earthworm, are used for locomotion and aid in the earthworm's ability to sense their environment (WormWatch Canada 2002). Tubercles (small protrusions) found along the body segments of *Lumbricus* allow them to be highly sensitive to chemicals in their environment making earthworms a very good test organism since they not only possess the ability to sense unfavourable environmental conditions but with the aid of their setae and a peristaltic wave of the musculature of their body, move away from such situations (Collier 1937; Stephenson *et al*. 1998).

Figure 8: Characteristic flattened posterior of *Lumbricus terrestris*

Earthworms respire through their skin and therefore need a moist environment (25-30%) to keep their skin damp (Lowe and Butt 2005). If the soil should dry excessively, they burrow deeper. If the soil becomes waterlogged, the dissolved oxygen is driven off and the earthworms cannot breathe. They will again migrate to more favourable conditions (Colorado State 2011).

1.12.3 Reproduction of Lumbricus terrestris

Being hermaphroditic, earthworms reproduce sexually with the mutual exchange of sperm during copulation. Unlike other genera where copulation usually takes place vertically below

ground, *L. terrestris* mate horizontally aboveground along the soil surface at night. This unusual behaviour can be seen in Figure 9. In the case of *L. terrestris*, after copulating the sperm can be stored up to eight months (Butt and Nuutinen 1998) and cocoons being produced up to twelve months after mating. Hatchability of the cocoons decreases to about 62% over the first five months after copulation, to 11% by the sixth month and then 0% after that (Butt and Nuutinen 1998). The average number of viable cocoons is five, with an average of three progeny per cocoon. *Lumbricus terrestris* matures in eight to sixteen months and reproduction is relativity slow (Butt and Nuutinen 1998; Environment Canada 2007a) leading to their undesirableness to researchers.

Figure 9: Photo of copulating *Lumbricus terrestris*

1.12.4 Ecotoxicological studies using Lumbricus terrestris

Earthworms are particularly desirable as research tools not only because they breathe through their skin and maintain a close physical proximity to the soil and its constituents but they ingest large quantities of the soil and potential contaminants (Banks *et al*. 2006; Kinney *et al*. 2008; CEPA 2009). They have been increasingly used over the past twenty years in ecotoxicological studies for soil restoration and biomonitoring with the species *Eisenia fetida* being the favoured test species (Lowe and Butt 2005; Frund *et al*. 2010), since the OECD Guidelines for Earthworm Acute Toxicity Testing were established in 1984 using this species (Wiles *et al*. 2008). Toxicity bioassays such as EPA/600/8-87-011 (1987), or EPA/600/R-94/024 (2004), or Environment Canada's EPS1/RM/33 (1997), have existed for assessing stressors to the aquatic biota for some time, but an avoidance bioassay for the soil to evaluate

hazardous waste would have great potential (Yeardley *et al.* 1996). Earthworms are also of interest for the role they play in the biomagnifications of inorganic and organic soil pollutants such as mercury, PAHs, pesticides, and PBDEs (Kinney *et al.* 2008; Yasman and D'Souza 2010). Unlike the plants, earthworms offer a different route of entry for contaminants. Metals and hydrophobic organic contaminants can be taken up by passive diffusion across the outer membrane of the earthworms from soil solution or by desorption of the compound as it passes through the gut of the organism (Banks *et al.* 2006) offering a more holistic approach to this investigation. Additionally, it has been concluded by many researchers that using the avoidance behaviour response of earthworms to sublethal concentrations of chemicals in soil can have ecological relevance (Yeardley *et al*. 1996; Environment Canada 2007a) since organisms will often show signs of behavioural responses at lower levels of stress than those detected by acute toxicity tests and in shorter duration. Also, reproduction would be a more sensitive indicator than mortality for these lower levels of pollutants (Yasman and D'Souza 2010). The work by Dittbrenner *et al.* (2011), like that of Evans (1947), used *Lumbricus terrestris* to examine their burrowing behaviour of earthworms. By means of X-rays, these researchers showed that burrows can be affected by contaminants. They found a significant linear decrease in the burrow volume with an increase in the concentration of the contaminant, imidacloprid, a neonicotinoid insecticide which is extensively used worldwide in agriculture (Dittbrenner *et al.* 2011). The work of McCarthy *et al.* (2003) (field), and Alvarez (2000) (laboratory), also used *L. terrestris* in their research on pulp mill biosolids-amended soils, they found no significant difference between treatment and reference field sites after 98 days, nor under laboratory conditions.

1.12.5 Relevance to Project

The role of earthworms within the soil has been known for more than a century (Butt and Girgoropoulou 2009). Therefore, it is important to determine if they are impacted by the land-application of biosolids. *Eisenia* species, favoured by government protocols, are agreeable to laboratory-rearing and are found across Canada within organic rich, decaying matter such as composts, manure piles, and gardens. These non-burrowing organisms are

epigean (rarely found in soil, but rather live on or close to the surface) and are not true soil dwellers (Crouau *et al.* 1999; CEPA 2009; Environment Canada 2007a). However these are not environments to which biosolids are land-applied. Thus, the superiority of *L. terrestris* which does exist in these environments is obvious, and were chosen for avoidance bioassays because of their sensitivity and the ease of detecting their behavioural responses (i.e. migratory avoidance in sub-acute bioassays, usually within 24-72 hours) (Environment Canada 2007a).

1.13 Plants

The following plants will be used in this investigation, and each will be discussed in turn.

Zea mays – Corn
Phaseolus vulgaris - Common Bean
Glycine max - Soya bean
Brassica rapa - Field mustard

1.14 Zea mays

Table 17 is the taxonomic key for *Zea mays*. Note that this plant is monocotyledon.

Table 17: Taxonomic Key of *Zea mays* (USDA *Zea mays*)

Kingdom:	Plantae
Subkingdom:	Tracheobionta(vascular plants)
Superdivision:	Spermatophyta (seed plants)
Division;	Magnoliophyta/angiosperms flowering plants)
Class:	Liliopsida (monocotyledons)
Subclass:	Commelinidae
Order:	Cyperales
Family:	Poaceae (grass)
Genus:	*Zea*
Species:	*mays L.*

1.14.1 Ecology of Zea mays

Corn, a monocotyledon, is believed to have been domesticated by the Mayans and Aztec civilizations in Central America more than 5600 years ago and spread through the rest of the Americans by the end of the 15th century and early 16th century (Purdue University 1999). Maize, as it is also known, was then introduced to the rest of the world after the Europeans

came to the Americas and today is the second most widely grown crop globally (FAO 2009b) followed by sugar cane.

Sixty-five days after germination, most corn plants with silk start producing seeds and after approximately one hundred and twenty-five days will be mature (Purdue University 1999; Iowa State 2007). Times can vary depending on the hybrid of the corn planted, the environment it is planted in, soil structure, nutrient availability, planting date (early or late spring affects the moisture content in the ground and the temperature of the soil, both of which affect germination) and location (as related to weather, which determines the amount of rain and sun) (Iowa State 2007). The corn is mature and can be harvested when the water moisture inside the kernels has been reduced to less than twenty percent (Dobermann and Walters nd).

1.14.2 Anatomy of Zea mays

Corn plants grow on a single stock with a distinctive growth pattern of broad leaves 50-100 cm long and 5-10 cm wide altering along its length (Figure 10, left). The erect stem at maturity can reach heights up to 2–3 m (Purdue University 1999). The female inflorescences or cluster of flowers are tightly covered by many layers of leaves and form the ears as they develop and grow, about 3 mm a day (McWilliams *et al*. 1999). The pale yellow silks that emerge from the tops of the ears are elongated stigmas, and once pollinated, each produce a kernel of corn (McWilliams *et al*. 1999) and darken as they mature (Figure 10, middle) (Purdue University 1999). The male inflorescence (Figure 10, right) is found as a tassel at the apex of the stem and releases pollen from the anthers once they have matured in the warm, dry weather. Corn pollen is anemophilous (dispersed by the wind) and has a high settling velocity. It therefore lands within fifteen meters of the tassel and pollinates the silk of a different plant (McWilliams *et al*. 1999; Purdue University 1999; Thomison nd). In field conditions, at least 97% of the kernels are pollinated by other plants in the vicinity (Thomison nd). Depending on the variety, the kernels, once mature, could be blackish, bluish-gray, red, white, or yellow (Purdue University 1999).

Figure 10: Image of *Zea mays*: young plant (left), Female inflorescence - silk (middle), Male inflorescence- tassels (right)

1.14.3 Ecotoxicological Studies using Zea mays

Diez *et al*. (2001) examined the effect of contaminants such as heavy metals, PCBs, phenols, and indols from pig slurry (manure) on *Z. mays* crops grown under forced irrigation in field conditions. They concluded that the heavy metals were not significantly different at 1.4 m soil depth. The phenols, indols, and PCBs initially found in the pig slurry were absent in the soil after five months and the germination, grain yield, plant yield, and nitrogen uptake of the corn plants were unaffected at the end of the growing season (Diez *et al*. 2001).

In 2004, Baek *et al*. determined that crude oil and oil components were phytotoxic to *Z. mays* at concentrations of 10,000 mg/kg, as were PAHs at concentrations between 10-10,000 mg/kg and this phytotoxicity increased with the number of rings in the chemical struchure but soil contaminated with 0-1000 mg/kg of aliphatic hydrocarbons were not toxic to these plants. *Zea mays* showed significant reduction in root development in as little as 1% (w/w) crude oil and no germination at all in 5% (w/w) oil contaminated soil.

Carbonell *et al.* (2011), under greenhouse conditions, used *Zea mays L*. to determine the input of metals to agricultural soil from municipal solid waste compost (application rate of 50 Mg/ha) and commercial NPK fertilizer (application rate, 33 g/plant, determined by the plant's nitrogen needs). Their findings showed that compost increased Cu, Pb, and Zn in the soil while the artificial fertilizer increased Cd and Ni and decreased Hg levels. This group

also found that the roots acted as a barrier to the uptake of Cr, Hg, Ni, and Pb thus lowering the translocation of these heavy metals to the aerial parts of the plants. They also found that Cd, Cu and Zn were translocated from the root to other parts of the plant. This correlated to the highest metal concentrations being found in the roots of the *Zea mays* plants and no significant difference of metal uptake being found between the two treatments (Carbonell *et al.* 2011).

1.14.4 Relevance to Project

Terrestrial plants are not only important as a food source to humans, but they also provide food and shelter for many mammalian and avian species. Additionally, they act as air pollution filters while their roots prevent siltation and soil erosion. *Zea mays* was the second most abundant crop produced in the world in 2009 (FAO 2009a). The world production that year was 817 million tonnes with the United States being the leading producer (333 MT) and Canada ranking eleventh producing 9.6 MT (FAO 2009b). It is important to determine if the land-application of biosolids has an impact on this globally-important crop.

1.15 Glycine max

Shown in Table 18 is the taxonomic key for *Glycin max. G. max* is a dicotyledon.

Table 18: Taxonomic Key of *Glycine max* (USDA *Glycine max*)

Kingdom:	Plantae
Subkingdom:	Tracheobionta (vascular plants)
Superdivision:	Spermatophyta (seed plants)
Division;	Magnoliophyta/angiosperms (flowering plants)
Class:	Magnoliopsida (dicotyledons)
Subclass:	Rosidae
Order:	Fabales
Family:	Fabaceae (pea family)
Genus:	*Glycine*
Species:	*max*

1.15.1 Ecology of Glycine max

Glycine max, or soya beans, are native to East Asia and in China. They were initially used to add nitrogen to the soil as well as being a food source for the past 5000 years (USSEC 2008). For cultivation of this annual legume to be successful, a climate that has hot summers (mean temperatures of 20 °C to 30 °C) is needed (Willis 1989; Soya 2008) because this plant must reach at least the first trifoliolate stage before it can be induced to flower (McWilliams *et al*. 2004). If the temperature falls outside this range, the growth is stunted dramatically since length of daylight and temperature control the flowering of soya beans (McWilliams *et al*. 2004; Soya 2008). While *G. max* can grow in a wide range of soils, its optimum growth is in moist alluvial soil (soil containing fine particles of silt and clay with larger particles of sand and gravel) with good organic content (McWilliams *et al.* 2004). Like other legumes, soya beans have the ability to establish a symbiotic relationship with the bacterium *Bradyrhizobium japonicum* (syn. *Rhizobium japonicum*; Jordan 1982) to perform nitrogen fixation (Kuykendall *et al.* 1982; Hungria *et al*. 1998; Encyclopaedia Britannica nd).

1.15.2 Anatomy of Glycine max

Modern crop cultivars of *G. max* generally reach a height of 1-2 m and take 80–120 days to grow from sowing to harvesting (Pers. Comm. Jeff Robinson, Woodrill Farms). As the plant grows, trifoliate leaves are produced singularly at different nodes, alternating upwards along the stem (McWilliams *et al*. 2004) with small, inconspicuous, self-fertile flowers formed in the axil of the leaf. Thus, these self-pollinators do not need the aid of wind or insects to reproduce. The flowers produced can either be white, pink, or purple (McWilliams *et al*. 2004). As the seeds mature, the plant goes through senescence; growth slows down, photosynthesis declines, and eventually the leaves of the plant dry and fall off the stock. This allows the plant to direct its energy into the further development of the protein-rich seeds (Willis 1989). The seeds of the soya bean plant form in a hairy pod (Figure 11) that grows in clusters of 3–5, each 3–8 cm long and usually containing 2–4 seeds ranging from 5–11 mm in diameter (Willis 1989). Once the hull of the soya bean is mature, it is water-resistant to protect the cotyledon and hypocotyls (Meyers *et al.* 2007). If the hull becomes cracked, the

seed will not germinate (Pers. Comm. Jeff Robinson, Woodrill Farms). The hull of a soya bean pod could be a variety of colours ranging from yellow, green, blue, brown, black, or mottle depending on the variety of the plant (Meyers *et al.* 2007).

Figure 11: Pod developing on a soya bean plant

1.15.3 Ecotoxicological Studies using Glycine max

Argentina in one of the world's leading producers of soya beans and with such high global demand continuing, there is a need to use less desirable land for growing this important crop such as near vehicular traffic and industrial production (Salazar *et. al* 2012). These researchers worry since *Glycine max* can transfers heavy metals from the soil and that the potential is present to also take up other toxic elements. Salazar *et al.* (2012) showed that concentrations of Cd, Pb, and Zn in soya beans did pose a toxicological risk for consumers and the rhizosphere was the important soil zone when considering food safety due to the bioaccumulation of toxic metals.

Rodriguez *et al*. (2011) wanted to determine if global increase of atmospheric CO_2 along with the increased emissions of heavy metals from industry in recent decades would have an impact on crops and thus food safety. They examined the root, stem, and seeds of *G. max* plants grown in different concentrations of soil amended with fly ash (particulates generated

as the result of combustion) with either 400 ppm (current global levels) or 600 ppm CO_2 (possible future conditions). Trace elements of Br, Co, Cu, Fe, Mn, Ni, Pb, and Zn were analysed to determine if translocation was taking place. All elements except Pb were in higher concentrations in the plants with the 600 ppm CO_2 treatment and Br, Co, Cu, Fe, Mn, and Pb were found in highest concentrations in the root (for all levels of fly ash). Translation to the seed was observed for Ni and Pb at 600 ppm CO_2 and moderate to high levels of fly ash (Rodriguez *et al*. 2011).

Wu *et al*. (2010) grew *Glycine max* in biosolids and wastewater irrigation and examined plant tissue and soil for particular PPCPs. They found the soya beans grown in the biosolids treatment had higher concentrations of some PPCP than those grown in the wastewater irrigation treatment and felt this was due to loading. After sixty days, carbamazepine, triclosan, and triclocarban had translocated through the root to the above-ground portion of the plant with carbamazepine having the highest concentration (216±75ng/g) in the leaves of soya beans that had biosolids treatment. This group also noted that the concentration in the soil of carbamazepine, triclosan, and triclocarban had decreased significantly by 38, 80 and 37% respectively over 110 days and concluded that this was probably due to degradation and plant uptake of the compounds over time.

1.15.4 Relevance to Project

Glycine max is a food crop of global importance. In 2009 it was the tenth most abundant crop produced (223 million tonnes) with the United States being the world leader in production (producing 91.4 million tonnes), followed by Brazil and Argentina, with Canada ranking seventh (providing 3.5 million tonnes to the global market in the same year) (FAO 2009a; FAO 2009b). In 2009, over 200 million tonnes of soya beans were produced globally (FAO 2009a). Therefore, if this important food crops were to be negatively impacted by the land-application of biosolids, it would have detrimental global implications. *Glycine max*, being a predominant crop in Southern Ontario, was chosen for use in further bioassays to examine root nodules.

1.16 Phaseolus vulgaris

Table 19 below provides the taxonomic key for *Phaseolus vulgaris*, a dicotyledon that is being used in this thesis.

Table 19: Taxonomic Key for *Phaseolus vulgaris* (USDA *Phaseolus vulgaris* 2008)

Kingdom:	Plantae
Subkingdom:	Tracheobionta (vascular plants)
Superdivision	Spermatophyta (seed plants)
Division;	Magnoliophyta/angiosperms (flowering plants)
Class:	Magnoliopsida (dicotyledons)
Subclass:	Rosidae
Order:	Fabales
Family:	Fabaceae (pea family)
Genus:	*Phaseolus* (bean)
Species:	*vulgaris L.*

1.16.1 Ecology of Phaseolus vulgaris

Phaseolus vulgaris, the common bean, is an herbaceous, annual dicotyledon. It was domesticated for its edible bean in ancient Mesoamerica more than 7,000 years ago and is now grown worldwide in both dry and green bean forms in sub-tropic and temperate zones spanning 52°N to 32°S latitude (van Schoonhoven and Voyset 1991; Graham and Ranalli 1997). The leaves of this annual plant are occasionally used as a vegetable, and the straw for fodder (Al-Qawrn 2009). Like *Glycine max*, *P. vulgaris* is classified as a legume (Tantawy *et al*. 2009) and acquires its nitrogen through the symbiotic relationship with the nitrogen-fixing bacteria *Rhizobia* (Graham and Ranalli 1997). *P. vulgaris* can tolerate a wide range of environmental conditions except a very wet climate and has a low tolerance for frost. It grows between altitudes of 50 and 3000 m with average daytime temperatures of 14 to 26° C and an annual precipitation of 400-1600 mm/year (Debouck 1994) with soil pH between 5.5-7.5 (Purdue 1998). Like most flowering plants, it is sensitive to red or far red light (Reed *et al*. 1993; Spectrum 2008). Therefore, the light not only controls many physiological reactions, such as germination, stem elongation, and flowering, but also determines how crowded the plants are and induces the plant to grow taller or fuller accordingly (Reed *et al*.

1993). These plants, as well as being self-pollinating, germinate and mature very quickly, thus making them a desirable plant for study. *Phaseolus vulgaris* can reach physiological maturity in sixty to sixty-five days after planting in a warm temperate regions or two hundred days when planted at cooler elevations since their development is dependent on the growing conditions (temperature, amount of light, and water availability) (Debouck 1994; Graham and Ranalli 1997). In Canada, *Phaseolus vulgaris* can be planted in mid-May and be harvested by the beginning of September (Government of Saskatchewan 2009). The variety used in this study takes approximately eighty days to reach physiological maturity (Pers. Comm. Twig and Tree, Wellandport, Ontario)

1.16.2 Anatomy of Phaseolus vulgaris

Phaseolus vulgaris can either produce an erect stem with branches or a twisted vine-like stem with reduced branching. Therefore, the height at maturity varies greatly (from 20-60 cm for the bush variety to 2-3 meters for the vine variety) (Debouck 1994). *P. vulgaris* is epigeal (the cotyledons emergence above the surface of the ground after germination) and as such, the cotyledons turn green and commence photosynthesis once above ground (Graham and Ranalli 1997). In the initial development of the plant, the hypocotyl emerges and pushes its way up through the soil in a distinct, bent hairpin shape. The hypocotyl arch (or Crozier's hook) will straighten out once it emerges from the soil due to phototropism (Graham and Ranalli 1997) and can be used as a measurable growth parameter in bioassays. *Phaseolus* produce alternate leaves with trifoliate leaflets along the main stem. The flower develops twenty-eight to forty-two days after planting depending on growth conditions (Graham and Ranalli 1997) producing pods of varying colours depending on the species (Figure 12). Seeds that are produced are smooth and usually slightly elongated, varying in weight between 50mg/seed to 2000 mg depending on the variety (Graham and Ranalli 1997).

Figure 12: *Phaseolus vulgaris* from green planter bioassay showing the developing been pods

1.16.3 Ecotoxicological Studies using Phaseolus vulgaris

The use of higher plants such as *Phaseolus* in environmental risk assessment has only recently gained the attention of the scientific community (Gong *et. al.* 2001; McCarthy *et al*. 2003). Gong *et al*. (2001) did not recommend the use of *P. vulgaris* due to its low sensitivity to mineral oil (one of their contaminants) but many other researchers have found it useful. Maliszewska-Kordyback and Smreczak (2000) in a laboratory study examined the ecotoxicological activity of PAHs using several plant species including *P. vulgaris.* Their findings showed that concentrations of the PAHs below 10mg/kg stimulated the early developmental stages of the plants while concentrations above 100 mg/kg had adverse effects on later growth parameters (root length, stem length, wet weight, and dry weight). McCarthy *et al.* (2003) used *Phaseolus vulgaris* in a field study to examine pulp mill biosolids and found no significant difference in germination, flower development, root length, or F_1 germination rates when compared to reference soil. Additionally, Tantawy *et al.* (2009) used *Phaseolus vulgaris* as a test species to evaluate Milagrow, a natural extract from the pollen of

cabbage used as a foliar fertilizer. They evaluated plant growth parameters (such as height, number of leaves, wet and dry weights, and pod yield) and found improvements, with a concentration of 5 g/100L showing the best improvement over reference plants.

1.16.4 Relevance to Project

Plaseolus vulgaris, although not a crop that would have biosolids applied to it, was chosen as a test organism since it is also grown in southern Ontario, and is similar to *Glycine max* in plant size and structure (Graham and Ranallii 1997) but has a shorter life cycle (Government of Saskatchewan 2009) and has a larger seed (two to three times larger). Therefore, seed size can also be evaluated. Even though *Phaseolus vulgaris* is also a legume like *Glycine max,* only *G. max* was used in the bioassays to examine root nodules since *P. vulgaris,* being a crop that can be consumed raw, would not normally have biosolids applied to them.

1.17 Brassica rapa

Table 20 below is the taxonomic key for *Brassica rapa*. As can be seen here, it is also a dicotyledon.

Table 20: Taxonomic Key for *Brassica rapa* (USDA *Brassica rapa* 2008*)*

Kingdom:	Plantae
Subkingdom:	Tracheobionta (vascular plants)
Superdivision:	Spermatophyta (seed plants)
Division;	Magnoliophyta/angiosperms(flowering plants)
Class:	Magnoliopsida (dicotyledons)
Subclass:	Dilleniidas
Order:	Capparaless
Family:	Brassicaceas/Cruciferae (mustard family)
Genus:	*Brassica*
Species:	*rapa L.*

1.17.1 Ecology of Brassica rapa

Brassica rapa, or field mustard shown in Figure 13, is a biennial herb and ruderal (a plant species that is first to colonize disturbed lands) and has therefore been used as a 'cover' crop to hold the soil and prevent erosion (University of Wisconsin 1990). The origin of the *Brassica* family is unknown but is thought to have originated somewhere between the Eastern Mediterranean and Eastern China. The turnip, one member of the *Brassica* family, were found in the times of Alexander the Great (356-323 BC) (Toxopeus and Bass 2004). The taxonomy of *Brassica rapa* is perplexing and there have been many classifications proposed, each trying, unsuccessfully, to capture the wide variety of the taxa in one system. Since it comprises many different crops (including kale, broccoli, cabbage, turnip, and rape), it has proven to be a very difficult family to classify (Toxopeus and Bass 2004). The genus *Brassica* is comprised of various species of plants with economic significance (Musgrave 2000).

Figure 13: Photo of *Brassica rapa* on Day 15

Brassica rapa can tolerate annual precipitation of 3.5 to 41.0 dm, annual temperatures between 3.6 to 27.4°C, and a soil pH of 4.2 to 7.8 (Duke 1983). Being entomophilous (pollinated by insectes), the tiny yellow flowers produced are pollinated by bees (University of Wisconsin 1990).

1.17.2 Anatomy of Brassica rapa

The seeds of *Brassica rapa* are quite small (~ 1mm in diameter) and produce a plant approximately 20 cm in height on a single stock that is mature in 28 days with seeds maturing in 40-48 days (University of Wisconsin 1990). This very rapid lifecycle makes *Brassica rapa* an ideal organism to use if impact on future generations is also of interest as is the case in this study

1.17.3 *Ecotoxicological Studies using Brassica rapa*

The OECD Guidelines for Testing of Chemicals (2003) uses *Brassica rapa* along with many other species in their Vegetative Vigor Test to assess the potential effect to the plant caused by the test chemical being applied to the above ground portion of the plant (i.e. leaves and stem). Ceric (2001) and McCarthy *et al.* (2003) both used *Brassica rapa* as a test organism for their ecotoxicological bioassays using full life-cycle tests to examine pulp mill biosolids and found no significant difference compared to Wilke *et al.* (2008) who used *Brassica rapa* in a 14-21 Day bioassay to determine seedling emergence and biomass production for the testing of hazardous wastes. They found adverse effects (growth reduction) at higher levels of Cu (20g/kg) in one of their test samples. In 2009, the Californian Office of Environmental Health Hazard Assessment added *Brassica rapa* as a species in soil toxicity testing in their government protocols (CEPA 2009).

1.17.4 Relevance to Project

Brassica rapa was chosen as a plant for our bioassays not only because it can be found in ditches and roadsides all across Ontario but because it has been extensively studied and developed by Dr. Paul Williams of the University of Wisconsin and has very detailed

documented growth stages (Williams 1990). Additionally, its fast lifecycle allows for appropriate assessment of different development stages. Although *Brassica* is not a plant that would have biosolids applied to it, it was also chosen for this study due to its seed size. Unlike the seeds of other crop plant, *Brassica rapa's* seeds are tiny (~1 mm in diameter) and the effects, if any, of biosolids being applied needs to be determined.

1.18 Summary

In summary, the many organic and inorganic chemicals that come from industry and municipalities that eventually enter a WWTP have the potential to partition into the organic-rich biosolids and thus be land-applied to agricultural fields. As shown earlier, there is much research on fate and transport of these contaminants but little on impact. Therefore, there is a critical need for a holistic approach to assess if the land-application of biosolids impacts the indigenous terrestrial biota of the agricultural biome and thus to determine if land-application is a sustainable practice.

As a result, the overall objective of this thesis is to determine if the land-application of biosolids to agricultural biomes is a sustainable practice. This is to be accomplished by means of a holistic approach using selected, environmentally-relevant, indigenous, terrestrial organisms that are important to the Southern Ontario agricultural region. The organisms that were selected are: *Folsomia candida, Lumbricus terrestris, Zea mays, Glycine max, Plaseolus vulgaris, and Brassica rapa.* Additionally, upon examining government protocols, it was discovered that they were inadequate for the scope of my thesis. Therefore, a further objective of this thesis was to develop useful protocols and test them using the individual indigenous terrestrial biota mentioned above for use in a laboratory setup that simulated as closely as possible the natural environment.

2. **Methodology – Protocol Development**

This section describes methodologies utilized in this study to ascertain if there is any effect (negative or positive), to terrestrial organisms due to the land-application of biosolids on agricultural lands. Figure 14 is a diagrammatic representation of the organisms and each of the various bioassays used in this thesis.

One of the primary objectives of this thesis is protocol development, and therefore the methodology section was written in a format similar to that of government protocols.

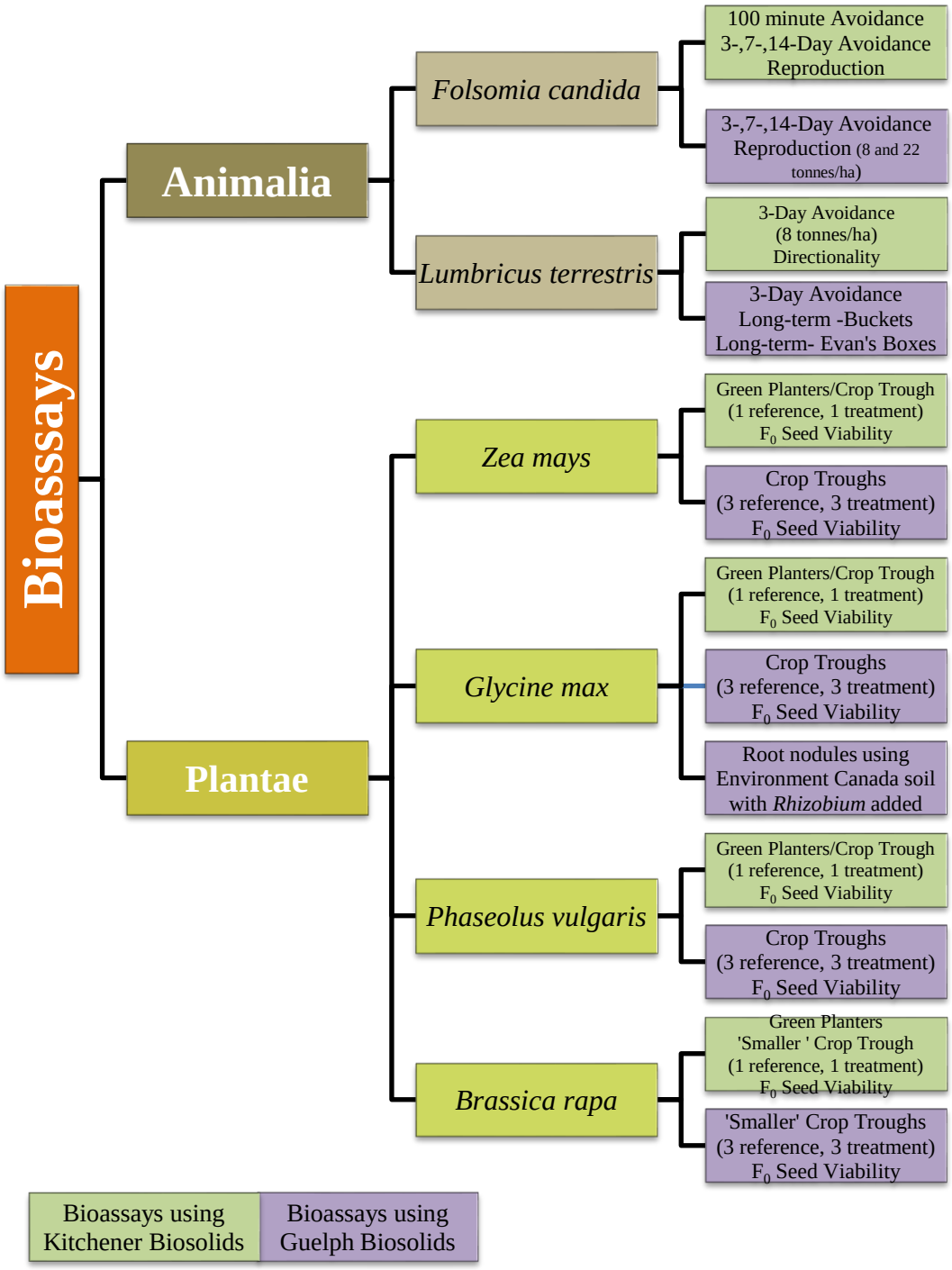

Figure 14: Organism and bioassays used to assess for impact using biosolids from two different sources

2.1 Ryerson Protocol for Cleaning Glassware and Other Objects used in Bioassays

2.1.1 Material and Equipment

Non-phosphate detergent, Extran (purchased from VWR Scientific)

Basin of adequate size to completely submerge items being washed

Hydrochloric Acid (HCl) 10% (v/v)

Acetone/Hexane (purchased from VWR Scientific)

Distilled water

2.1.2 Cleaning Protocol (Modified from Environment Canada)

Note: Prior to use in experiments, all glassware and other material must to be thoroughly cleansed

1. In a basin large enough to contain the items to be washed, a solution of dechlorinated municipal drinking water (DMDW) with a sufficient amount of non-phosphorous continuing detergent such as Extran was made according to the manufacturer's instructions
2. Each item was submerged in the soapy water and allowed to soak for at least 15 minutes
3. After the time had elapsed, each item was thoroughly rinsed with DMDW while finger scrubbing to ensure the removal of all residue
4. Next, each item with acid-washed in dilute HCl (10% v/v) by soaking for 10 minutes to remove any residue of scale, metals or bases, then rinsed with deionized water (three times)
5. To ensure no remaining organic compounds were present, the items were rinsed once using full strength acetone (in a fumehood). If an oily residue was noticed, this last step was performed using high-quality hexane instead
6. Each item was again rinsed with deionized water (three times) and allowed to dry in an inverted position

Figure 15 is a diagrammatic representation of the method section for *Folsomia candida*, to provide an overview of the many aspects of culturing and age synchronizing that were required prior to the bioassays commencing.

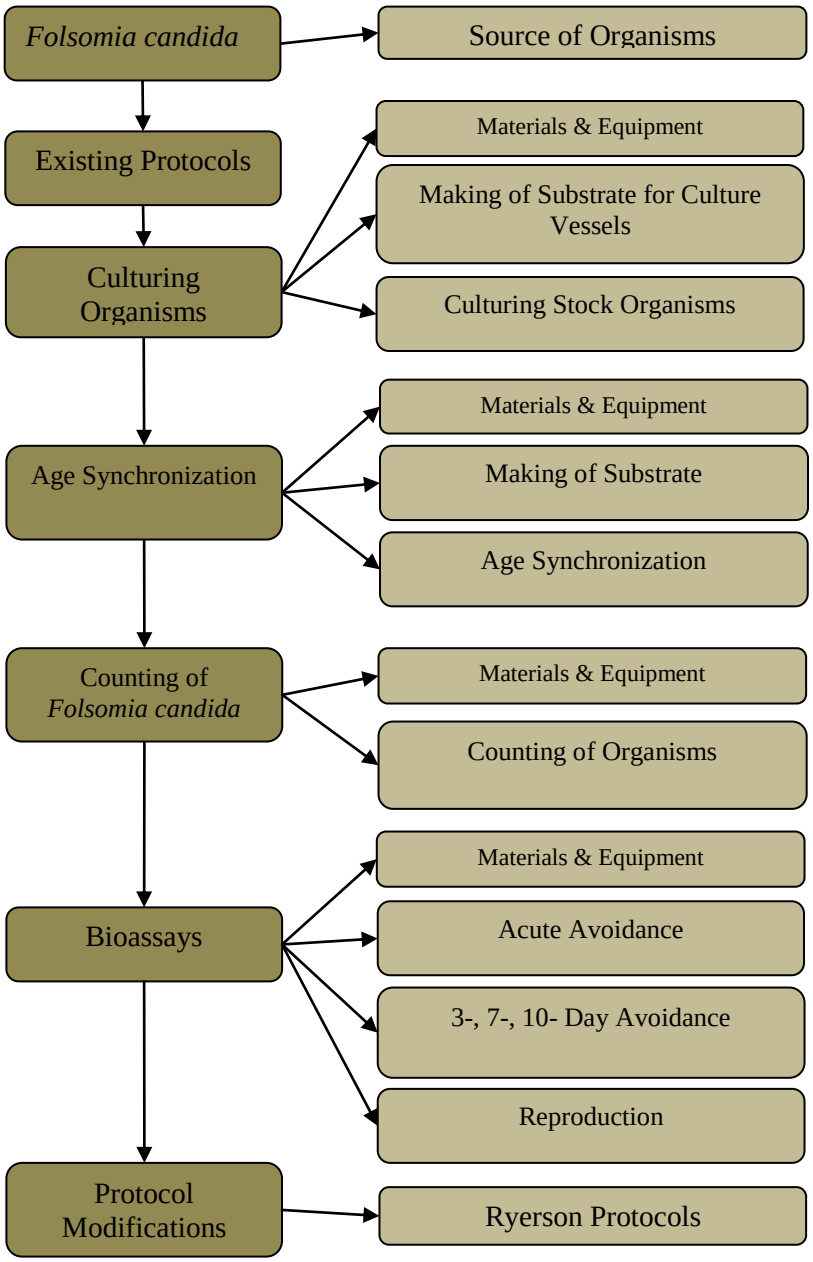

Figure 15: Flowchart for *Folsomia candida* Methodology Section (pages 81-92)

2.2 *Folsomia candida* (Springtails)

2.2.1 Source of Organisms

An initial culture of *Folsomia candida* was obtained from the Science & Technology Branch, Environment Canada, Ottawa courtesy of Juliska Princz, Head of Soil Toxicology Laboratory Biological Methods Division. Our laboratory's starter culture was a pure culture and verified as being *Folsomia candida.* This culture was the source of all organisms used in these bioassays.

2.2.2 Existing Protocols

To assess if the land-application of biosolids have any impact on *Folsomia candida* the following protocols were examined. From Environment Canada Standard Operating Procedures SOP 15.29/1.0/S, 15.30/1.0/S, 15.32/1.0/S; Draft copy of Environment Canada RM/47 2007 (provided by Richard Scroggins, Chief, Biological Methods Division, Environment Canada) since their established government protocols were not published at the time this investigation was being carried out. The International Standards Operation (ISO) #11267 (1999), Organization for Economic and Cooperative Development (OECD) pre-validation (2005) and test draft guidelines (2008) and research by Wiles and Krogh (1998), Hopkins (1997), Fountain and Hopkin (2005), Aldaya *et al.* (2006), Crouau and Moia (2006), and Campiche *et al*. (2007) were also used for guidance. After the Ryerson protocols were established, OECD published their Guidelines for Testing Chemicals using a Collembolan reproduction test (#232 September 7, 2009), which again follow similar procedures as those sources mentioned above.

2.2.3 Culturing of Organisms (Adopted from OECD and Environment Canada's SOPs)

2.2.3.1 Materials and Equipment

Culturing Chamber - Transparent vessels (34.2 cm x 20.9 cm x 11.8 cm) with tight fitting lid purchased from Canadian Tire lined with activated charcoal and Plaster of Paris

LePage Poly Plaster of Paris

Activated Charcoal - (decolourized powder, 375 µm mesh)

Fleischmann's Quick Rise Instant Granulated Dry Yeast (purchased from any grocery store)

2.2.3.2 Making Substrate for Culture Vessels or Bioassay Chambers

The charcoal in the substrate aids in the absorption of gases such as ammonia while the Plaster of Paris provides a platform upon which the springtails can lay their eggs and be easily manipulated. In addition, the Plaster of Paris locks in moisture thereby maintaining the humidity in the culture vessels (providing the vessel is maintained properly by keeping it hydrated). The activated charcoal is included not only to absorb waste gases and excreted products from the white springtails but to also make them easier to discern against the resulting black surface

Note: The quantity of reagents used here makes enough substrate for one 34.2 cm x 20.9 cm x 11.8 cm culture vessel, 7 glass Petri dishes for age-synchronizing or 15 Mason jars for bioassays. The amounts can be modified as needed by using the specified ratio of 1:8 (activated charcoal:Plaster of Paris) to obtain a thin layer of about between 1-2 cm in the bottom of the containers.

1. In a fume hood while wearing a dust mask, 240 grams of LePage Poly Plaster of Paris and 30 grams of activated charcoal were weighed out using a top-loading balance, then added to a 1-Liter plastic bottle with a tightly sealed lid
2. After shaking vigorously for 2 minutes to obtain a uniform mixture, the lid on the jar was slightly loosened and the dust allowed to settle before opening all the way
3. 200 mL of deionized water were added to the container and shaken vigorously until all reagents were mixed evenly, about 30 seconds
4. Description given here are for the culture vessel, but the same method applies for the other vessels except the contents were divided among the containers to produce a thin, 1-2 cm layer, in the bottom of each. Working quickly because the mixture begins to solidify with the addition of water and mixing, the contents were poured

into the bottom of the transparent culture vessels avoiding getting any on the sides of the vessel as this will dry and fall to the surface, potentially harming the Collembola. Immediately after pouring, the bottom and sides of the vessel were tapped to release any air bubbles. This created an even, smooth surface without crevasses (thus ensuring the egg clutches could be easily seen and assessed)

Note: it was better to pour the mixture in the middle of the container, since this led to a more even distribution throughout the container and less chance of getting it on the walls

5. The lid was replaced on the culture vessel and the Plaster of Paris and charcoal mixture allowed to solidify on a level surface for about 2 hours. The surface was then washed with deionized water to remove any residue (at least three times or until the water ran clear). While washing, edges and surface of the substrate were gently rubbed with a finger to help remove any residue or rough edges
6. The pH of the substrate was measured to ensure that it was between 6.0-7.0 by pressing a strip of pH paper against the substrate after the last wash while the surface was still moist
7. If the container was not to be used right away, a 2.5 cm layer of deionized water was left on the surface of the substrate to prevent it from drying out and to maintain the moisture content within the substrate and the container was stored at room temperature until needed

2.2.3.3 Culturing Stock Organisms

Organisms were introduced into the culture vessel by either tapping them from their previous container into the new one or by 'blowing' them with the aid of a pasture pipette and bulb. Alternatively, a wetted paint brush could be utilized to gently pick up individual springtails and move them. The method that was used depended on the quantity of organisms needing be transferred and the accuracy of the count required. Whichever method is used, care must be taken not to harm these delicate organisms.

1. 50-100 springtails were transferred into the prepared culture vessels by using either a wetted fine-tipped paint brush to pick them up or a glass pipette and bulb to gently 'blow' them from one container into the other
2. The culture was fed with 0.1 g (100 mg) of Fleischmann's Yeast in two separated piles (50 mg yeast for each pile) weekly. A few drops of DMDW to the surface of each yeast pile to moisten (prepared by bubbling air into 20 L carboys of tap water for 24 hours or until chlorine was absent as determined by analysis). And on subsequent feedings, old, unused food was removed at the same time. This maintains a fresh food supply and reduces the amount of spoilage by bacteria and fungi
3. The lid was tightly secured to maintain the desired relative humidity and prevent desiccation, as well as escape, of organism
4. Temperature was maintained at 20 ± 2°C with a relative humidity near 100% (monitored using a digital thermometer that also read humidity). In our lab, this was achieved by using a small "wine fridge"

   Note: Since springtails are easily dehydrated, resulting in their death, the relative humidity in each vessel must be maintained to ensure optimal environmental condition. Optimal humidity is achieved by keeping the Plaster of Paris moist. The addition of several drops of DMDW to the surface of the substrate or misted along the walls of the vessel and allowed to run down to the surface was required weekly (or more frequently if the substrate became dry, indicated by turning a lighter grey colour). The Plaster of Paris was sufficiently moistened when it no longer quickly absorbs water and was over-saturated when there was standing water. Water was continuously added to the substrate until it just began to collect on the surface
5. The culture was maintained in low lighting conditions (5.2 -11.2 μmol/m^2/s for cool white fluorescent) for better reproduction. *Folsomia* are blind, they do not *need* light, but can sense it (Hopkin 1997)
6. The culture vessels were aerated weekly or more frequently if fungal growth became a problem. This was accomplished at the time of feeding and re-hydration of the substrate. The condensate on the lid was allowed to drip back onto the surface of the

substrate being sure not to unduly interfere with the organisms and not to drip directly onto the egg clutches. The lid was used to gently fan the air across the top of the vessel to ensure the complete exchange of air all the way to the bottom of the chamber, at the same time being careful not to disturb the organisms. The lid was then left off for several minutes, being careful not to allow any organisms to escape. The height of the chamber makes escape difficult but not impossible

7. A weekly check of the health of the culture was made. Actively moving organisms and the presence of egg masses was indicative of a healthy culture. Also disappearance of the previous week's food is also a good indicator
8. When the culturing vessel became overcrowded (greater than 2-3 organisms per cm^2) which happened approximately every three months, the population needed to be reduced by removing organisms (either for experimental use or to start a new culture) since the collembolans will reduce egg production in a crowded environment. Moving to fresh containers induces oviposition (the laying of eggs)

2.2.4 Age Synchronization (Modified from OECD, Environment Canada's SOPs)

2.2.4.1 Materials and Equipment

Glass Petri Dishes (9 cm diameter x 1.5 cm deep) lined with 0.5 cm layer of activated charcoal and Plaster of Paris

2.2.4.2 <u>Age Synchronization</u>

For experimental use, the age of the organisms used must be known, and must be between 10 and 12 days old. Removing the adults after 2 days ensures the synchronization of the hatching of the juveniles to within 48 hours. Starting with a laboratory culture of *Folsomia candida* that is reproductively mature (approximately 22 day old organisms), indicated by egg masses evident on the floor of the culture chamber, ensures the success of the age synchronization process.

1. Adult springtails (~ 50 organisms) were transferred from the culture vessel to several Petri dishes prepared with Plaster of Paris: charcoal substrate (Section 2.2.3.2) using a

fine-tipped paint brush wetted with DMDW or by gently blowing with a Pasteur pipette. The number of age-synchronized organisms needed for the bioassay will determine how many Petri dishes needed to be set up

2. Springtails were fed with 1 mg of Fleischmann's yeast (pile was located in the center of the Petri dish) and moisten with one drop of DMDW
3. Organisms were left to oviposit for 48 hours under same conditions of, light, temperature and humidity as during culturing.

   Note: Once set up, the Petri dishes should be disturbed as little as possible since bumping of the container will induce stress on the organisms and affect reproduction rates.
4. After this time, Petri dishes were checked to determine if any eggs (small white spheres about 80-110µm in diameter) had been laid on the substrate surface
5. If clutches of eggs were observed, all adults were removed from the Petri dishes and returned to the culture vessels
6. The eggs in the Petri dishes were left to hatch (approximately 7-9 days)
7. Two days after the first hatching was observed, un-hatched eggs were removed using the flat edge of a scupula. The remaining culture was then ~ 48 hours old and now said to be 'Age Synchronized'
8. After 10 to 12 days elapsed from hatching, the juvenile springtails were ready for use in bioassays

2.2.5 Counting of Folsomia candida (Modified from OECD and Environment Canada's SOPs)

2.2.5.1 Materials and Equipment

Bromophenol blue (Dilute solution)

Digital Camera

Image J Software by NIH Images (counting software, author Wayne Rasband, National Institute of Health, Maryland, USA)

2.2.5.2 ***Method for Counting Organisms***

1. The bioassay vessels containing soil were flooded with DMDW comprising a small amount of Bromophenol Blue. The amount of Bromophenol blue added, and thus the intensity of the colour produced, is dependent on the level of contrast desired between the white springtails and the colour of the soil (which varies) (Figure 16)

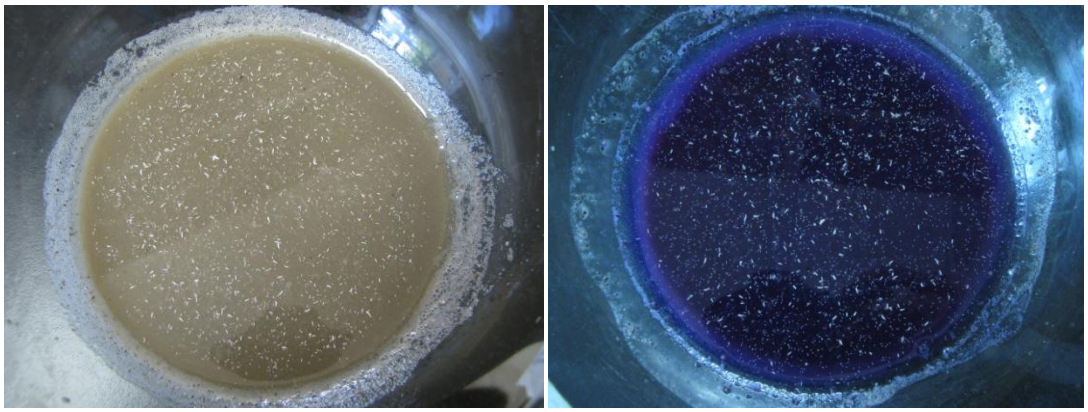

Figure 16: No Bromophenol blue added (image on the left): Bromophenol blue added (image on the right)

2. Contents of the vessel were stirred very gently with a plastic spatula to ensure that all of the springtails were released from the soil. The vessel was set aside for a few minutes to allow the soil to settle to the bottom and springtails to rise and float on the surface of the water due to their hydrophobic exoskeleton. The Bromophenol blue added contrast to the water and made it easier to count the white springtails against the brown soil (Figure 16)
3. Depending on the test that was being performed, the number of organisms present could be too numerous to count. If this was the case, the contents of the vessel was divided into several glass bowls having a diameter about 15 cm to increase the surface area and thus act to 'dilute' the organisms (Figure 16)
4. To each bowl, more Bromophenol blue was added to the water and the above steps repeated
5. Several digital pictures of each bowl were taken to provide an average when counting

6. For images with low numbers of organisms, a pen that counts with each tap on the page (purchased from VWR International) or for larger numbers, Image J was used to count the number of organisms in the digital photographs

2.2.6 Bioassays

Two different avoidance bioassays were performed: i) 100-minute avoidance bioassays using Petri dishes (Method #1) and ii) 3,-7,-14-Day avoidance bioassays using Mason jars (Method #2)

2.2.6.1 Materials and Equipment

Avoidance Chamber

Glass Petri dishes (9 cm diameter, 1.5 cm deep)

Mason Jars (125 mL, 5 cm diameter) purchased from Canadian Tire or any grocery store then lined with activated charcoal and Plaster of Paris

Divider made from plastic Quilters template sheets cut to fit diagonally across the Mason jars lined with activated charcoal and Plaster of Paris

Reproduction Chambers

Biosolids obtained from either Kitchener or Guelph WWTP

Reference soil (obtained from OMAFRA research farm in Stratford Ontario) (approximately 700 g for one avoidance and reproduction bioassay)

Treatment soil (same reference soil as above with biosolids added at a rate of 22 tonne/ha on a dry weight basis, unless otherwise indicated). The actual dry weight of biosolids to be added is determined for each new batch of biosolids used. See Appendix I for calculations (approximately 700 g of soil was needed for one avoidance and reproduction bioassay)

2.2.6.2 Acute Avoidance Bioassays - Method # 1

The 100-Minute Avoidance bioassay for *Folsomia candida* gives a quick and easy assessment of potential impact and can be carried out while waiting for results from the

longer duration bioassays. The endpoint examined was the location of the springtails at the specified time point during the bioassay.

1. Nine cm diameter filter paper was cut in half and placed on the bottom of ten cleaned, Petri dishes with a 2 mm gap in between the two halves. Each Petri dish counted as one replicate
2. One filter paper was covered with a paste made for reference soil and the other piece of filter paper was covered with a paste made from treatment soils maintaining the 2mm gap in between the two halves. The filter paper gave the soil something to adhere to and prevented it from mixing during the bioassay
3. Into the gap of each Petri dish were placed five randomly selected age-synchronized springtails, at 1 minute intervals. This allowed time for making observations between each of the ten different Petri dishes
4. The springtails' location (either on the reference side or on the treatment side) was observed and recorded every 20 minutes in each of the Petri dishes for a total of 100 minutes, recording the location of each of the five springtails in each dish at each time interval

2.2.6.3 3-7-14 Day Avoidance Bioassay - Method #2

The amounts indicated here are for the particular batch of biosolids used at this time. For calculation on determining the amount needed for different batches of biosolids, see Appendix I. The endpoint observed for these bioassays was the location of the springtails after the specified time and noted as either being in the reference or treatment.

1. Ten Mason jars were prepared with 1 cm of Plaster of Paris and activated charcoal as described in Section 2.2.3.2
2. 75 g of reference soil were mixed with 14.3 mL of DMDW to account for the water content in the biosolids and 75 g treatment soil were prepared by mixing 75 grams of reference soil with 14.6 mL of biosolids

3. Plastic dividers made to fit diagonally across the jars were inserted and 15 g of reference soil was added to ½ of the jar and 15 g treatment soil to the other ½ of the jar and the process was repeated for all 10 Mason jars. (Figure 17). Each jar was labelled accordingly indicating which side was reference and which was treatment

   Note: the soil was compacted slightly to allow the springtails to be easier to observed on the surface and less able to 'crawl' within the soil

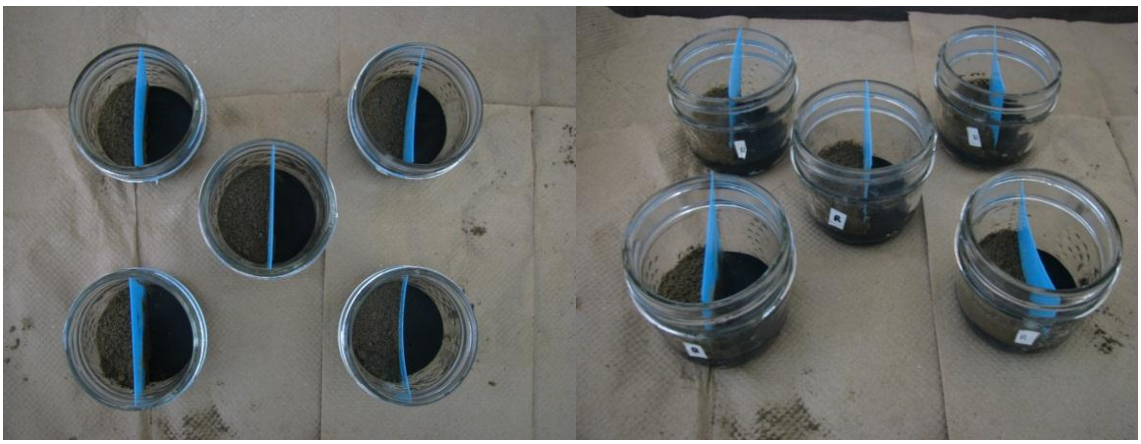

Figure 17: Avoidance chamber setup for *Folsomia candida*

4. The dividers were carefully removed so as to not disturb or mix the soils
5. Ten 10-12 day old age synchronized *Folsomia candida* were gently added to the centre of each jar. Jars were sealed tightly to maintain moisture content of the soil

   Note: to aid in the accuracy of the counting, counting springtails were initially transferred into an empty Petri dish. When the appropriate number was achieved, all springtails were then transferred to the test vessel. This ensures the accuracy of the number of organisms in each jar
6. Jars were placed in a dark location and left undisturbed for the duration of the bioassay (either three, seven, or fourteen days). The test vessels were not fed during this time period

7. After the appropriate time (three, seven, or fourteen days) elapsed, the dividers were replaced along the center line in each of the jars and the springtails were counted visually and recorded as either being on the reference or on the treatment soil
8. After all ten organisms had been accounted for, the divider was again removed and the jars resealed and returned to the dark to await the next time period. Additionally, during the counting, the test vessels were aerated at the same time

2.2.6.4 Reproduction Bioassay

The biological endpoint examined in this bioassay was the number of live springtails present at termination (39 days); the distinction between juvenile and adult was not made. Before commencing the bioassay, moisture content, pH, as well as the water holding capacity of the soil was determined as described in government protocols such as Environment Canada's RM 43.

1. 150 g of the reference soil were mixed with 28.7 mL of DMDW to account for the water content in this batch of biosolids. As well, 150 g of the autoclaved reference soil was prepared in the same manner. (Autoclaved soil was used in the initial test to ensure that there was not any interference in the bioassay from *Folsomia* that could naturally be present in the soil)
2. 150 g treatment soil were prepared by mixing 150 g of reference soil with 29.1 mL of biosolids
3. 30 grams of the reference soil were then placed into each of the five Mason jars already prepared with a 1 cm layer of Plaster of Paris and charcoal (described above, Section 2.2.3.2). The procedure was repeated for the autoclaved soil and treatment soil

   Note: the soil was compacted slightly so that the springtails are easier to observed and to give a flat surface to lay their eggs on
4. Ten 10-12 day old age-synchronized springtails were transferred into each of the test vessels with soil

   Note: to aid in the accuracy of the counting, the springtails were first transferred while counting into an empty Petri dish. When the appropriate number was achieved, all

springtails were added to the test vessel. This ensured the accuracy of the number of organisms in each jar and was repeated for all jars

5. The test organisms were fed with 2 mg of Fleischmann's yeast which was hydrated with 1 drop of DMDW
6. All jars were sealed tightly to maintain the high humidity in the vessel and prevent the escape of any organisms, and left undisturbed in a dark location. Temperature was maintained at 20 ± 2 °C
7. Twice weekly, the jars were aerated, and 2 mg of fresh yeast were added to each vessel every two weeks for the duration of the bioassay (39 days)
8. At the completion of the bioassay, the number of live organisms was determined using the method previously described in Section 3.2.5. The distinction between juvenile and adult springtails was not made. Due to the large number of organisms that were produced in these longer time frames, the dilution of the vessels is highly recommended to aid in accurate counting

2.2.7 Protocol Modification- for Folsomia candida

In the case of the springtails, government protocols were not available at the time this research was carried out. However SOPs were adapted. Although the ISO 11267 guidelines of 1998 were referenced in many articles, these guidelines were not themselves accessible without a high cost. The OECD guidelines were not finalized until September of 2009 and Environment Canada's, although dated September 2007, was not published until early 2009.

The present ISO guideline 11267 was one of the first for the collembolan *Folsomia candida* and has been applied successfully for toxicity testing in soil since its release in 1999. However this methodology pertains exclusively to bioassays where *F. candida* are in contact with contaminated pore water in soil (Crouau *et.al*. 1999). Therefore, many researchers have altered this protocol in their research to account for such changes as pH, organic matter as well as moisture levels of the soil. The alterations that were made for this research are as follows.

2.2.7.1 Extended the Duration of the Reproduction Bioassay

Existing government protocols were 28 days long. Thus, at the completion of these bioassays, the original 10-day old springtails were now 38 days old, sexually mature (which occurred around Day 14 of bioassay) and able to lay two batches of eggs. On Day 28, these eggs would have hatched and the juveniles would be approximately 4 days old, and very small (0.5mm long) (Fountain and Hopkin, 2005) making them difficult to count. By extending the reproduction bioassay to 39 days (11 more days) the juvenile springtails were now 15 days old and not sexually mature (so not laying eggs of their own). However, these juvenile organisms were now much larger (between 1.5 and 3 mm long) and thus easier to discern (and count) in the soil and therefore a more accurate count can be made.

2.2.7.2 Removing Light

Reproduction bioassay carried out in the government protocols used a 12 hr light:dark light cycle with 400-800 lux illumination. This light cycle was to prevent the springtails from escaping and to keep them below the soil surface (OECD Version 3.4, 2008). This light:dark cycling condition was not utilized in the newly-developed Ryerson Protocol since *Folsomia*, being blind, do not need light (while they can sense light) and lay more eggs in total darkness (Fountain and Hopkin 2005). Since light inhibits reproduction (and this was a criteria being evaluated), environmental conditions that support this biological function were utilized. Therefore, reproduction bioassays were carried out under dark conditions.

2.3 *Lumbricus terrestris* (Earthworms)

Figure 18 is a diagrammatic representation of the method section for *Lumbricus terrestris*.

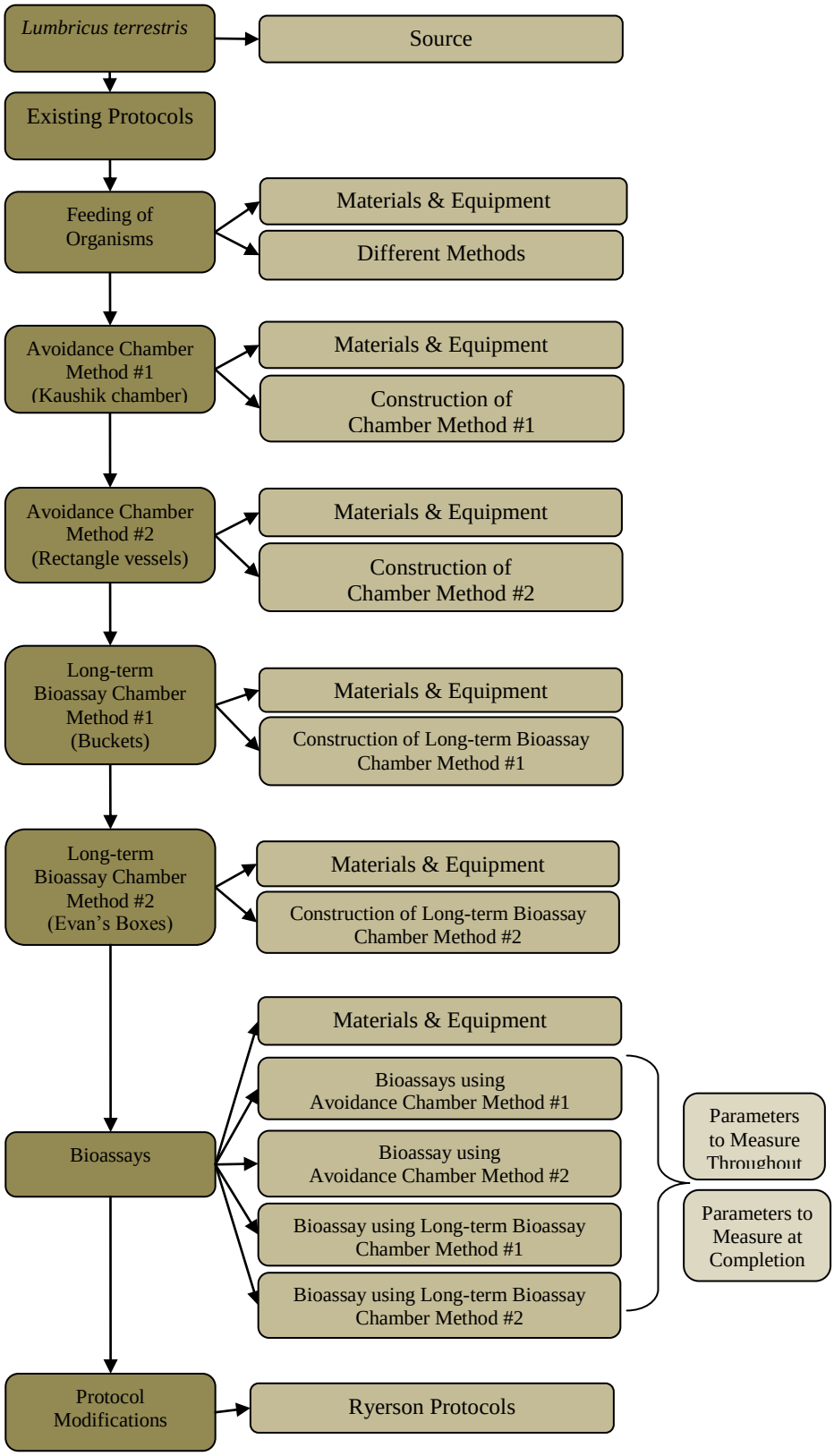

Figure 18: Flowchart for *Lumbricus terrestris* Methodology Section (pages 96 – 117)

2.3.1 Source of Organisms

Adult *Lumbricus terrestris* were purchased from Wards Scientific, St. Catherine, Ontario where their taxonomy has been verified. Individual organisms had a wet weight between 3-10 g.

2.3.2 Existing Protocols for Lumbricus terrestris

To assess if the land-application of biosolids had any impact on *Lumbricus terrestris*, the following methodologies were examined. The European OECD #207 (1984) examined acute toxicity and #222 (2004) reproduction bioassays. ISO 11268 (1993) described a method for acute toxicity testing using artificial soil, while USEPA #712-C-96-167 (1996) described sub-chronic toxicity testing, and Environment Canada's RM 43 (2007) is a protocol assessing soil toxicity. All of these government protocols use *Eisenia sp.* which are not an environmentally-relevant organism for this work. The exception is that Environment Canada does mention *Lumbricus terrestris* for use in their avoidance bioassays but not for reproduction. The following papers were also examined; Evans (1947; 1948), Stephenson *et al.* (1998), Alvarez (2000), McCarthy *et al.* (2003), Lowe and Butt (2005). Again, with the exception of Alvarez and McCarthy, the focus was on *Eisenia* species for their ecotoxicological studies.

2.3.3 Feeding of Lumbricus terrestris

2.3.3.1 Materials and Equipment

Quaker Oats™

Composted vegetable matter

Fresh and dried leaves

Note: Feeding of the earthworms took place every 14 days. Various methods were tested to determine the most favourable feeding method. Feeding methods tested are listed as follows:

2.3.3.2 ***Oatmeal***

Two tablespoons of cooked Quaker Oats™ (3-5 minute cooking variety) as per Environment Canada protocol EPS1/RM/43 – June 2004 (with June 2007 amendments) was added to a depression in the soil then covered with a thin layer of soil to minimize microbial growth.

This method was discarded because the oats became mouldy very quickly. There was evidence of feeding by the earthworms; however, there was continuous soil loss in attempts to control the mould and mites. This method might be better employed in winter months when excess humidity from the air is not an issue, thus circumventing the mould issue. This method was also discarded because buried food in not the preferred feeding method of *Lumbricus* but only for *Eisenia* (Environment Canada 2007a; Ayers 2010; Frund *et al.* 2010).

2.3.3.3 ***Composted Vegetable Matter***

Supplement feedings with dried compostable vegetable matter that did not contain peels, stones or seeds was prepared by macerating contents in a blender until smooth with the addition of DMDW if necessary. After blending, the contents were spread onto a tray and dried at 105°C overnight or until the consistency of dried pabulum. Once dried, this organic matter was crumbled and then sprinkled on the soil surface according to feeding frequency previously determined through observation of the culture over time. Extra food was stored in the refrigerator for up to seven days. (Method adapted from Environment Canada protocol EPS1/RM/43 - June 2004 (with June 2007 amendments)).

This method was also discarded due to the fungal growth that appeared on the soil surface, and the soil loss that resulted from trying to control the problem. Again this method might be better suited to winter months when the air is drier and humidity is not excessive.

2.3.3.4 Fresh Leaves Picked Daily

Fresh Leaves – (Maple, *Acer sp*.) leaves were collected daily from the Ryerson Quad (high traffic urban area) during summer months and acid-washed (three times) and dried. The leaves were placed on the soil surface and the earthworms were allowed to feed on them.

Leaves proved to be a highly effective method as the feeding frequency and midden (worm excrement) production was more readily observed when compared to the previous methods. The source of leaves was undesirable as they could be contaminated with air-borne pollutants in the downtown Toronto area. Thus this method was discarded.

2.3.3.5 Dried Leaves obtained from Non-Urban Source

Dried leaves – A mix of dried leaves, predominantly Sugar Maple (*Acer saccharum*) were collected during November 2008 from a non-urban, private residence on Georgian Bay. No pesticides were reported to be used on these trees or the surrounding area (although the possibility of long-range transport of contaminants was not discounted). The leaves were applied directly to the soil surface and misted with DMDW. To supplement the leaves, corn meal was also sprinkled on the surface of the soil as this is the food choice at the worm breeders and is known to help increase reproduction (Pers. Comm. Wards Scientific). This was deemed to be the most effective feeding method and was used for all bioassays pertaining to *Lumbricus terrestris*.

2.3.4 Avoidance Chamber Method #1 (Kauschik Chamber)

2.3.4.1 Materials and Equipment

High Density Poly Ethylene (HDPE) bucket, 18.9 L capacity with 28.7 cm internal diameter

Acute Avoidance Chamber Apparatus, refer to Figure 19 below for dimensions (adapted from Environment Canada Protocol EPS 1/RM 43 – June 2004 (with June 2007 amendments))

Acrylic pieces 100 mm x 80 mm to insert along inner wedges to stop earthworms from moving between chambers after completion of test

2.3.4.2 <u>Construction of Avoidance Chambers - Method #1</u>

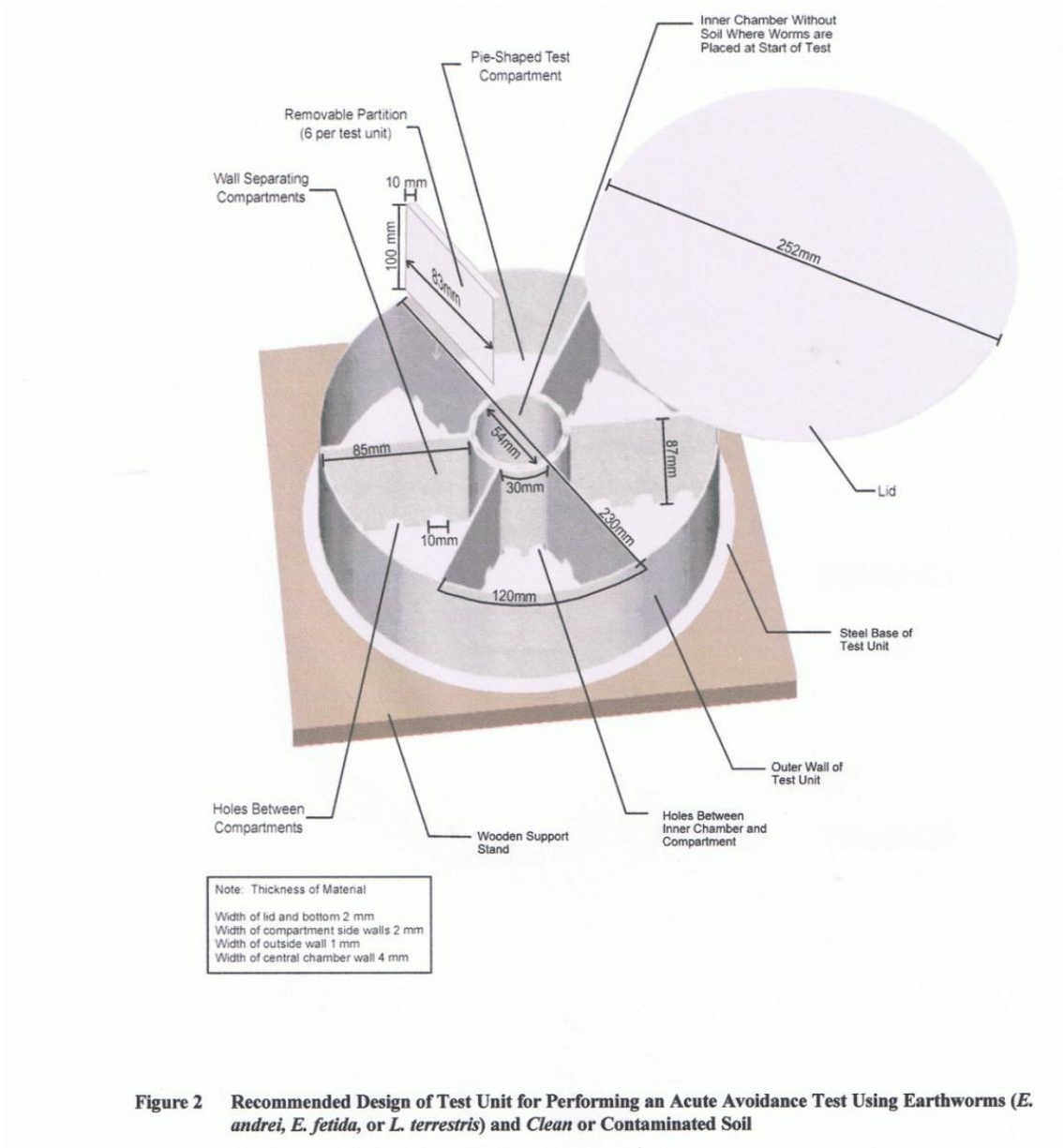

Figure 2 Recommended Design of Test Unit for Performing an Acute Avoidance Test Using Earthworms (*E. andrei, E. fetida,* or *L. terrestris*) and *Clean* or Contaminated Soil

Figure 19: Layout of Kaushik Avoidance Chamber (Source: Environment Canada EPS 1/RM/43 – June 2004 (with June 2007 amendments)

Avoidance chambers as described by Environment Canada EPS 1/RM/43 – June 2004 (with June 2007 amendments) were constructed with the following alterations:

1. Bottom 30 cm of a 18.9 L high density polyethylene (HDPE) bucket (for the walls and bottom of the chamber) were used instead of a wooden support (which could be easily contaminated) and steel
2. Removable floors were added and placed in each wedge to aid in the removal of the soil from each chamber at the completion of the bioassay
3. The depth was increased to 30 cm due to the fact that *Lumbricus* are vertical dwellers and need a depth greater than the prescribed 10 cm
4. Length of partitions were adjusted to fit the bottom of the pail (now 28.7 cm and not 23 cm). (Figure 20)
5. Holes in the bottom of partitions between wedges were increased to 15 mm instead of 10 mm (*Lumbricus terrestris* can be up to 10 mm in diameter as adults)

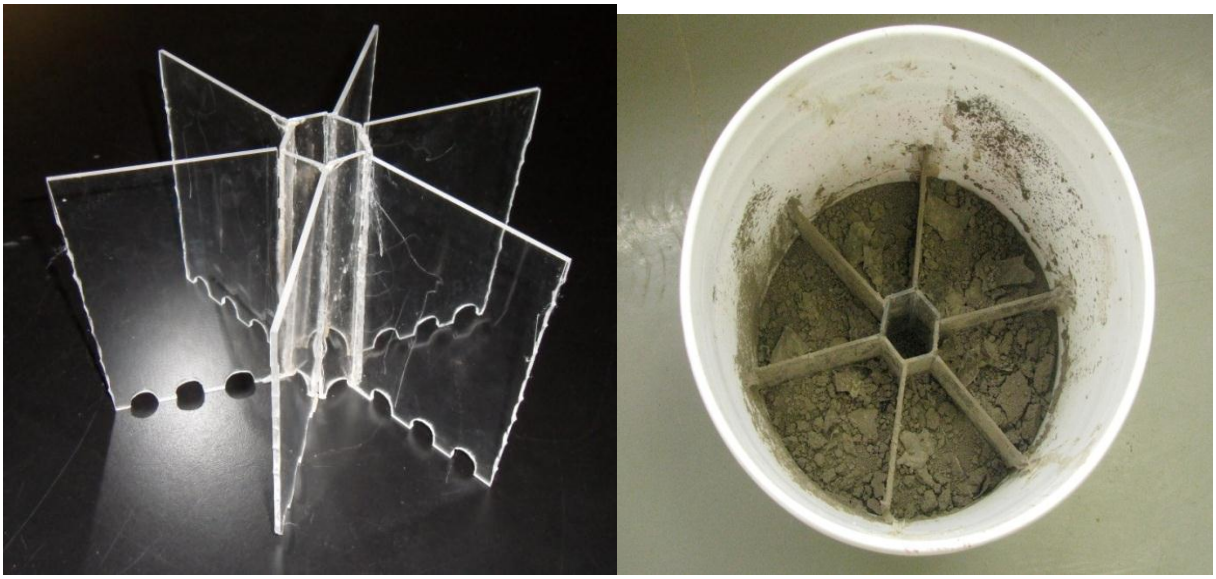

Figure 20: Insert (left) for Altered Avoidance Chamber (right)

2.3.5 Avoidance Chamber - Method #2 (Rectangular Vessels)

2.3.5.1 <u>Materials and Equipment</u>

Transparent vessels (34.2 cm x 20.9 cm x 11.8 cm) purchased from Canadian Tire

Plastic divider made from quilters templates (34.2 cm long and 11.8 cm tall)

Cheesecloth or other suitable material for covering chambers to prevent the earthworms from escaping, while allowing air exchange and a method to secure in place over the opening (string was used in these bioassays)

2.3.5.2 <u>Construction of Avoidance Chamber -Method # 2</u>

1. Containers 34.2 cm x 20.9 cm x 11.8 cm were utilized for the avoidance Chamber in Method # 2 with no adaptations needed
2. Plastic dividers were cut to fit lengthwise in the chamber and divide the reference and treatment soil (Figure 21)

Figure 21: Avoidance Chamber Method # 2

2.3.6 Long-term Bioassay Chamber-Method #1 (Buckets)

2.3.6.1 <u>Materials and Equipment</u>

High Density Polyethylene (HDPE) buckets, 18.9 L capacity, inner diameter of 28.7 cm with 10 to 15 5mm holes drilled in the bottom to allow for drainage and air exchange to the bottom

Inert circular marker made from quilter template plastic with same diameter as the bucket and 1 cm wide (28 cm outer diameter x 27 cm inner diameter)

Cheese cloth and method to secure in place over the opening of the buckets (string or elastic bands were used in these bioassays)

2.3.6.2 Construction of Long-term Bioassay Chamber - Method # 1

Expanding on the work that previously took place in Dr. McCarthy's research lab by R. Alvarez, 18.9 L HDPE buckets with an inner diameter of 28.7 cm and a depth of 30 cm were used. Each bucket had fifteen 5 mm holes drilled in the bottom and a 2.5 cm layer of large gravel added to the bottom, to allow for drainage and air exchange. Additionally, thin, inert 'washers' were used to separate the top 15 cm of soil in each of the buckets from the remaining soil below but still allowing the earthworms mobility throughout the soil column. Therefore, when the buckets were dismantled at designated time internals, the treatment soil and the reference soil could be separated.

2.3.7 Long-term Bioassay Chamber-Method #2 (Evan's Buckets)

2.3.7.1 Materials and Equipment

Evan's boxes - 100 x 20 x 8 cm constructed of Plexiglas with a hinge down the long side to create a 'door' so that the Evan's boxes, when laid on their side, can be opened and the contents examined

Transparent vessels (34.2 cm x 20.9 cm x 11.8 cm) purchased from Canadian Tire

2.3.7.2 Construction of Long Term Bioassay Chambers - Method #2

Building on the glass design that was first introduced by A.E. Evans in 1947 to observe the burrowing behaviours of earthworms, protocols were developed instead of using the 500 mL Mason jars with 300 mL of soil that was prescribed in government protocols. These larger vessels are more representative of the natural habitat of *Lumbricus.* The following describes the construction of the newly designed Evan's boxes:

1. Evan's boxes were constructed of Plexiglas to final dimensions of 100 cm tall x 20 cm wide and 8 cm thick Plexiglas adhesive was used to meld the seams and attach the 30 cm x 30 cm base (to support the column when upright) and hinge. The hinge was added to one of the long sides to permit the chamber, when lying on its side, (Figure 22) to be opened to allow observations to be made without destroying the soil column or unduly disturbing the earthworms

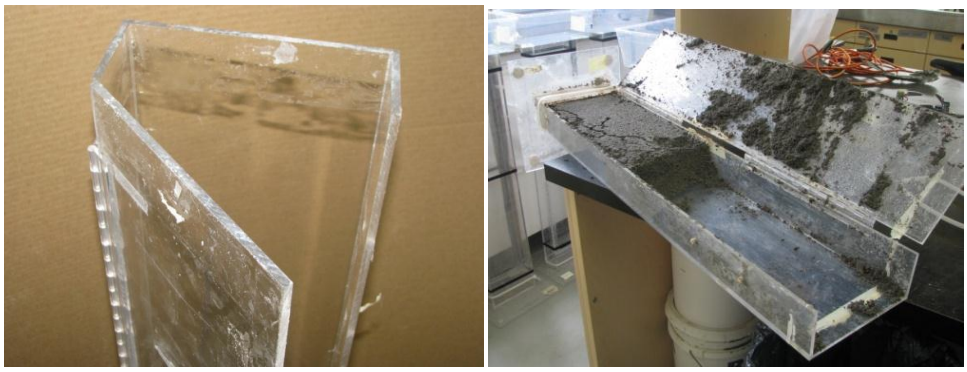

Figure 22: illustration of the hinge design to permit the opening of the chamber

2. 7.5 cm lip was constructed at the bottom beyond the hinge to hold sand for drainage. (Figure 23). Sand was used instead of gravel in this case to lessen the amount of settling

Figure 23: Sand layer at the bottom of the chambers for drainage

3. The unsealed corner opposite the hinge was sealed using a thin layer of plastic with heavier duct tape on the back. The plastic prevents the duct tape from sticking to the Plexiglas and possibly causing contamination due to the glue, while the duct tape provides support, strength, and durability. Three strips of Velcro strips were tightly wrapped around the bottom, center, and top of the chamber to hold the 'door' closed against the pressure of the soil inside during the bioassay (Figure 24).

Figure 24: Evan's Box sealed and ready for filling with soil

4. 34.2 cm x 20.9 cm x 11.8 cm transparent mating chambers were added to the top of the Evan's boxes to provide greater surface area since *Lumbricus terrestis* mate horizontally on the surface of the soil. An opening, the same dimensions as the top Evan's boxes, was cut in the bottom of these chambers and the now formed 'flaps' were used to secure the chamber to the top to the Evan's Box (Figure 25).

Figure 25: Mating chamber added to the top of the Evan's Boxes

5. Two dowels, each individually cut to provide a tight fit (one lengthwise and one width wise) for the particular Evan's Box, were inserted into the top of the chambers to hold the flaps open and prevent earthworms crawling between the Plexiglas side wall and the flaps at the bottom of the mating chamber (Figure 26)

Figure 26: dowels to secure the mating chamber to the top of the Evan's boxes

6. Small (5-7mm) ports were drilled along the narrow sides of the Evan's boxes so soil moisture readings could be taken at various levels throughout the bioassay. Additionally,

if the soil became too dry, water could be injected through these ports at different depths instead of trying to moisten a 100 cm column of soil from the surface.

7. When bioassays were underway, black fabric or plastic was wrapped around the Evan's Box (but not the mating chamber) to simulate the below ground environment (Figure 27)

Figure 27: Finalized setup of the Long-Term Bioassay Chambers - Method #2 (Evan's Boxes)

2.3.8 Bioassays using Lumbricus terrestris

2.3.8.1 <u>Materials and Equipment</u>

Soil moisture meter

Soil moisture probes

Biosolids obtained from either Kitchener or Guelph WWTP

Reference soil obtained from OMAFRA research farm in Stratford Ontario, (approximately 200 L for both the avoidance and long term bioassays)

Treatment soil (same reference soil as above with biosolids added at a rate of 22 tonne/ha dry weight unless otherwise indicated). The actual dry weight of biosolids to be added is determined for each new batch of biosolids used. See Appendix I for calculations. (Approximately 200 L for both avoidance and long term bioassays)

Note: The $CaCl_2$ slurry method as described in government protocols such as Environment Canada RM 43 was used to determine the pH of the soil prior to being used in the bioassays, and was adjusted if necessary to a pH 6.0 ± 0.5 using calcium carbonate as needed.

2.3.8.2 Avoidance Bioassays Method #1 (Modified from Environment Canada)

1. All material to be used in the bioassay has been cleaned following the Ryerson Cleaning Protocol
2. The avoidance wedge was placed in the bottom of the bucket followed by positioning the false bottoms to each segment
3. Each alternating sections were filled with reference soil that has been adjusted with water to account for the moisture in the biosolids (see Appendix I for calculations) and the remaining alternate sections with treatment soil. Alternating wedges had reference soil and three with biosolids treated soil
4. A pre-weighed earthworm (using a top-loading balance) was placed in the central empty chamber and allowed to enter a wedge of the avoidance chamber of its own accord. If entry has not taken place within 30 minutes, the worm was removed and replaced with another. Due to the negative phototactic nature of earthworms, they seek the darkness of one of the chambers
5. Step 4 was repeated selecting similar weight earthworms until all 10 earthworms had been added
6. The chamber that each earthworm initially entered was recorded along with the length of time

7. The avoidance chamber was covered with cheese cloth and left undisturbed for the duration of the bioassay (72 hours). Earthworms were not fed during this time
8. Temperature was monitored daily around the chamber using the digital thermometer without disturbing the earthworms
9. Upon completion of the bioassay, plastic pieces were quickly and carefully inserted along the walls of the wedges to prevent the earthworms from moving between chambers (the chamber being disturbed will cause the earthworms to move about regardless of the soil content and thus lead to erroneous results if they were not prevented from enter different wedges)
10. The soil was removed from each wedge with the aid of the false bottom one at a time and examined for the presence of earthworms. All findings were recorded, noting the section from which the earthworms were recovered, as well as any notable endpoints (mortality, pathological symptoms)

    Note: if an earthworm was found between wedges at the termination of the bioassay, the wedge that the anterior portion of the earthworm was in was used for counting (it was also indicated that the earthworm was between wedges)

Avoidance Bioassay Method #1 was unsuccessful due to poor design for accommodating *L. terrestris*. Therefore, this method was discarded for use in determining avoidance behaviours and thus assessing impact from biosolids.

2.3.8.3 Avoidance Bioassay Method #2

1. All material to be used in the bioassay had been cleaned using the Ryerson Cleaning Protocol
2. The plastic divider was placed length-wise down the center of the avoidance chamber. The left half was filled with reference soil that has be adjusted with DMDW to account for the water content in the biosolids and the right half with treatment soil. (Figure 28, left)

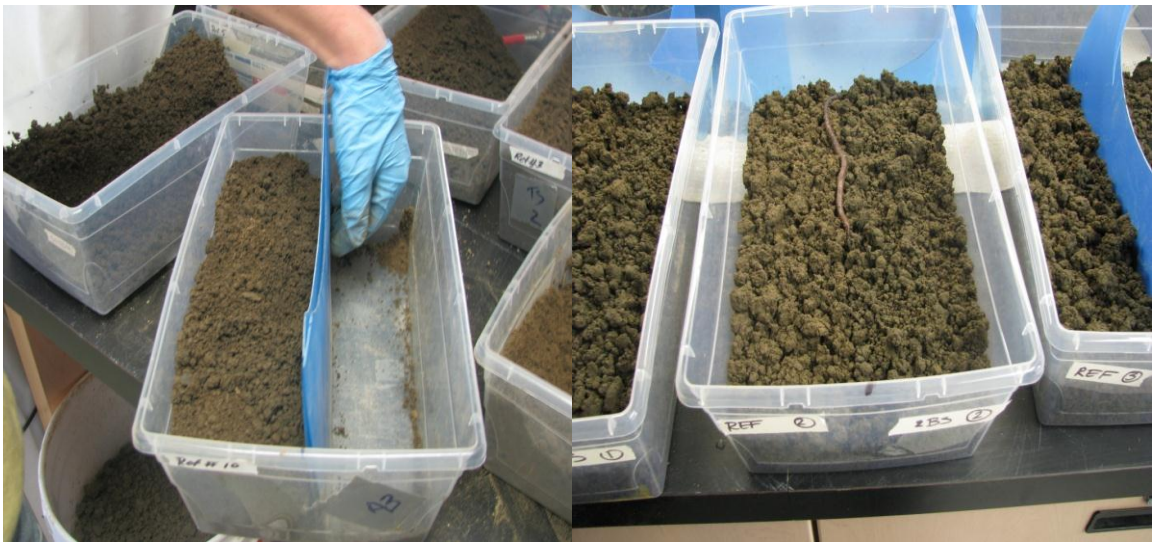

Figure 28: Avoidance Bioassay - Method #2. Reference soil being added (left), divider removed and earthworm placed along interface prior to making it way into the soil (right)

3. Both sides were labelled to signify their contents and the top edge of the avoidance chambers was marked to indicate the location of the divider
4. The divider was gently removed, being careful not to disturb or mix the soils
5. The above procedure was repeated with the other nine avoidance chambers
6. Earthworms of similar weight were selected for the avoidance bioassay. One pre-weighed earthworm was placed along the centre division of each avoidance chamber between the two soil types (Figure 28, right) and allowed to enter the soil of its own accord. If entry had not taken place within 30 minutes, the worm was removed and replaced with another. Which treatment each earthworm entered was recorded along with the length of time to do so
7. The avoidance chambers were covered with cheesecloth and left undisturbed for the duration of the bioassay (72 hours). Earthworms were not fed during this time
8. The temperature was monitored using the digital thermometer around the chamber without disturbing the earthworms
9. Upon completion of the bioassay, the plastic divider was gently slotted back into place between the two soils at the mark previously indicated to prevent the earthworms from moving between treatments.

Note: If the chamber is disturbed, the earthworms tend to move about irrespective of the soil contents, leading to erroneous results

10. The soil was removed from reference side first and examined for the presence of the earthworm. If the earthworm was not found in the reference side, then treatment soil left in the avoidance chamber was examined for the presence of the earthworm. Care was taken to make sure that cross-contamination of the soil did not take place when going from treatment to another reference
11. All findings were recorded, noting which soil type that the earthworm was located in, as well as any notable endpoints (mortality, pathological symptoms). If the earthworm was found partially in the two soils, the result was recorded as to which soil the earthworm's anterior end was in since this convention would indicate which direction the earthworm was moving towards at the time the bioassay was terminated
12. The avoidance chambers were reassembled as previously described by returning the reference soil (using the divider to keep the two soil types separate) to the left half of the avoidance chamber and returning the same earthworm to the soil surface as before being sure not to cross contaminate the soils
13. For the second half of the bioassay, the avoidance chambers were rotated 180° but otherwise treated exactly the same as the first half of the bioassay. This step was to eliminate any directional factors that might arise from the rectangular shape of the avoidance vessel and the earthworms. Since the same soil and same earthworm were used in both parts of the bioassay with the only difference being the reference soil was either on the right or left, directional factors should be eliminated

2.3.8.4 <u>Method for Long-Term Bioassay Method # 1 (Buckets)</u>

This includes the acute and chronic exposure bioassay (30, 60, and 90 days)

Note: once set up, the experiment is left for the duration (90 days). It was dismantled and examined at each time interval. The location, health, and any other noticeable results were recorded then reassembled for the next time point. Any dead earthworms were removed at this time. The condition of the soil was also examined at the same time.

1. All material to be used in the bioassay had been cleaned following the Ryerson Cleaning Protocol
2. A layer of drainage gravel, approximately 2.5 cm deep, was added to the bottom of the buckets to prevent water pooling and anoxic conditions developing
3. Due to the fact that the reference soil was obtained from a farmer's field, it was carefully examined prior to the bioassay to remove any pre-existing earthworms, cocoons and foreign matter. This was achieved by finely sifting the soil, then rehydrating the soil back to field conditions to account for moisture lost during sifting (~7 on the soil moisture meter)
4. To one of the reference buckets and one treatment bucket, a soil moisture probe (purchased from Gemplers, Madison, WI) was buried according to manufacture's instructions into the bottom reference soil layer, just above the gravel layer
5. All ten buckets were filled with 30 cm of reference soil and the buckets were lightly tapped on the floor to evenly distribute the soil column, while ensuring not to pack too firmly

<u>Reference Long-Term Bioassay Chambers Only</u>

6. For the reference buckets, the top 15 cm of soil was removed and mixed with enough DMDW to account for the moisture content in the biosolids and set aside. This amount depended on the particular batch of biosolids being used. (See Appendix I for calculation). The soil and water were thoroughly mixed to ensure that all large chunks of soil had been broken up
7. Ten randomly selected, pre-weighed earthworms (each between 3-10 g determined using a top loader balance) were added to the remaining reference soil in the bottom of the bucket. The earthworms were observed until they have all burrowed in the soil

   Note: Earthworms that did not burrow into the soil within 30 minutes of being added to the surface were removed and replaced with another earthworm.

8. Once all of the earthworms had successfully burrowed, an inert circular marker (about 1 cm wide), was placed on top of the soil. This marker denotes the interface between the reference layer and the treatment layer and was used to facilitate the separation of these layers when the buckets were dismantled to determine survivorship and partitioning of the earthworms between the layers
9. The reference soil that had be adjusted for moisture content to match that of the biosolids, was returned to the bucket, recreating the top 15 cm of the soil profile
10. This procedure was repeated with the four remaining reference buckets

Treatment Vessels for Long-Term Bioassay Chambers Only

11. For the biosolids treatment buckets, the top 15 cm of soil was removed and enough biosolids were added to obtain a dry weight rate of 22 tonnes/ha and set aside. (See Appendix I for calculations). The biosolids were thoroughly mixed with the soil to ensure that all the large chunks of soil had been broken up and that there are no 'pockets' of biosolids.
12. Ten randomly selected, pre-weighed earthworms (each between 3-10 g determined using a top loader balance) were added to the remaining reference soil in the bottom of the bucket. The earthworms were observed until they all burrowed in the soil

    Note: Individuals that did not burrow into the soil within 30 minutes of being added to the surface were removed and replaced with another worm.
13. Once all the earthworms had successfully burrowed, an inert circular marker (about 1 cm wide) was placed on top of the soil. This marker denotes the interface between the reference layer and the treatment layer and was used to facilitate the separation of these layers when the buckets were dismantled to determine survivorship and partitioning of the earthworms between the layers
14. The soil with the biosolids added was returned to the bucket, recreating the top 15 cm of the soil profile
15. This procedure was repeated with the four remaining treatment buckets

For All Long Term Bioassay Chambers

16. The surface of all ten buckets was covered with an equal amount of dried leaves and misted with DMDW, then covered with cheese cloth and randomly placed in a cool location with the temperature maintained at 15°C ± 2° C (using a digital thermometer to monitor) where it was quiet and the earthworms were not disturbed
17. A dark environment (5.4-10.8 μmol/m^2/s for a 16:8 hr light:dark cycle) and air temperature, relative humidity was maintained. Soil moisture readings were taken daily via the buried probes. The soil surface was moistened as needed to maintain moist soil conditions with DMDW
18. Earthworms were fed every 14 days by applying a layer of crushed dried leaves, sufficient to cover the whole surface of the soil, in each of the buckets and misted with DMDW. The amount needed was determined by observations of the culture and what was being consumed, but ensuring that all buckets were treated equally. Supplement feeding was achieved by sprinkling a dusting of cornmeal on the surface of the soil. Figure 29 illustrates the setup for Methdo #1 Longt term bioassay

Figure 29: Long Term Bioassay Method #1 setup

2.3.8.5 Method for Long Term Bioassay Method # 2 (Evan's Boxes)

1. Chambers were assembled as described in Section 3.3.7 and then filled with reference soil to within 10 cm of the top. The mating chambers were put into place ensuring the flaps were on the inside of the Evan's boxes. The two pieces of dowelling were inserted against the flaps to secure the unit in place

2. Five chambers were filled the remainder of the way with reference soil that had DMDW added to account for the water content in the biosolids (see Appendix I for calculations) leaving the top 5 cm of the mating chamber empty to prevent earthworms crawling out, and to provide space for feeding. The remaining five chambers were filled in a similar fashion with treatment soil. Thus, there was now a 15 cm layer of biosolids (or reference soil) on top of a deeper column of reference soil as is the case in the environment
3. Five adult *Lumbricus terrestris* weighing between 3-10 g each were added to the surface of each chamber and allowed to burrow. The five earthworms in each of the ten chambers were previously selected so that combined weight from all the earthworms in each of the chamber had approximately the same initial biomass
4. The time taken to burrow was recorded. If a worm did not burrow within 30 minutes, it was removed and replaced with another
5. Once all worms had burrowed, an initial supply of food was provided as described in Section 2.3.3.5. (Figure 30).

Figure 30: Feeding of Earthworms in Ryerson Long-Term Bioassay Chambers. Image shows the mating chambers atop the Evan's Boxes

6. The chambers were wrapped using either dark fabric or plastic to simulate being underground. The sides of mating chambers were not covered in this manner but left clear since this portion is to simulate above ground conditions

7. Cheese cloth was secured over the top of the mating chambers to prevent earthworms from escaping (Figure 31, right) while allowing air exchange. Chambers were placed in a closed off section of the laboratory where the temperature was maintained using an air-conditioner at 18-20°C

Figure 31: Long-term Bioassay Method #2 (Evan's Boxes) setup. Image on the left shows the soil column with one in the forefront covered with black cloth, and uncovered one showing burrowing of earthworms. Image on right shows covered mating chambers

8. Natural lighting was provided and cultures were fed dried leaves and corn meal bi-weekly
9. Observations were made at this time with regards to soil moisture, food supply, and overall condition of the chambers
10. The earthworms were examined according to the schedule in the next section
11. To examine the chambers at the end of the shorter duration bioassays (i.e. the 7-day and 28-day bioassays), the dark covering was gently removed and with the aid of a flashlight,

the location and survivorship of the earthworms within the chambers could be determined visually. Light from the flashlight caused the earthworms to move about and thus aid in their discovery. The location and number of earthworms present was noted at this time

12. At the end of the longer term bioassays (60 and 90 days), the chambers could be dismantled. After the dark covering was removed, the mating chamber was then carefully removed being sure to prevent any soil falling from the opening at the bottom. Next the Evans box was gently laid on its side on a bench (Figure 22, right). The Velcro straps were undone and the door carefully opened
13. Thorough observations of the soil profile would now be made (number of earthworms alive, health, location, burrow pattern and weight and presence/number of any cocoons) without unduly disturbing the soil column
14. After examination, the chamber was be reassembled and set back up for the next time point

2.3.8.6 <u>Parameters to Measure throughout the Experiment</u>

After 7 days acute (lethality) bioassay – number of organisms present

After 30 days acute (lethality) bioassay - number of organisms present

After 90 days chronic (growth) bioassay - number and weight of each organism present as well as number/presence of any cocoons.

2.3.8.7 <u>Parameters to Measure at Completion of Each Bioassay</u>

Mean number of both live and dead earthworms

Pathological symptoms such as erythema, ulceration, lesions, discolouration

Behavioural responses including, but not limited to, tactile response, locomotion, lethargy, casting, midden productions, and appearance of non-burrowing earthworms

2.3.9 Protocol Modification –Lumbricus terrestris

As mentioned earlier, all government protocols favoured the use of *Eisenia* species since they are easy to culture under laboratory conditions. However, since this organism was not

environmentally-relevant to this work, *L. terrestris* was chosen because it was, although it is harder to culture. Other modifications that were made to government protocols are discussed below.

2.3.9.1 Avoidance Bioassays Vessels

The earthworm vessels prescribed in government protocols were inadequate. The protocol for earthworm avoidance bioassay given by Environment Canada use the Kaushik chamber which is shown in Figure 20, with reference and treatment soil added to alternating pie wedges. As can be seen in their figure heading, the Environment Canada RM 43 protocol does indicate that *Lumbricus terrestris* are to be used with this apparatus. Alterations were made to the avoidance chamber that was constructed following Environment Canada guidelines as seen in Figure 32 making the overall height larger (150 mm instead of 100 mm), the wedges longer (115 mm instead of 83 mm) and making larger diameter holes at the bottom (15 mm instead of 10 mm). Even with these alterations to their design, the *Lumbricus* were not able to easily move about within this chamber configuration.

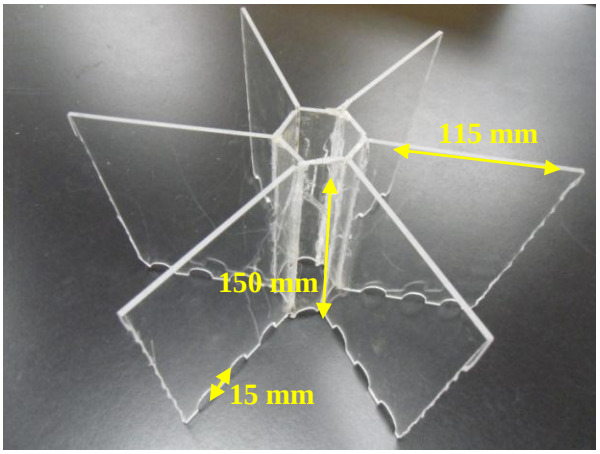

Figure 32: Government Protocol Designed Avoidance Chamber insert (Modified from Environment Canada RM 43)

Consequently, this design was discarded and the newly designed avoidance chamber was successfully used instead. This chamber was 34.2 cm long, 20.9 cm wide and 11.8 cm in depth, allowing plenty of room for the earthworm to freely move about. The chamber was

divided lengthwise with reference soil on one side and treatment on the other. To account for any directional factors that might arise from corners of a rectangle vessel (that would not be present in a circular vessel) due to the earthworm possibly seeking a place to escape the contaminant (Yeardley *et al.* 1996), the bioassay was repeated using the same worm; first with the reference soil on the right, then repeating the bioassay this time with the reference on the left. During the bioassay, the chambers were covered with cheese cloth to allow ventilation. Other researchers such as Yeardley *et al.* (1996) used a similar setup, but their vessels were 7.5 x 15.0 cm circular evaporating dishes with a soil depth of 2.5 cm containing 10 earthworms each.

2.3.9.2 Using Different Long Term Bioassays Vessels

For longer term bioassays, OECD adds ten earthworms to 1-2 L glass vessels with five to six cm (~500 g) of soil, while Environment Canada prescribes 500 mL Mason Jars with 350 mL of soil (either reference or treatment) (Figure 35), each with three adult worms. The worms are left in these vessels for the duration of the bioassay (twenty-eight days for OECD, fourteen days for EC). While OECD recommends *Eisenia*, Environment Canada does include *Lumbricus* in these bioassays. Consequently these vessels did not offer the needed room and therefore it is difficult to ascertain if results were from the treatment or the lack of physical space.

Figure 33: Government Prescribed Vessels for Earthworm Bioassays

These vessels were first replaced with 18.9 L HDPE buckets in Method #1 containing approximately 20 L of soil which allowed for the needed room for the earthworms, but did not offer a means to unobtrusively observe them. Based on the 1947 work by A.C. Evans from the Department of Entomology at Rothamsted Experimental Station in England (the longest running agricultural research station in the world), the Evan's Boxes were redesigned to be larger both in height and width (Evans 1947). These chambers are now 100 cm in height by 20 cm wide and 8cm deep constructed of Plexiglas instead of glass to be more stable and most noteworthy, the addition of horizontal space at the top to allow for mating. These redesigned chambers now offer a means to easily observe the *Lumbricus* below ground without unduly disturbing them by simply removing the dark covering. Additionally, the needed space for them to mate above ground was provided, an opportunity that was not considered in any of the previously published government protocols.

2.3.9.3 Duration of Earthworm Bioassays

As in government protocols, the 48-hour for *Eisenia* (72-hour for *Lumbricus)* avoidance bioassay was performed (but not using the Kaushik chamber, but instead using the Ryerson Avoidance Chamber). In addition, 30-day acute (lethality), and 90-day chronic (growth) bioassays were also performed again using the *Lumbricus*, instead of the 28-day and 48-day as prescribed in OECD protocols.

2.4 Plants

The plant genus and species that were utilized in these bioassays were *Zea mays* (corn), *Glycine max* (soya bean), *Phaseolus vulgaris* (common bean) and *Brassica rapa* (field mustard). Figure 34 is a diagrammatic representation pertaining to the bioassays with regards to the plants.

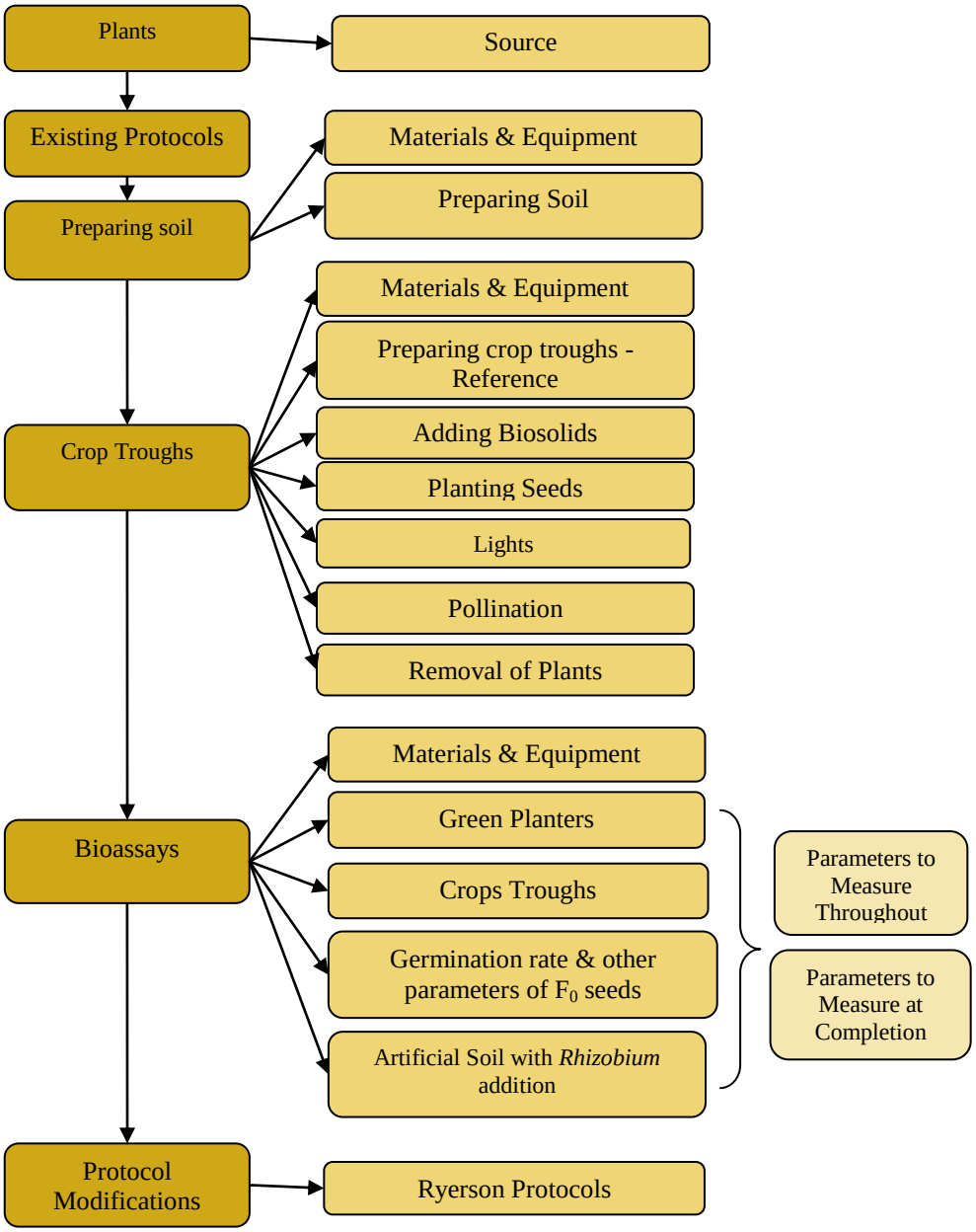

Figure 34: Flowchart for Plants Methodology Section (pages 122-150)

2.4.1 Source of Organisms

***Zea mays* kernels** (Non BT field corn seed DKC 39-45 treated with poncho 250) were obtained from Woodrill Farms, Guelph, Ontario, a seed supplier for the farmer in that area. These seeds are Non BT and therefore do not have the gene to be resistant to *Bacillus thuringiensis*. DKC is a brand name for this variety of seed and Poncho 250 is an insecticide applies to the seeds to protect them from corn root worm. (Pers. Comm Jeff Robinson Woodrill Farns)

***Glycine max* seeds** (OAR Lakeview non-GMO soya bean) were also obtained from Woodrill Farms, Guelph, Ontario. OAR means that this variety of seed was developed at Ontario Agriculture Research center; Lakeview is the brand name. Non-GMO indicates that the seed in is its native state and was not genetically modified and is not resistant to Roundup (Pers. Comm Jeff Robinson Woodrill Farns).

Phaseolus vulgari seeds were purchased from Tree and Twig, a reputable and well established seed distributor in Wellandport, Ontario

Brassica rapa seeds (Wisconsin Fast Plant) purchased from Carolina Biological, North Carolina.

All seeds were guaranteed to be >97% viable and in order to maintain their viability were stored in their original paper packages (if provided), in the dark, in a sealed container at 4±2°C until they were needed for the bioassays.

2.4.2 Existing Protocols

To assess if the land-application of biosolids have any impact on the four plant species used in this study, the following methodologies were examined. Environment Canada RM 45 (2007) Emergence and Growth of Terrestrial Plants, OECD # 208 Guidelines for Testing Chemicals and # 227 (2003) Guidelines Seedling Emergence and Growth, ISO 11269 Part 1 (1993) Method for the Measurements of Inhibition of Root Growth and Part 2 (1995) Effects of Contaminated soil on the emergence and early growth of higher plants, USEPA # 712-C-96-363 (1996) Ecological Effects Test Guidelines again a seedling emergence test. As well as the government protocols, research papers such as McCarthy *et al.* (2003), Ceric (2001), and

information from University of Wisconsin, Madison, Department of Plant Pathology on *Brassica rapa* were also examined.

To attempt to faithfully reflect our natural environment in a laboratory setting, several factors needed to be taken into consideration. Parameters such as a source of uncontaminated reference soil, providing wind (fan), rain (watering using inverted Buchner funnel to simulate rain droplets), sun light (choosing the best possible light source) as well as controlling humidity were included in the Ryerson Protocols for the plants.

The plant bioassays were carried out in custom-made crop troughs. Transparent vessels were used for the *Brassica* due to their much smaller size. As well, green gardening planter boxes were also used with all the plants.

2.4.3 Preparing Soil for Plant Bioassays

2.4.3.1 Materials and Equipment

Reference Soil (obtained from Stratford, Ontario from an OMAFRA research agricultural site) was nutrient rich (Pers. Comm. Michael Payne, OMAFRA) Perth Clay Loam, which is Grey Brown Podzolic that is known not to have biosolids or fertilizers applied to it (Pers. Comm. Michael Payne, OMAFRA) (Appendix II)

2.4.3.2 Preparing Soil

1. Larger roots, twigs and other plant material that might be present in the reference soil obtained from OMAFRA field site as well as any live organisms were removed
2. The soil was loosely sifted to remove large pieces and to obtain a fairly uniform grain size
3. If moisture in the soil had been depleted due to the sifting, it was replaced prior to use in experiments. This was accomplished by adding DMDW to the soil to reach a moisture content similar to that of the soil in the field (80% or 8 on the soil moisture meter)

2.4.4 Preparing Green Planters Boxes

2.4.4.1 Material and Equipment

Green Planter Boxes 10.2cm x 10.2cm x 12.7cm (Figure 35), obtained from Sheridan Nursery

2.4.4.2 Preparing Green Planters Boxes

No special preparation was needed to prepare the green planter boxes. Reference and treatment soil were prepared as for the crop troughs. See Methodology below starting with section 2.4.5.

2.4.5 Preparing Crop Troughs

2.4.5.1 Materials and Equipment

Gravel of varying sizes

Soil moisture meter

Soil moisture probes

Crop troughs 120 x 30 cm (for *Zea mays*) and 90 x 30 cm (for *Glycine max* and *Phaseolus vulgaris*) made from plywood with a height of 35 cm lined with ethylene propylene diene monomer (EPDM) pond liner, without algaecide (used to prevent leaching into the soil from building materials). These units were constructed with casters to aid in their continuous mobility which enabled them to be randomly moved about under the lights and to be pulled out for examination and to make measurements (Figure 35)

Transparent vessels (34.2 cm x 20.9 cm x 11.8 cm in size) purchased from Canadian Tire (for *Brassica rapa*) (Figure 35)

Figure 35: Vessels used for plant bioassays. Crop trough used for larger plant (left) smaller vessels for *Brassica* (center), and green planter boxes (right) used for all plants genera

Reference Soil

Each 120 cm crop trough requires 108 L of reference soil.

Each 90 cm crop trough requires 81 L of reference soil.

Each 34.2 cm x 20.9 cm x 11.8 cm vessel requires 7 L of reference soil

Each 10.2cm x 10.2 cm x 12.7 cm green planter box requires 800 mL of soil

Biosolids obtained from either Kitchener WWTP or Guelph WWTP

Treatment soil is the same soil as the reference soil with biosolids added to an application rate of 22 tonnes/ha on a dry weight basis. For the batch of biosolids obtained from Kitchener WWTP:

Each 120 cm crop trough requires 10.95 L to obtain this application rate.

Each 90 cm crop trough requires 8.136 L of biosolids. For each new batch of biosolids used, the amount needed to give an application rate of 22 tonne/ha on a dry weight basis needs to be determined. For this methodology, refer to Appendix I on how to obtain this application rate

Each 34.2 cm x 20.9 cm x 11.8 cm vessel requires 0.73 L of biosolids to obtain an application rate of 22 tonne/ha dry weight. NOTE: due to the smaller depth of these vessels, the biosolids were applied to a depth of 5cm instead of 15cm as

in the crop troughs and the amount of biosolids added was calculated accordingly

2.4.5.2 <u>Preparing Crop Troughs - Reference</u>

When setting up the crop troughs, a natural representation of the soil profile (Figure 36) was replicated as closely as possible under laboratory conditions.

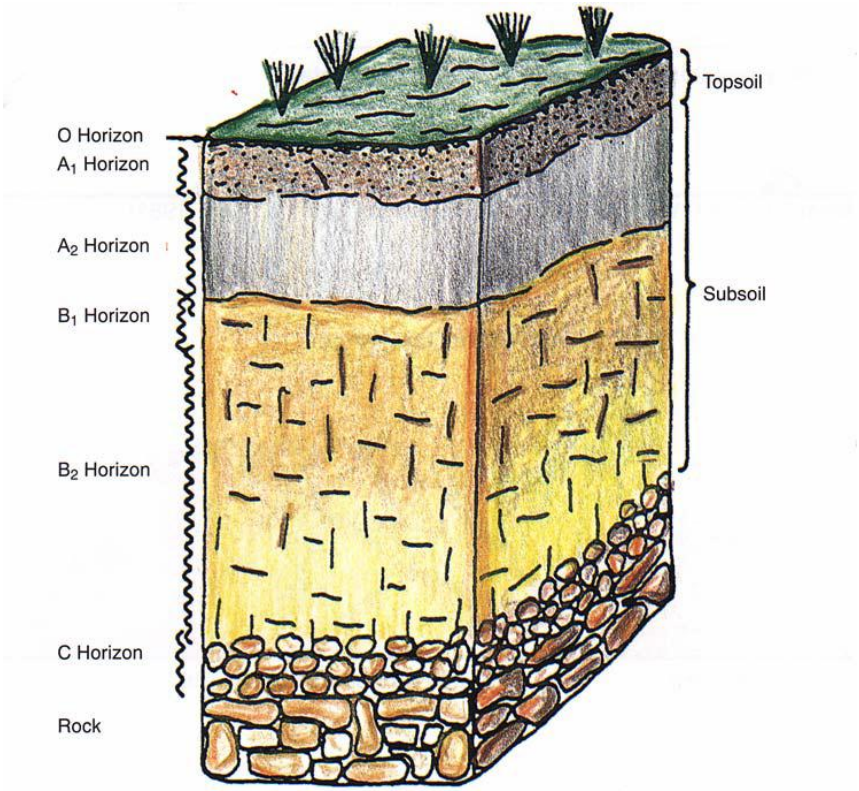

Figure 36: Illustration of the zones in a soil profile (Source: Google images)

1. 2.5 cm of large gravel (purchased from Home Depot or Rona) was layered in the bottom of each of the crop troughs. This provided for drainage and represented the 'rock' layer of the soil profile. Next, a 1.5 cm layer of small gravel (purchased from the same places) was added. The smaller gravel on top prevents the soil from settling in between the space of the larger gravel and represented the C horizon in the soil profile. (Figure 37).

Figure 37: Gravel added to the bottom of the crop troughs. Right images shows the smaller gravel filling in around the large gravel to prevent settling of soil

2. One reference trough and one treatment trough for each genus was used to monitor the moisture of the soil using buried soil moisture probes (Figure 38). The first probe, prepared as per manufacturer's directions, was buried just above the gravel layer and the other end of the cable was kept outside the crop trough so that it could be attached to the meter when moisture readings were needed. These units work by measuring soil water tension (in centibars, cbar). The higher the reading (i.e. the tension) the dryer the soil (Mueller 2003).

Figure 38: Soil probes by Watermark which are buried at specified depths

3. 30 cm of sifted reference soil was next added to each crop trough. The bottom 15 cm represented the B horizon while the top 15 cm which is where the treatment will be added, represented the A horizon. The O horizon (top layer containing plant life) was removed to prevent competition with the crops that are to be grown.

<u>To Reference Trough Only</u>

4. Water was added to the soil of reference crop troughs due to the aqueous nature of the biosolids, and thus all crop troughs were treated equally (in terms of moisture content and handling). To accomplish this, the top 15 cm of soil from the reference crop trough was removed to a large Rubbermaid® tote designated for reference soil use only. An appropriate amount of water was added to account for the water in the biosolids and depended on the particular batch of biosolids being used. A sample calculation is provided in Appendix I
5. The mixing of water to the soil was accomplished by removing soil from the trough in stages and alternately added to the Rubbermaid® tote with the previously determined amount of water. The water and soil were thoroughly mixed together after each addition using gardening utensils (trowel and small rake) designated specifically for reference soil use. Before being returned to the crop trough, the second soil moisture probe was buried, as per manufacturer's directions, at the interface now created between the bottom 15 cm of reference soil and the top 15 cm of reference soil
6. Depending on the type of biosolids (moisture content), the soil at this stage may be very moist. As the soil dried, if needed, mixing of the top layer was continued to aid in drying and to break up any large chucks of dirt
7. The third probe was buried just below the surface of the soil (Figure 39). Each cable was labeled appropriately

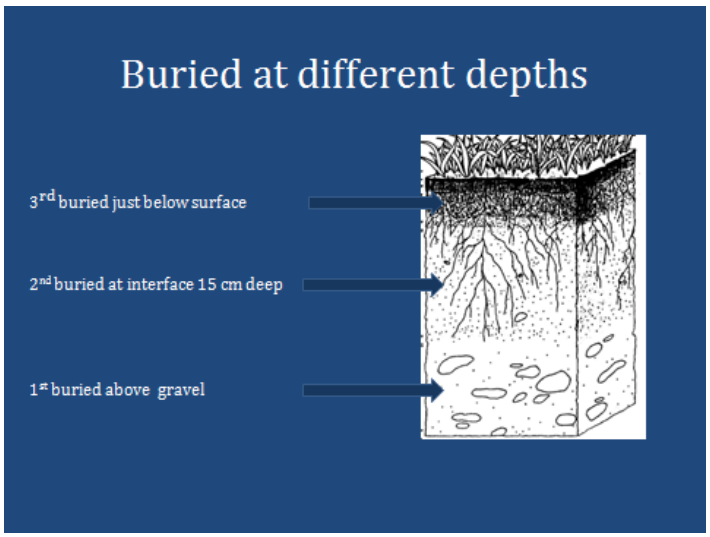

Figure 39: Location of buried soil moisture probes in the soil profile (Source: Modified from Hendricks 1985)

NOTE: The first probe was buried at the C horizon to ensure that water was getting all the way through the troughs and that there was not an excess pooling at the bottom in the gravel and the crops being over watered because, if this was the case, water would be pooling here at this depth. Moreover, these probes are used to ensure that enough water is added to maintain the proper moisture content all the way to the bottom of the root zone. The second probe was buried just below the biosolids/soil interface (i.e. at 15cm depth) between the "A" and "B" horizons. The objective of the second probe was to compare how much water passed through the biosolids, since it is known that biosolids have a greater capacity than soil alone to hold water (Pers. Comm. Michael Payne, OMAFRA). With a set in the reference troughs and another in the biosolids troughs, this can be monitored. The third probe was buried at the surface near the initial root zone to determine what moisture was available to the roots and thus the plant. The correct moisture reading depends on the soil type and for the soil obtained from the OMAFRA site, it needs to be 30-60 cbar and is the usual range for soils (except heavy clay soils which would then be in the range of 60-100 cbar) (Mueller 2003).

8. To determing moisture readings, the hand-held meter was attached to each of the probes in turn via a cable and values were recorded. The top probe was used for monitoring

moisture and the troughs are watered according to the moisture content in the reference trough

9. A second type of soil moisture meter was also used as a comparison because if the buried probes 'dry out' they no longer function correctly and then could not be relied on for moisture measurements. Figure 40 is an illustration of the meter used in this research. This instrument measures the humidity of the soil using a scale from 0 (dry) to 10 (saturated), with a reading of between 6 and 8 being acceptable for most plants (Soil Moisture Meter nd) as water needs are species-dependent. The main function of this type of unit is to prevent over or under watering of the plants. The drawback of this meter is that it can only measure the top 20 cm of the soil profile, (the length of the probe)

Figure 40: Soil Moisture Meter (Source: Soil Moisture Meter nd)

2.4.5.3 Adding Biosolids

1. Treatment troughs were prepared as described in Section 2.4.5 with soil moisture probes being buried at the same depth as the reference trough
2. For the biosolids crop troughs, the top 15 cm of soil was removed and, alternating with the appropriate amount of biosolids instead of water, they were added in the same manner described for reference troughs, using a different bucket and mixing utensils designated for biosolids use only (Figure 41). The amount of biosolids needed is determined per batch and an example calculated is given in Appendix I).

Figure 41: Mixing of biosolids into soil to make treatment soil

3. Once the soil had been mixed thoroughly with the biosolids, the soil was returned to the crop trough. Again, using utensils that were designated for biosolids use only, (to prevent cross contamination), the top soil was continuously turned over to aid in drying before the seeds were planted

   Note: in practice, biosolids are usually added to the fields in the fall prior to frost and the seeds normally are not planted until spring. Thus, the soil in our experiment is allowed to decrease in moisture for a few days before seeds are planted to simulate what actually takes place in the field.

2.4.5.4 Planting Seeds

All reference crop troughs were planted first to prevent any cross contamination from the biosolids to the reference soil.

1. Starting with the reference crop troughs, in the 120 cm crop troughs, five *Zea mays* kernels were planted at a depth twice the length of the seed with 25 cm distance between each seed. A 10 cm buffer was left at each end of the crop trough
2. In the 90 cm crop troughs, five *Glycine max* seeds were planted at a depth twice the length of the seed with 18 cm distance between each seed. A 9 cm buffer was left at each end of the crop trough

Note: The seed casing of *G. max* were examined to ensure that they were not cracked as these seeds will not germinate

3. In the second set of 90 cm crop troughs, five *Phaseolus vulgaris* seeds were planted at a depth twice the length of the seed, with 20 cm distance between each seed. A 5 cm buffer was left at each end of the crop troughs
4. Steps 1-3 was repeated for the biosolids crop troughs
5. In the five 34.2 cm x 20.9 cm x 11.8 cm transparent vessels, ten *Brassica rapa* seeds were planted in two rows at a depth twice the length of the seed, with 7.5 cm distance between each seed and leaving 7.5 cm at each end of the box
6. Step 5 was repeated for the biosolids vessels

2.4.5.5 Light

Custom built light banks were built that were 122 cm wide x 244 cm long and adjustable in height (Figure 42) which each housed sixteen 121.9 cm T8 VitaLux lights bulbs. The four different plant species were each randomly arranged under different light banks and the height was adjusted as the plants grew.

Figure 42: Images of custom made light banks for growing terrestrial plants

2.4.5.6 Pollination

Zea mays is pollinated by the wind. To simulate wind dispersion, a fan was randomly placed around the crop troughs making sure not to cross pollinate between the reference and the treatment plants. *Glycine max* and *Phaseolus vulgaris* are self-pollinators so nothing additional needed to take place with these crops. *Brassica rapa* is pollinated by *Bombus sp*.

(Bumblebees) and other insects (Williams 1990) so to replicate *Brassica* pollination in the lab, sterile cotton swabs were utilized.

1. During this process, reference and treatment *Brassica rapa* plant containers were separated to prevent cross-contamination
2. Starting with the reference plants, a clean sterile cotton swab was used to gently brush against the anther portion of the stamen to collect pollen grains on the swab (Figure 43). A visual check for the yellow dust against the white swab was performed to ensure that pollen grains had indeed been collected
3. Moving to another flower, the pollen grains were deposited on the stigma
4. This procedure was repeated several times to ensure that all flowers of all reference plants were pollinated
5. Using a new clean cotton swab, the above procedure was repeated with the treatment plants
6. All vessels were randomly returned under the lights

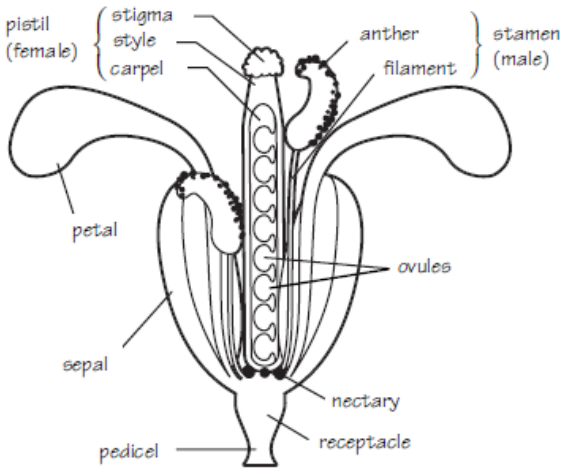

Figure 43: Anatomy of *Brassica rapa* flower (Source: Wisconsin Fast Plants 1998)

2.4.5.7 Removal of Plants at Termination of Bioassay

1. At the termination of the bioassay, the soil was thoroughly moistened to make removal of the plants and root system easier
2. Using a gardening trowel and measuring 10 cm out from the stem of each plant for *Zea mays*, 5 cm for *Glycine max* and *Phaseolus vulgaris* and 3 cm for *Brassica rapa*, each plant and root system was gently removed. It was impossible to remove all of the lateral roots due to their fine delicate nature but by measuring the specified radius around individual plants ensured that all plants were treated equally and the root mass obtained was the same for each
3. Any excess dirt was removed with gentle tapping and the plants and roots were allowed to air dry for 24 hours, after which time any additional loose dirt was carefully removed
4. The plant was divided between shoot and root material indicated by the mark on the plant where it was either in or out of the soil; below soil was the root mass, above the soil was the shoot mass. Using an analytical balance, the dry-weight of all the root and shoot biomass was determined

The number of pods and seeds was counted and using an analytical balance, the weight of the pods and seeds were determined

2.4.6 Plant Bioassays

For the initial bioassays using Kitchener biosolids, two crop troughs of the appropriate size for the genus planted were used; one for the reference soil and one for treatment soil. For subsequent bioassays using Guelph bioassays, 6 crop troughs were used; three for reference conditions and three for treatment soil conditions. Additionally, **t**o ensure that the crop trough itself is not interfering in the bioassay in any way, a parallel experiment was run the first time through using garden planter boxes. This is to ensure that there was not something from the construction material of the crop troughs interfering with the bioassays.

2.4.6.1 Materials and Equipment

Green planter boxes (prepared as described Section 2.4.4)

Crop troughs (prepared as described in Section 2.4.5

Custom-built light banks to hold 16 bulbs and span an area of 120 cm x 240 cm

Light bulbs (sufficient to provide 300 ± 100 μmol/m^2/s at the soil surface). After extensive research, the T8 VitaLux lights bulbs 121.9 cm in length purchased from MT-DTC were chosen for use in these bioassays

Digital thermometer providing minimum/maximum reading with built in humidity sensor purchased from Accutest, Toronto

Quantum Light Meter capable of reading photosynthetically active radiation (PAR) in μE/m^2/s purchased from Spectrum Technologies, Plainfield, Il. (Field Scout model # 3415FSE was used in our lab)

Digital micrometer purchased from Canadian Tire

Household Fan (45 cm diameter) purchased from Canadian Tire

2.4.6.2 Bioassay for Growing Plants in Green Planters

Treatment soil for the green planter is reference soil with biosolids added at a reduced amount to account for the smaller volume of soil in the planter boxes. Instead of calculations being based on a depth of 15 cm as is the case in the crop troughs, it was instead based on a depth of 5 cm.

1. All planter boxes were cleaned according to Ryerson Cleaning Protocol
2. 40 small planter boxes were filled almost to the top with 10 cm of reference soil
3. To 20 of these planter boxes (reference), the top 5 cm of soil was removed and mixed with the appropriate amount of DMDW to account for the water in the biosolids. To determine the amount needed refer to Appendix I. This value depends on the particular batch of biosolids being used

4. Once the soil had been mixed thoroughly with the water using mixing utensils specific for reference soil, it was allowed to 'dry' before being crumbled and returned to the green planter boxes
5. To the remaining 20 planters (treatment), biosolids was added following the above procedure, but instead adding biosolids and using utensils specific for biosolids to prevent cross contamination
6. Starting with the reference soil boxes to ensure no cross-contamination, 2 *Zea mays* seeds were planted at a depth of twice the seed length into five of the 20 reference boxes
7. Step 6 was repeated for *Glycine max* and *Phaseolus vulgaris*. For the *Glycine max* seeds, the casings were checked to ensure they were not cracked since if cracked, they will not germinate
8. Step 6 was repeated for *Brassica rapa*, except 4 seeds were planted
9. The above planting of seeds was repeated using the twenty biosolids green planter boxes
10. Moisture content of the soil in all the small planter boxes was checked
11. Since the biosolids are known to hold water (Pers. Comm Michael Payne, OMAFRA), the reference soil was used as a guide to determine the moisture content and the addition of DMDW to all the small planter boxes was based on the reference soil moisture readings
12. All green planter boxes were randomly placed under the light banks. Soil moisture content was maintained at 80% throughout the experiment (as determined by the reference soil boxes) and periodic light measurements were taken around the planter boxes with the light meter. During watering, planter boxes were randomly rotated to ensure equal light is obtained throughout the experiment
13. Each day the following measurements were taken and recoded:
    a) minimum and maximum temperature using digital thermometer (with built-in humidity sensor)
    b) minimum and maximum relative humidity
    c) soil moisture (using both soil moisture probe and soil moisture meter)

14. Initially, daily observations, then as the plants matured, weekly observations, of the following parameters were made:
    a) rate of germination
    b) shoot length
    c) leaf length
    d) leaf width
    e) number of leaves
    f) any other growth parameters that are applicable
15. After completion of the bioassay, the dry weight of the root and shoot biomass for each plant was measured using an analytical balance as well as the number and weight of pods and seeds if present (again using an analytical balance)

2.4.6.3 Bioassay for Growing Plants in Crop Troughs

The following methodology applies to all plants species. In the case of *Brassica rapa*, due to the small size of these plants, the bioassays were carried out in smaller vessels rather than the large crop troughs.

1. Crop troughs were assembled as previously described and arranged randomly under the light banks
2. Sheets of cardboard covered in tinfoil were placed around the edges of the light banks to reflect light back towards the plants and increase intensity (Figure 44). Initial light readings using the Quantum light meter were taken

Figure 44: Sheets of tinfoil covered cardboard to reflect light back towards the plants. Also notice the three green cables from the buried moisture probes

3. Using the reference soil as a guide, moisture content was determined using either the buried moisture probes or the moisture meter and troughs were watered accordingly. Biosolids are known to hold the water (Pers. Comm, Michael Payne, OMAAFRA), thus, the reference troughs were used to determine when and how much water to provide. DMDW was added to all the crop troughs to maintain a soil moisture content of 80% throughout the bioassay to match the moisture content of the soil collected from the field
4. Watering of the crop troughs was initially done using an inverted 15 cm diameter Buchner funnel attached to a funnel holding the required amount of water vial Tygon tubing as seen in Figure 45. This method imitated the natural process of gentle rain, and thus the plants themselves received water as is the case in nature and not just the soil. When the plants were much larger and stronger, a garden watering can is used with a 'rainspout' type nozzle

Figure 45: Watering crop troughs via inverted Buchner funnel

5. A gentle breeze was provided for the growing plants by randomly placing a fan-around the crop troughs (Figure 46). This prevented the development of weak, spindly stems and stocks. As well, in the case of *Zea mays*, the wind assisted in pollination

Figure 46: Fan use prevents spindly stem and stocks

6. To raise the humidity to approximate field levels, (< 50%) (Environment Canada 2007b) a bucket of water was placed in front of the fan to help alleviate lack of sufficient hydration when the plants are grown indoors in the dry winter months

7. Periodically, light measurements were taken around the crop troughs with the light meter and the crop troughs were randomly rotated to ensure equal light was obtained throughout the experiment
8. Daily measure of the following parameters were made
   - minimum and maximum temperature
   - minimum and maximum relative humidity
   - soil moisture
9. As the plants grew bigger, measurements of growth parameters were done weekly. See below for the parameters recorded for each different genus.

   NOTE: as the plants senescence (period between maturity and death), they dry and become brittle making it more difficult to take measurements as seen in Figure 47 of the soya bean plants. Only those measurements that could be taken without damaging the plant in any way should be taken after this point

Figure 47: Soya beans senescence, making it difficult to continue with measuring

10. As the plants grew taller, the light banks were raised to accommodate their growing height. Since each of the different genera were grown under different light banks, this was altered as necessary throughout the bioassay to accommodate their individual lifecycles
11. Bioassays were completed when each crop plant reached maturation (seeds are mature and able to be planted). Further study on these seeds was carried out and the growth of the F_1 generation was observed

2.4.7 Parameters to Measure throughout the Experiment – Zea mays

The following growth stages were monitored throughout the life cycle of *Zea mays*; i) rate of germination (VE), ii) percent germination, iii) the length and width of all the leaves (V2 to R5) using a digital micrometer, iv) number of leaves at different stages, v) date to tassel formation (R1), vi) date to ear formation (R2), vii) plant height (initially using micrometer then as plant gets larger, using a tape measure). In these and other plant diagrams, 'V' refers to the vegetative stage of plant growth, 'E' indicates when emergence takes place, 'R' refers to the reproductive stage, and 'H' indicates the plant is ready for harvesting. For *Zea mays*, refer to Figure 48 from New South Wales Department of Agriculture for an illustration of the changes that took place across the life cycle of this plant. Table 21 provides details of the changes taking place at the different growth stages.

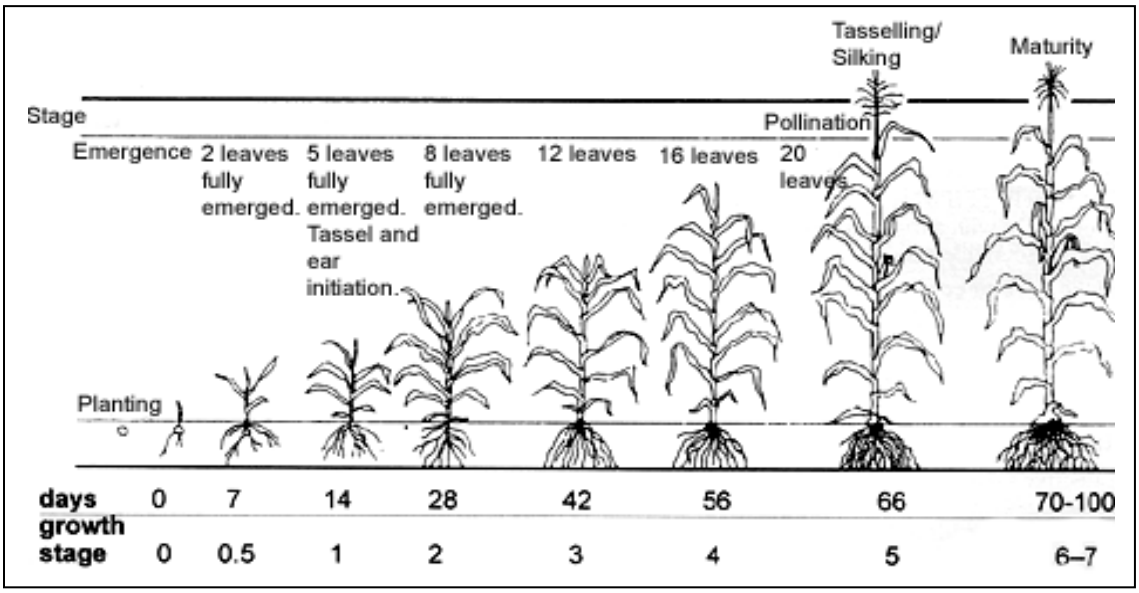

Figure 48: Developmental stages of *Zea mays.* (Source: Bechingham 2007)

NOTE: Days indicated in figures are approximate and are for climatic zone of the image source, in this case, New South Wales. For Ontario, the growing season for corn is approximately 125 days.

Table 21: Summary of the Key Developmental Stages of *Zea mays* (Dobermann, Walter nd)

VE	Emergence
V1	First leaf (rounded leaf)
V2	Second leaf
V3	Third leaf
V6	Sixth leaf. Growing point and tassel are above soil surface and stalk is beginning a period of increased elongation. The nodal root system is now major functioning root system. Sets of roots elongating from the three to four lowest nodes.
V(n)	nth leaf
VT	Tassel. Last branch of tassel is completely visible and silks have not yet emerged. Begins 2-3 days before silk emergence. Plants have almost attained full height.
R1	Silking, begins when any silks are visible outside the husks.
R2	Blister, 10-14 days after silking. R2 kernels are white on the outside and resemble a blister in shape. Endosperm and its now abundant inner fluid are clear in colour and the tiny embryo can be seen upon careful dissection.
R3	Milk, 18-22 days after silking. R3 kernels are yellow on the outside, and the inner fluid is now milky white due to accumulating starch. Silks are brown and dry or becoming dry
R4	Dough, 24-28 days after silking. Milky inner fluid has thickened to a pasty consistency.
R5	Dent, 35-42 days after silking. All or nearly all kernels are dented or denting and the shelled cob is dark red in color.
R6	Physiological maturity, 55-65 days after silking. All kernels on the ear have attained maximum dry matter accumulation. Black or brown abscission layer has formed. Black layer formation occurs progressively from the tip ear kernels to the basal kernels of the ear. Husks and many leaves are no longer green. Average kernel moisture content at R6 (black layer formation) is 30-35%.
H	Final harvest. Kernel moisture content is below 20%.

2.4.8 Parameters to Measure throughout the Experiment - Glycine max

The following growth stages were monitored throughout the life cycle of *Glycine max*: i) rate of germination (ve), ii) percent germination, iii) the length and width of all the leaves (v2 to r7) using micrometer then, as plant grew larger, using a tape measure, iv) number of trifoliates, v) date to flowering (r1), vi) date to pod formation (r2), vii) plant height initially

using micrometer then as plant gets larger, using a tape measure. Refer to the following diagrams from North Dakota State University and University of Illinois Extension, Agriculture Departments respectively in Figure 49 and 50 for the plant anatomy and life cycle stages of *Glycine max*. Table 22 lists the key developmental stages of soya beans that were observed. In Ontario, the growing period for *Glycine max* is approximately 100 days, which would represent R8 in Table 22.

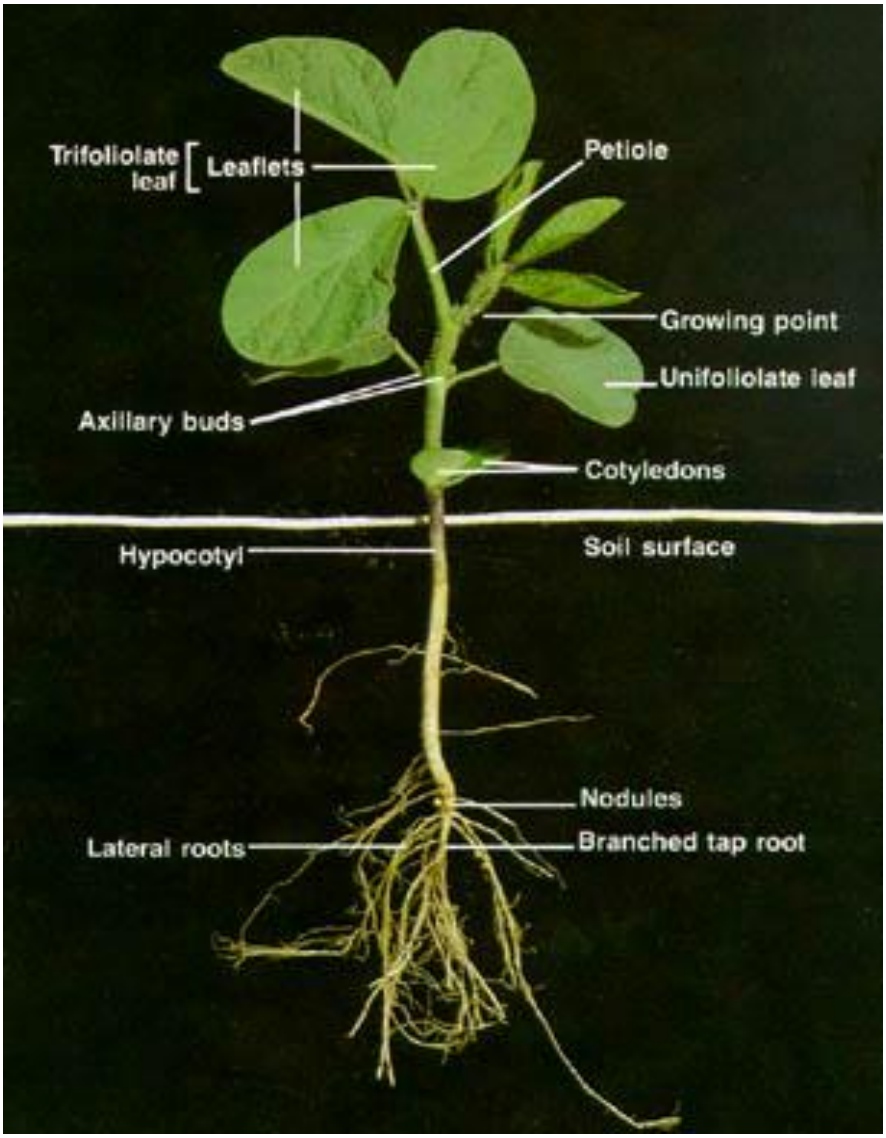

Figure 49: Plant anatomy of *Glycine max*. (Source: McWilliams *et al.* 1999)

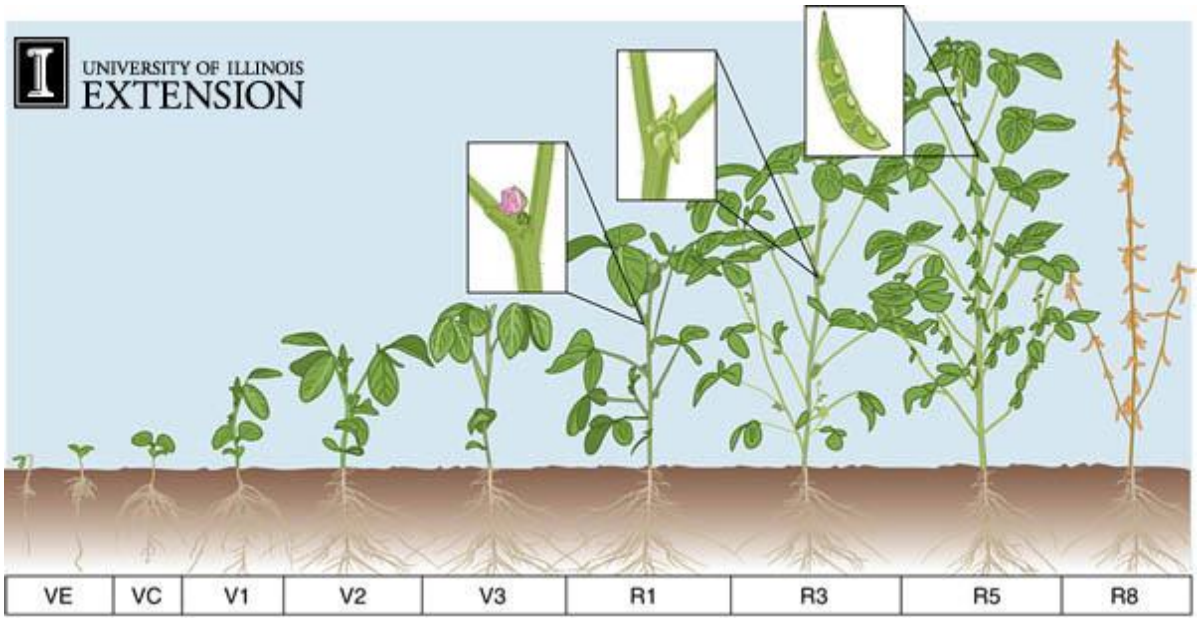

Figure 50: Developmental Stages of *Glycine max* (Source: University of Illinois Extension). R8 = ~100 days.

Table 22: Summary of Key Developmental Stages of *Glycine max* (McWilliams *et al*. 2004)

VE	Emergence
VC	Cotyledon stage
V1	First trifoliolate
V2	Second trifoliolate
V3	Third trifoliolate
Vn	Nth trifoliolate
V6	Flowering will soon start
R1	Beginning bloom, first flower
R2	Full bloom, flower in top 2 nodes
R3	Beginning pod, 3/16” pod in top 4 nodes
R4	Full pod, 3/4” pod in top 4 nodes
R5	1/8” seed in top 4 nodes
R6	Full size seed in top 4 nodes
R7	Beginning maturity, one mature pod
R8	Full maturity, 95% of pods on the plant are mature

2.4.9 Parameters to Measure throughout the Experiment - Phaseolus vulgaris

The following growth stages were monitored throughout the life cycle of *Phaseolus vulgaris*: i) date of germination (recorded as the emergence the hypocotyl with cotyledons breaking through the soil surface) (~Day 9), ii) percent germination, iii) length and width of all the leaves, iv) number of leaves, v) date to bud formation, vi) flowering (~Day 51, 61), vii) date to pod formation (~Day 71), viii) shoot length (total plant height) initially using micrometer then as plant gets larger, using a tape measure. For *Phaseolus vulgaris* refer to Figure 51 from the transparencies of Biology of Plants, Worth Publishing for the diagrammatic representation of these growth stages, and Table 23 from R. Hall, The Bean Plant, Compendium of Bean Diseases, for a description of the physiological changes taking place throughout the growth of *P.vulgaris.* Again these dates are approximate and depend on where, geographically, the plates are grown. In Ontario, *Phaseolus* take about 80 days to mature.

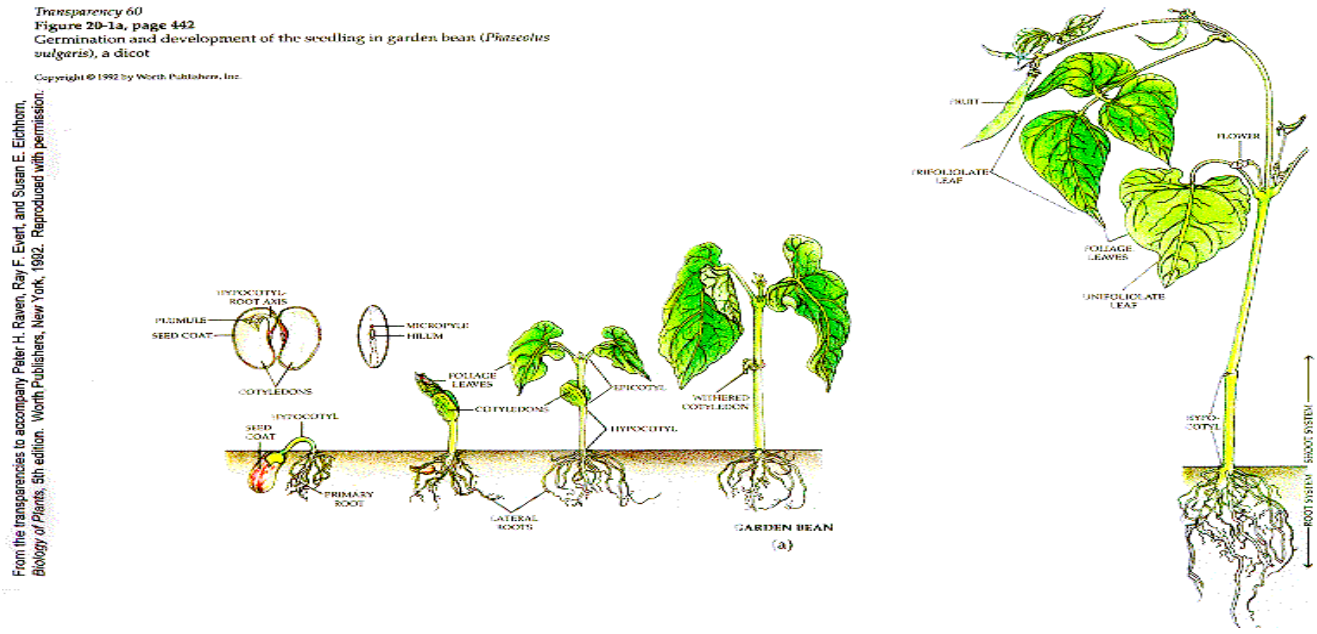

Figure 51: Developmental Stages of *Phaseolus vulgari.* (Source: Raven *et. al.* 1992)

Table 23: Key Developmental Stages of *Phaseolus vulgaris* (Hall 1991)

V1	*Emergence:* from the appearance of cotyledons on the soil surface to the unfolding of primary leaves
V2	*Primary leave:* from the full unfolding of the primary leaves to the unfolding of the first trifoliate leaf
V3	*First trifoliate leaf:* from the full unfolding of the first trifoliate leaf to the unfolding of the third trifoliate leaf
V4	*Third trifoliate leaf:* from the full unfolding of the third trifoliate to the appearance of the first floral bud or raceme
R5	*Preflowering*: from the appearance of the first floral bud or raceme to the opening
R6	*Flowering*: from the opening of the first flower to the expansion of the ovary after fertilization
R7	*Pod development*: from the expansion of the ovary to the elongation of the pod to its full size before increase in seed weight
R8	*Pod filling*: from the beginning of seed weight and size increase to the development of pigmentation of seeds and onset of leaf senescence
H	*Harvest maturity*: from initiation of senescence to complete senescence and drop in seed moisture to about 15%

2.4.10 Parameters to Measure throughout the Experiment – Brassica rapa

The following growth stages are monitored throughout the life cycle of *Brassica rapa*; i) days to germination, i) percent germination, iii) days to formation of flower buds, iv) days to pollination, v) days to pod formation, vi) length and width of all the leaves using a micrometer, vii) number of leaves at various stages throughout the lifecycle, viii) shoot length (total plant height) using a micrometer. Figure 52 shows the lifecycle of Brassica.

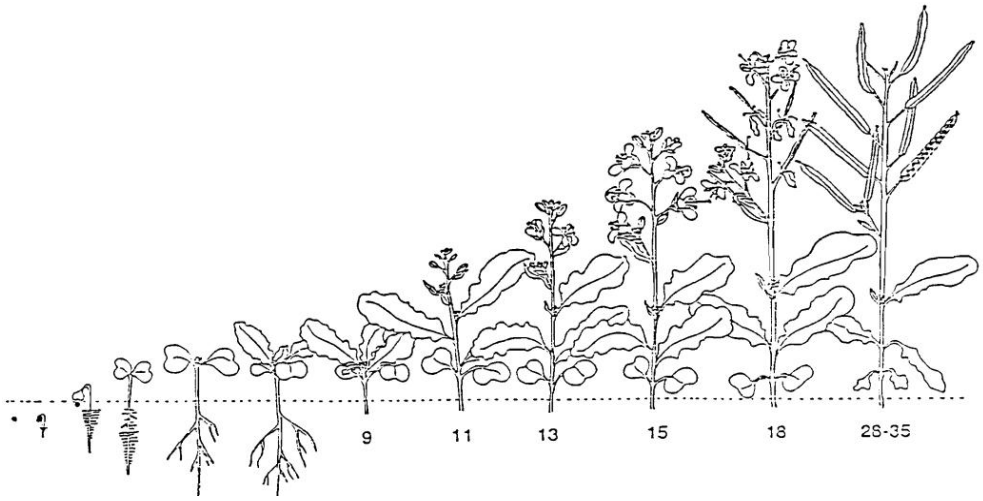

Figure 52: Developmental Stages of *Brassica rapa*. (Source: Williams 2007)

The following information in Table 24 is from University of Wisconsin, Madison, Department of Plant Pathology. This instruction manual was published by Carolina Biological Supply Company, Burlington, North Carolina and lays out in detail the various development stages of *Brassica rapa*.

Table 24: Summary of Key Developmental Stages of *Brassica rapa*

Day 1-3	Day 1, the embryonic root (radicle) emerges from seed. Day 3, seedlings emerge from the soil. The two seed leaves (cotyledons) appear and the embryonic stem (hypocotyl) begins to extend upwards. The chlorophyll and purple anthocyanin pigments (if present) are readily visible
Day 4-9	True leaves start to develop by Day 5. Cotyledons continue to grow larger. By Day 8 flower buds begin to appear at the growing tips of the plants
Day 10-12	The plant grows larger. The stem elongates between nodes (point of attachment of leaves) and leaves and flower buds raise to a height well above the leaves
Day 13-17	The flower buds open and reveal the internal structure of the flower. The various parts, such as the pedicel, receptacle, sepals, petals, stamens (anthers and filaments), pistil (stigma, style, and ovary) can be identified. At this stage, pollen is viable and pollination should be initiated. The stigmas are receptive to pollen for 2-3 days after flower opening. Therefore cross pollinate on Days 13, 15 and 17 can be achieved by rotating a bee thorax (or sterile cotton swab) over the flowers to pick up the pollen and then distributing the pollen to different plants in the same fashion. On the last day of pollination (Day 17), pinch off all unopened buds and side shoots
Day 18-22	The flower petals start to drop off, and pods elongate and swell. The endosperm and embryo development of the seeds has started and will continue to Day 34-36
Day 23-36	The development of the embryo is complete; seeds are formed with seed coats. The ovary walls and related structures have by now developed into large pods (silique) and pods begin to dry. On Day 36, plants are no longer watered and the ripening process continues. The pods turn yellow, embryo dehydrates and seed coat turns brown
Day 36-40	Plants are left to dry and by Day 40 the pods are removed. When pods are brittle, they can be rolled between the finger and thumb to harvest the seeds

2.4.11 Parameters Measured at Completion of Experiment for all Plant Genera

For each of the plant bioassays, the following parameters were measured at the completion of the plant's lifecycle: total plant height using a tape measure, total number of leaves, dry mass of shoot and root measured using an analytical balance, number and weight of pods and seeds (using an analytical balance for the weight), as well as width of the stem 2 cm above the soil using a micrometer.

2.4.12 Method to Determine Germination Rate and other Parameters of F_0 Seeds

After the seeds have been air dried from the parent plants, these F_0 seeds are checked for viability.

1. Planter boxes were cleaned according to Ryerson Cleaning Protocol and were distinguished if they were previously used for reference or treatment
2. Forty green planter boxes were filled with fresh reference soil. Ten green planter boxes for each species were planted with either two randomly selected seeds from the parent plants grown in reference soil into each (planting as described previously) or seeds from parent plants grown in biosolids for a total of five reference and five treatment green planter boxes for each genus
3. All forty green planter boxes were randomly placed under a light bank, watered, and monitored and measured as described above. Germination and other growth parameters were recorded if applicable

2.4.13 Further Investigation of Land-applying Biosolids

Further investigation of the impact of land-applying biosolids was carried out using government protocol artificial soil. This bioassay was carried out using only *Glycine max*. Additionally *Rhizobium japonicum* was added to the soil to take a closer look at root nodule formation and distribution. Even though *Phaseolus* is also a legume like *Glycine max*, *G. max* being the more predominant crop in Southern Ontario was chosen to be used in further bioassays to examine root nodules.

2.4.13.1 Materials and Equipment for Artificial Soil Bioassay

Rhizobium japonicum obtained from Woodrill Farm in Guelph Ontario. This bacterium has been inoculated into peat as a means of aiding its distribution into the soil

Artificial soil (used by Environment Canada, OECD, USEPA, ISO, ASTM) consisting of:

10% *Sphagnum sp*. peat (sieved with a 2mm mesh screen), purchased from Sheridan Nurseries, Mississauga, Ontario

20% kaolin clay (particles <40µm), purchased from Pottery Supply House, Oakville, Ontario

70% grade 70 silica sand (particle size between 50-500 µm) purchased from Bell & Mackenzie Co. Ltd. Hamilton, Ontario

and measured on a dry weight basis

2.4.13.2 ***Bioassay with Artificial Soil Planted Using Glycine max Inoculated with Rhizobium japonicum***

The artificial soil is nutrient-poor; therefore, minimal growth was expected and any detrimental or positive effect of the biosolids would be easier to then ascertain.

1. Soil was prepared in large quantities using a cement mixer and adding: i) two parts kaolin clay, ii) seven parts silica sand, and iii) one part *Sphagnum sp*. peat. The artificial soil was thoroughly mixed until a uniform mixture was obtained
2. $CaCO_3$ was added to the dry mixture if needed to adjust the pH of the final hydrated soil to 6.0 - 7.5. (A sample of the final soil was tested and $CaCO_3$ added to the large batch accordingly, if needed)
3. DMDW was gradually added with continuous mixing until a uniform colour and texture was reached. Crop troughs were prepared as in Section 2.4.5 using this artificial soil in place of the reference soil. Six crop troughs (three for reference and three for treatment bioassays) were prepared
4. *Glycine max* seeds were examined to ensure that the shell casing was not cracked since these seeds will not germinate.
5. Thirty seeds were gently placed into a 150 mL glass beaker which contained 1 cm of the *Rhizobium japonicum* in peat (Figure 53) and with a very gently motion, the seeds were swirled until lightly coated. All that was needed is for the seeds to have a fine dusting or few tiny specs of the dark peat on them. The bacterium was present in the peat and

transfers to the soil when the seed is planted ensuring the presence of the bacteria in the soil and then later, potential development of the root nodules

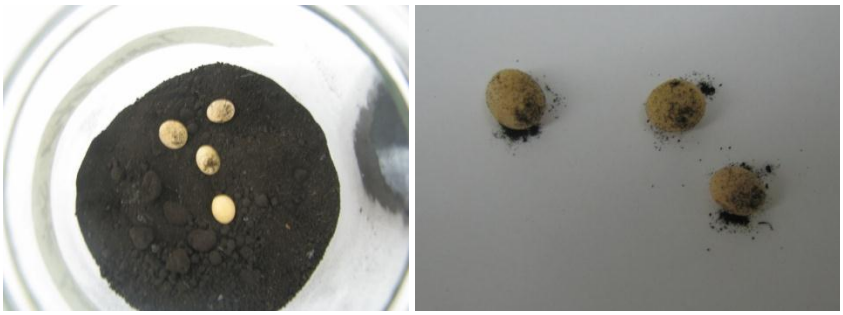

Figure 53: *Glycine max* seeds coated with *Rhizobium japonicum* in peat

6. Seeds were planted in the crop troughs as described in Section 2.4.5.4 and the troughs were randomly arranged under the light banks and the bioassay was carried out as described in Section 2.4.6.3
7. At the termination of this bioassay, the soil was thoroughly moistened to make the removal of the plants and root system easier. A radius of 5 cm around the stem of each plant was measured and then, using a gardening trowel, each plant and root system was gently removed. It was impossible to remove all of the root system due to their fine delicate nature, but measuring a constant radius around individual plants ensures that the root mass obtained is the same for each
8. The bulk of the soil was gently removed and the whole plant (shoot and root mass together) was laid onto a paper liner and allowed to air dry for 24 hours. Any remaining soil was very gently removed the following day, being careful not to disturb any root nodules that may be present. The presence, location, and quantity of any root nodules was observed and recorded
9. Plants were divided between shoot and root material indicated by the mark on the plant where it was either in or out of the soil; below soil is the root mass, above the soil is the shoot mass. Dry shoot and root mass was determined using an analytical balance along with the number and weight of any pods and seeds

2.4.14 Protocol Modification – Plants

The existing government protocols were inadequate for assessing the land-application of biosolids for the following reasons. Environment Canada RM 45 (2007) tests for the emergence and growth of terrestrial plants for either fourteen or twenty-one days and only looked at grass and vegetable crops, not crops that biosolids would be applied to them. While OECD # 208 and # 227 guidelines (2006, 2003) did include plants that were of interest to this project, (such as corn and soya beans), they were only interested in the early stages of growth, and observed for 28 days, or approximately one quarter of the plants life cycle. OECD Guideline #227 looked at the application of the toxicant to a young plant through spraying, as would be the case for herbicide or pesticide, this procedure would not be applicable to this research since in this case the 'contaminant', biosolids, are applied to the soil prior to the seeds being planted. The International Standards Organization (ISO) 11269 Part 1 (1993) looks at inhibition of root growth and Part 2 (1995) examed effect of contaminants on early growth of plants, i.e. emergence, again not looking at life cycle or viability of offspring. The USEPA # 712-C-96-363 (1996) used 5-Day seed germination tests, using *Lactuca sativa* (lettuce) as well as root elongation over 14 days duration (USEPA 1994a), not whole lifecycles and not environmentally-relevant crops. Also, the small lettuce seeds may be compacted by addition of the biosolids.

In this research, the four different plant species were grown to maturity; the complete life cycle was observed (up to 120 days for some species) thus allowing for many other parameters to be measured and compared. These include, time-to-flowering and time-to-seed production, the total number of leaves produced, as well as leaf size, shoot and root biomass, and length of mature plants. Furthermore, the seeds produced from the F_0 plants were dried, planted and observed for germination, germination rate, and growth to see if there was any impact on successive generations (i.e. F_1 plants).

2.4.14.1 Larger Test Vessels

To perform these longer duration bioassays, larger vessels were needed to accommodate the size of the full grown plants. While the government protocols became more descriptive with time, (i.e. 15 cm diameter pot size as prescribed by OECD guideline; nonporous containers specified by the USEPA using sterilized standardized soil; 1 L polypropylene beakers with 0.5 L of soil as prescribed by Environment Canada), these vessels were still inadequate. For this work, much larger crop troughs were therefore constructed. The troughs were either 120 x 30 x 30 cm containing 110 L of soil for Zea *mays* plants 90 x 30 x 30 cm containing 85 L of soil for both *Glycine max* and *Phaseolous vulgaris*, or 34.2 cm x 20.9 cm x 11.8 cm containing 7 L of soil for the *Brassica rapa*.

2.4.14.2 Source of Water Used

This research used dechlorinated municipal drinking water instead of deionized water as prescribed in government protocols since dechlorinated water would more closely resemble the rain water that the organism would be exposed to in nature.

2.4.14.3 Wind

The addition of a source of wind was not provided for in any of the other protocols, government or otherwise. To ensure that the plants did not develop weak and spindly stocks or vines due to lack of lignin or hemicelluloses production (Scheller and Ulvskov 2010), a breeze needs to be provided during plant development. This was accomplished by randomly placing a table fan as shown in Figure 54 around the troughs and running the fan during the day or night for varying time intervals as well as lengths of time. The fan also plays another key role in pollination especially for the anemophilous plants such as *Zea mays* that need wind to accomplish fertilization.

Figure 54: Providing wind for the growing plants

2.5 Other Abiotic Parameters Needing Consideration

2.5.1 Lighting

Light is another abiotic parameter that also needs to be addressed given that light is needed not only for the growth of the plants, but also to signal germination (Koning 1994). Since these bioassays are to simulate an outdoor growing environment as much as possible inside a laboratory setting, a method of providing light that would represent the quality of the sun's light needs to be considered. Light has three properties that affect the growth of plants; i) quantity (the intensity of the light) which can be controlled by the number of bulbs provided, ii) quality (the wavelength/colour reaching the plant) which can be controlled by the selection of the type of bulb used, and iii) duration (or photoperiod, the length of time the plant is exposed to the light) which can be controlled by using a twenty-four hour timer attached to the lights (Spectrum Technologies 2008). Light *intensity* is a measure of brightness at the source (measured in watts) where light *irradiance* is a measure of the amount of light striking a surface (measured in $\mu E/m^2/s$) (Spilatro 1998). One einstein is one mole of photons irrespective of the frequency and is named in honour of Albert Einstein who in 1905 introduced light quanta (photons) and explained the photoelectric effect (Cerny 2000). The microEinstein (µE) (not an official SI unit of measure) equals one-millionth of an

einstein per square-meter per second (Cerny 2000) and is used when studying photosynthesis. The µE measures the energy that a plant uses for its photosynthetic processes and is termed photosynthetically available radiation (PAR). This radiation is between 400-700 nm as seen in Figure 55. (Spilatro 1998). PAR, measured in $\mu E/m^2/s$ can be determined using a quantum meter (which was used for this research) or for a more accurate measurement, a spectroradiometer (Cerny 2000).

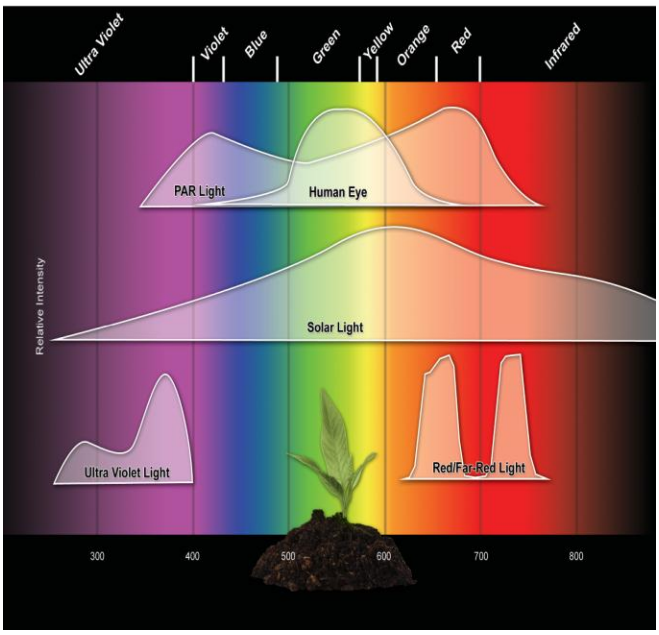

Figure 55: Photosynthetic active region of plants (Source: Spilatro 1998)

2.5.1.1 <u>Light and Germination</u>

In terms of seed germination, light plays a vital role. The embryos of seeds with thin seeds coats (as in this study) use either the presence of light or its absence to trigger germination (Koning 1994). Small, shallow-buried seeds like the *Brassica* seed's use the light that penetrates the soil to signal the initiation of germination thus allowing the cotyledon to surface and start photosynthesising before the seed's reserves run out (Koning 1994). If the seeds are buried too deeply, they will not germinate (Koning 1994). The opposite is true for the larger seeds like corn and the legumes. These seeds have evolved a reservoir of food available to them when they are underground and thus require darkness before they will

initiate germination (Koning 1994). The advantage of this method is that the shoot does not act as an indicator to predators indicating a rich food source before the plant gets a chance to develop.

2.5.2 Types of Light bulbs

Several types of bulbs were researched in this study for the spectrum of light produced, amount of heat generated, lifespan of bulb, and overall performance. In the end, the Vita-Lite bulbs by Dura-Test that have been used by the USEPA and Environment Canada were chosen for this research (Lewis *et al.* 1994; Environment Canada 2007b). Figure 56 compares the spectrum of natural sunlight (top image) to that of the Vita-Lite bulbs (lower left) and the cool white florescent bulbs (lower right). From this it can be seen how much closer to natural light (5500°K) the Vite-Lites bulbs (also 5500°K) are compared to contemporary bulbs (4200°K).

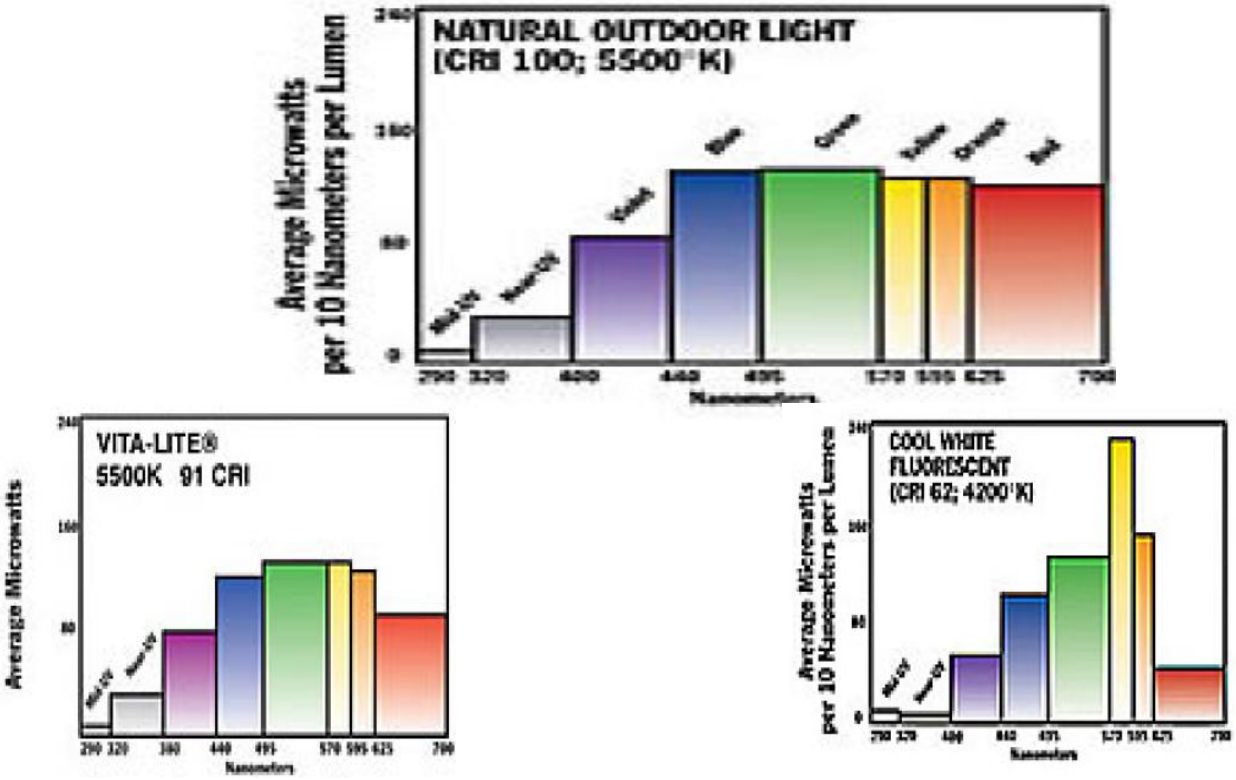

Figure 56: Various wavelengths of light needed for photosynthesis (Source: Spectrum Technologies 2008)

2.5.2.1 VitaLite Bulbs

These bulbs supply a crisp, full colour and ultraviolet spectrum in a fluorescent light that mimics the natural quality of outdoor light of the sun with a Colour Rendering Index (CRI) of 91 (whereas the sun has a CRI of 100) compared to a cool white fluorescent bulb that has a CRI of 62 (Vita-Lite nd). Another advantage of these bulbs over other types is that they do not have a drop off of light quality or quantity at the ends of the bulbs over time as happens with others (Pers comm. George Coutts, Duro-Test). Therefore, plants grown below the edges of the light fixtures receive the same quantity and quality of light as plants grown at the center for the whole life of the bulb. This cannot be said for the other types. These bulbs fit in conventional light fixtures and have a lifespan of 10,000-28,000 hours (Vita-Lite nd).

2.5.2.2 *High Intensity Discharge Bulbs*

Other, but less satisfactory options for light sources that were researched are the High Intensity Discharge (HID) grow lights and the T5 Fluorescent grow lights (Bustan Greenhouse 2008). The Mercury HID has a 13% efficiency rate of converting electricity into PAR. Metal Halide (MH) bulbs emit light in the blue spectrum, thus producing compact, green leafy growth, and are 20% efficient with a lifespan of 10,000 hours. Metal halide bulbs have a CRI of 39 for the fluorescent types and 18 for the incandescent types (Newman 2003; Bustan Greenhouse 2008). The High Pressure Sodium (HPS) HID bulb are the more efficient at 25%, have a longer life span (18,000 hours), and produces light with an orange/red spectrum which triggers hormones in the plant to induce flowers and budding (Newman 2003). Although HID bulbs are commonly used in greenhouses since they are mainly only used for starting plants, but are inadequate for the complete growth cycle needed in this research. Therefore, different types of HID bulbs would be needed to be used in conjunction to accommodate the at the different stages of the complete lifecycle of the plants. Another drawback of the HID bulbs besides their inefficiency at converting electricity to PAR, is the amount of heat that they generate (Bustan Greenhouse 2008; Newman 2003).

2.5.2.3 T5 Fluorescent Bulbs

The T5 fluorescent grow light are full-spectrum bulbs that have a very low lumen output (75-90 lumens per watt). Therefore, multiples need to be used and be placed very close to the plants (15 cm) to be effective (Bustan Greenhouse 2008). These bulbs are good for plants that only require low light, such as seedlings or herbs but are very poor for flowering and budding stages of a plant's development. Thus these bulbs, which are less effective than the HID lights (Bustan Greenhouse 2008), were not considered for use in this research.

2.6 Statistical Analysis

When choosing a method of statistical analysis to interpret data, one needs to consider the goal of the experiment and the type of data obtained (Motulsky 1995; Pers. Comm. Andrew Laursen, Ryerson University). When setting up bioassays, the independent variable is treatment (reference vs. biosolids) plants. Dependent variables for the animal bioassays include avoidance behaviour (for particular durations) and reproduction. Dependent variables for the plant bioassay are percent germination, germination rate, days-to-flowering, days-to-pod development, stem width, tassel height, leaf length, number of leaves, number of pods, weight of pods, number of seeds, weight of seeds, shoot weight, root weight, leaf area and plant height throughout the growth cycle (Pers. Comm. Andrew Laursen, Ryerson University). Many of the growth parameters were measured only once during the bioassay (such as percent germination and germination rate), but several variables, for instance number of leaves, leaf length, leaf area, and plant height, were repeatedly measured over the duration of the bioassay. All of the response variables are quantitative in nature (numerical) since actual measurements could be taken as opposed to qualitative (obtaining subjective observations). The germination results could be categorical or qualitative in nature if the outcome was to determine if the plant germinated or not thus generating a yes/no response. For this research, percent germination and when they germinated (rate of germination) was of greater importance (and also answers the question if they germinated) and were therefore used. The avoidance bioassays for the animal bioassays produced categorical data. For example, the earthworms were or were not found in the reference soil.

Due to space availability for most plant bioassays in this research, the number of replications (n) is three; consequently it is difficult to ascertain if the data are normally distributed and therefore conforming to the assumptions of a parametric test (Zar 1984; Rodger 2004). Although, since the other two assumptions (random, independent sampling and the homogeneities of variances) (Zar 1984) are more likely to be met, the data that were continuous in nature (e.g.; stem width, plant height, weight of seeds, and weight of pods) were analysed using parametric tests, specifically two-sample t-test comparing treatment means (Zar 1984; Motulsky 1995; Marion 2004). This is an appropriate analysis method since two groups were analyzed; reference and treatment (biosolids) (Marion 2004; Pers. Comm. Andrew Laursen, Ryerson University).

For the response variables that were discrete in nature (e.g. number of springtails present in the animal bioassays or percent germination, germination rate, days-to-flowing, days- to-pod development, number of leaves, number of seeds and number of pods in the plant bioassays), no assumptions were made regarding sample distribution and this data were analysed using non-parametric tests (Zar 1984; Motulsky 1995; Marion 2004). In these cases, the data were analysed using the Kruskal-Wallis analog of one-way analysis of variance (ANOVA) to determine if a significant difference existed between the reference and biosolids treatments (Zar 1984; Motulsky 1995; Marion 2004). Although non-parametric tests are less powerful, they are distribution-free and more robust than parametric tests (Zar 1984). Given this, non-parametric tests could be used to analyse any data set, but if the data support the three assumptions of parametric tests (as mentioned above), then the parametric test would be the more appropriate method to use since it offers more power to the analysis (Zar 1984). A Levene's test could be run to analyse the data to check for homogeneities of variance and thus support the use of parametric or non-parametric tests.

If the goal of the experiment is to compare three or more matched groups where a response variable is measured on the same test subject over a longitudinal axis (i.e. repeated measurements over time or space), a repeated measures-ANOVA (rm ANOVA) can be

applied (Motulsky 1995). This was the case for plant height throughout the duration of its lifecycle since the same plant was measured repeatedly over the duration of the bioassay. It is appropriate to use rmANOVA only where the repeated measures over time fit a linear model or can be transferred to fit a linear model (Pers. Comm. Andrew Laursen, Ryerson University). Plant height fits such an expectation; therefore, a rmANOVA was performed on these data.

For the animal bioassays involving the earthworms, the avoidance bioassays were analysed using Chi-Square tests. This statistical analysis is a nonparametric method (Key 1997) that is used when the data consist of frequencies (i.e. how many or how often the earthworms are found in the reference soil) and is compared to an expected value to determine if there is a significant difference between the observed and expected frequencies in one or more groups. In these bioassays, one would expect the earthworms to be found in the reference soil fifty percent of the time and in the biosolids amended soil fifty percent of the time if there was no preference exhibited by the earthworms. The formula to determine Chi-Square (X^2) is as follows;

$$\chi^2 = \sum_{i=1}^{k} \frac{(O-E)^2}{E}$$

Where O is the observed value and E is the expected value determined i to k times (Keys 1997). Using the appropriate degrees of freedom (here d.f. = 1), the critical X^2 value can be obtained from tables and ultimately determine if the calculated chi-square is greater than or equal to this critical value and thus determine if there is a significant preference shown for one soil type (indicating avoidance or preference for biosolids-amended soil).

Additionally, for the earthworm bioassays dealing with the directionality of rectangle vessels, a paired t-test was used to analyse this data. This parametric test is used to compare means of the same subject either over time as in before/after situations or in differing

circumstances/treatments (e.g. differences in bioassay set-up such as changing the orientation of where reference soil was located (left or right) when the same earthworm was used in repeated trials) (Zar 1994; McDonald 2009). This statistical test satisfies the null hypothesis if the mean difference between the pairs is not significantly different than zero.

3. **Results and Discussion**

3.1 Overview

The land-application of biosolids is a common practice around the world and has been so for centuries (Synagro 2002; O'Connor *et al.* 2005; Australian Water Association 2009; City of Toronto 2009, 2010a; OMAFRA 2010a). Biosolids have been used as a fertiliser, providing many micro and macronutrients for plants or as a soil amendment for the soil to aid soil structure and permeability (Butt and Nuutinen 1998; Banks *et al*. 2006; Wang *et al*. 2008). Biosolids, although regulated, are not without some perceived detriment. Any of the stressors that enter a WWTP have the potential to end up in the biosolids and, if the metals, nutrients, and pathogens are within limits set by the regulating bodies, could then be land-applied (Kinney *et al*. 2006; Smyth *et al*. 2007; Wu *et al.* 2008; Sabourin *et al.* 2009; Wang *et al*. 2009) since organic contaminants are not regulated. Many studies have been done to determine what is either in the sludge/biosolids or soil (Slone *et al*. 1998; Harrison *et al*. 2006; Kinney et al. 2006, 2008; Rogers and Smith 2007; Clarke *et al.* 2010) but there are few that assess impact of biosolids on the terrestrial biota. Thus, there is a need to determine if the land-application of biosolids to agricultural fields impacts the terrestrial biota and in turn assess whether this method of disposal for biosolids is a sustainable practice. The results presented here attempt to answer that question.

Figure 57 from Methodology (Chapter 2) was brought forward as an overview of the results that will now be discussed, starting with those obtained using Kitchener biosolids since this was the first source used, followed by the results using Guelph biosolids. Additionally, a chronological order in terms of Method development is also provided in the body of this section.

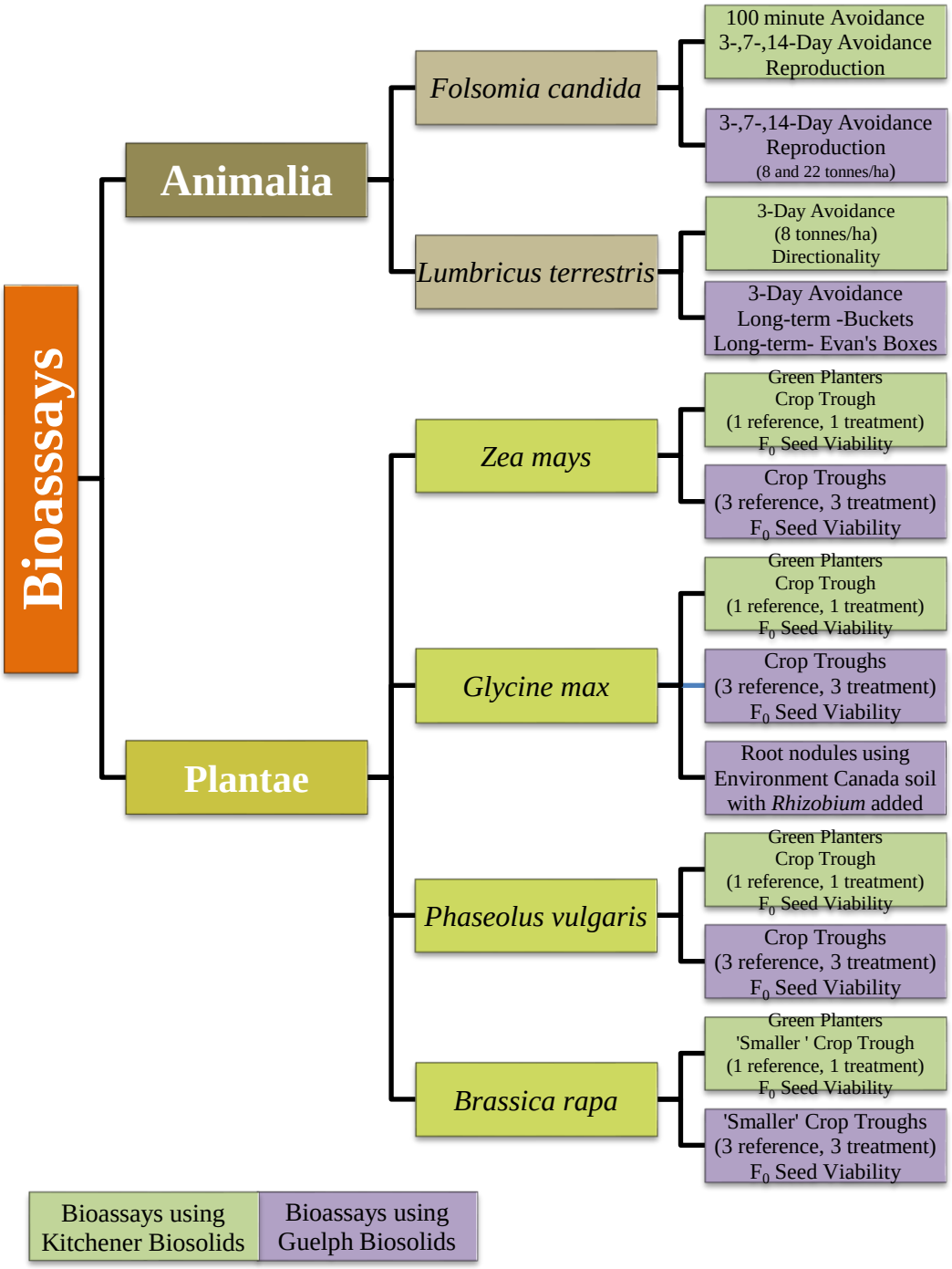

Figure 57: Organism and bioassays used to assess for impact using biosolids from two different sources

3.2 Kitchener Biosolids

The biosolids from Kitchener WWTP were processed using anaerobic digestion and dewatered by mechanical means. All bioassays were carried out using a worst-case scenario application rate of 22 tonnes/ha of biosolids on a dry weight basis unless otherwise specified. This amount was used at the suggestion of OMAFRA and MOE, the funding bodies for this research, as this represents the total amount of biosolids that can be applied over a five year period.

3.2.1 Animal Bioassays

For these bioassays, two different genera were used. They include *Folsomia candidia* (springtails) and *Lumbricus terrestris* (earthworms) which will each be discussed in turn.

3.2.1.1 Summary of Results Obtained for Avoidance Bioassays with Folsomia candida using Kitchener biosolids

***Folsomia candida* (springtails)**

- Avoidance Behaviour:
  - 100-minute
  - 3-day
  - 7-day
  - 14-day

Avoidance bioassays were performed in either a Petri-dish with five springtails for the 100-minute bioassays or in 125mL Mason jars with 10-day old aged synchronized springtails. Each of the longer duration avoidance bioassays consisted of five jars each containing fifteen springtails. Both the Petri-dishes and Mason jars were divided in half; one side contained reference soil and the other side contained treatment soil. At the end of the elapsed time, where springtails had compartmentalized was assessed.

For the avoidance bioassays, the results are represented as percentages of how many springtails were found in either the reference or treatment half of the vessel. When an impact was observed, as designated by the column in the tables under the heading "Impact of Biosolids", a positive effect of biosolids indicates that the springtails did not avoid the biosolids but instead spend significantly more time in the presence of biosolids. A negative impact of biosolids indicates the opposite; the springtail did avoid the biosolids. Since the bioassays were repeated several times, these repeats are designated as 'Trials' and within each Trial is a number of replicates as indicated in the Methodology. In the case of the springtails, there were five replicates.

Trial #1 and #2 of the longer duration avoidance bioassays (time frame measured in days) were performed using the same biosolids but different age (i.e. stored biosolids for several months at the laboratory), while Trial #3 and Trial # 4 were a different batch of biosolids. The 100-minute avoidance bioassays were all performed using the same initial batch of biosolids. Response variables that were discrete in nature (e.g. number of springtails present), were analysed using the non-parametric Kruskal-Wallis test and provided the 'H-value' listed in the tables (Table 25) (and for all other pertinent results throughout this section). The 100-minute bioassays either showed no significant difference (NSD) or that the springtails favoured the biosolids (Trial #1 and #3: $H=7.258$, $p=0.007$ and $H=4.192$, $p= 0.041$ respectively). As there does not seem to be any impact at the 100-minute level, further bioassays including this observation point were not performed. Over a longer period (3 and 7 days), there does seem to be an effect on the avoidance of springtails with laboratory stored biosolids (Trial #2 $H=6.707$, $p=0.010$, $H=8.534$, $p=0.003$, $H=13.741$, $p=0.000$ for the different durations) (Table 25). This could be a factor of storage (left in a sealed bucket) and the build-up of gases (possibly ammonia or sulphide) as opposed to an actual impact of biosolids. There was no significant difference with the freshly received biosolids represented by Trial #1 (Table 25).

Table 25: Results of *Folsomia candida* Avoidance Bioassays using Kitchener Biosolids analysed using the non-parametric Kruskal-Wallis test (NSD = No Significant Difference)

Analysis	Ref (%) mean	Ref S.D.	BS (%) mean	BS S.D.	H- value	p-value	Impact of Biosolids
100-min trial #1	28	11	72	11	7.258	**0.007**	positive
100-min trial #2	56	17	44	17	1.006	0.316	NSD
100-min trial #3	36	17	64	17	4.192	**0.041**	positive
100-min trial #4	40	25	60	25	1.006	0.316	NSD
3 day avoid trial #1	59	33	41	33	0.540	0.462	NSD
3 day avoid trial #2	61	16	39	16	6.707	**0.010**	negative
7 day avoid trial #1	52	17	48	17	0.101	0.751	NSD
7 day avoid trial #2	68	19	32	19	8.534	**0.003**	negative
7 day avoid trial #3	49	24	51	24	0.544	0.461	NSD
14 day avoid trial #1	59	17	41	17	1.866	0.172	NSD
14 day avoid trial #2	73	15	27	15	13.741	**0.000**	negative

3.2.1.2 Summary of Results Obtained for Reproduction Bioassays with Folsomia candida using Kitchener biosolids

***Folsomia candida* (springtails)**

- Reproduction

When using *Folsomia candida* in a bioassay, a measurable endpoint is needed. It is very difficult to measure their weight since it is roughly only 140 µg (Krogh 2008) and a sophisticated balance would be needed. Additionally, small particles of soil can be trapped between their legs thus affecting the final weight. Uncontrolled evaporation through the cuticle can also alter their weight if ambient humidity is not precisely controlled. If dry weights were to be used, it would require destroying the organisms. A more precise estimate

of their weight can be achieved by accurately measuring the length of single individuals, since the length of the collembola is proportional to the square root of the dry weight (Bengtsson *et al.*1983). Due to the difficulty in both of these procedures and the inherent errors in measuring the large numbers obtained in the reproduction bioassays, the biological endpoint used here was the number of organisms present. This number was determined by counting the individual organisms present at the completion of the bioassay. Since springtails are only approximately 0.4 -3.0 mm long and constantly moving, they are difficult to count in large numbers. Therefore in these cases, digital photography was used with the aid of an image analysis program, Image J, to count the organisms. As indicated by Krogh *et. al.* in their 1998 paper, this method is faster and provides a more reliable result. Due to their hydrophobic exoskeleton and the surface tension of water (Environment Canada 2007d; D'Avino *et al*. 2008), springtails float when their container is flooded, thereby facilitating the counting of the organisms. Bromophenol blue was added to the water for contrast thus aiding in the counting process since the white springtails show up better against the now darker background that is caused by the soil in the water.

Reproduction bioassays were carried out in 125mL Mason jars with 10-day old age-synchronized springtails. There were five reference jars and five treatment jars, each with ten springtails. At the end of the 39-day bioassay, the numbers of organisms present were counted without being distinguished by age.

Similar to Trial #1 and #2 of the avoidance bioassays, the reproduction bioassays were performed using the same biosolids but of different age (i.e. stored biosolids) while Trial #3 was a different batch of biosolids. In the reproduction bioassays, the figures represent the total number of organisms found. When an impact was observed, as designated by the column in the table under the heading "Impact of Biosolids", a negative impact of biosolids indicates that reproduction was hampered. Since a comparison of means was used to analysed the difference between reference and treatment, a 't-value' was provide as indicated in Table 26 (and all other pertinent tables in this section); additionally, a F-value obtained

from an ANOVA is present in the table when comparing the means of the reference, treatment and autoclaved soils.

When looking at the reproduction bioassays involving the autoclaved soil, it can be seen in Table 26 that there is no significant difference in the two reproduction bioassays between the three types of soil (i.e. reference soil alone, reference soil with 22 tonnes/ha on a dry weight bases of biosolids added or autoclaved reference soil) between the two trials ($F=0.310$, $p=0.739$ and $F=0.702$, $p=0.515$ respectively). The soil was autoclaved to ensure that there were no naturally occurring springtails present in the soil and that all counts came from the springtails that were added during the bioassay only. Had there been springtails originally present, the reference jars would have had a significantly larger number of springtails at the termination of the bioassays. As this was not seen, further bioassays including an autoclaved soil were not performed. In Trial #1 and #2 for the reproduction bioassays, there was not a significant difference observed (Trial #1 $t=0.498$, $p=0.632$ and Trial #2 $t=0.590$, $p= 0.573$) unlike in the avoidance bioassays where avoidance (Trial #2) may have been a function of storage of the biosolids and the build-up of gases. Activated carbon should have absorbed the NH_3 over the thirty-nine days of the reproduction bioassay, or it volatilized off during the multiple aerations and feedings that took place. Further research needs to be conducted in regards to storing the biosolids under laboratory conditions such as leaving the storage buckets opened during storage

For Trial #3 (a new batch of biosolids) there was a significant reduction observed in soils ith biosolids ($t=10.542$, $p=0.000$) (Table 26) thus emphasizing the need for analysis to be performed routinely at the WWTP. Although this is a statistically significant result, it must be remembered that it would be exceptional for springtails to be exposed to 22 tonnes/ha of biosolids under normal land-application conditions. Normal application rates are closer to 8 tonnes/ha on a dry weight basis, thus the methodology was modified to include this value in future work.

Table 26: Results of *Folsomia candida* Reproduction Bioassays using Kitchener Biosolids analysed using a t-test and ANOVA (NSD = No Significant Difference)

Analysis	Ref # present	Ref S.D.	BS # present	BS S.D.	t-value	p-value	Impact of Biosolids
reproduction trial #1	1588	503	1426	530	0.498	0.632	NSD
reproduction trial # 1 autoclaved soil	1698.2	614.2	-	-	ANOVA F=0.310	0.739	NSD
reproduction trial #2	3936	1025	3473	1425	0.590	0.573	NSD
reproduction trial # 2 autoclaved soil	3089	874	-	-	ANOVA F= 0.702	0.515	NSD
reproduction trial #3	1894	294	282	175	10.542	**0.000**	negative

3.2.1.3 Summary of Results Obtained for Avoidance Bioassays with Lumbricus terrestris using Kitchener biosolids

***Lumbricus terrestris* (earthworms)**

- Avoidance behaviour:
  - 3-day
- Directionality experiments

From a chronological stand point, this bioassay using Kitchener biosolids was performed *after* the bioassays using Guelph biosolids. Information gained from those results led to the application rate being decreased to 8 tonnes/ha instead of the 22 tonnes/ ha as in initial bioassays. As well, the avoidance bioassays for *Lumbricus terrestris* with Kitchener biosolids were performed using only Method #2 (the 34.2 cm x 20.9 cm x 11.8 cm transparent rectangle vessels) since it was deemed from earlier work, the most effective method due to the failure of *L. terrestris* to even enter the avoidance chamber of Method #1 (Kaushik chamber following Environment Canada's protocol). Therefore, Avoidance Bioassay Method #1 was discarded and Method # 2 was used for all further work.

In Avoidance Bioassay Method # 2, each of ten vessels were divided in half; one side contained reference soil and the other side contained treatment soil (in this case with an

application rate of 8 tonnes/ha of biosolids on a dry weight basis). One adult organism was placed along the center line of each and observed after 3 days. Therefore, for these bioassays a total of sixty replicates were used.

For the avoidance bioassays, the results represented if the earthworm was found in either the reference or treatment half of the ten replicate vessels. In the case where the values do not add up to 10, as in Trial #2 and #5, an earthworm escaped the avoidance chamber. If a worm was found equally between either the reference and biosolids halves of the avoidance chamber, then for the purpose of Chi-Square analysis, it was indicated as being present in both halves and indicated as a half in Table 27 Unlike in the springtail avoidance bioassay where ten organism were used, in these avoidance biassays, there was only one organism used in each vessel. Therefore, a Chi-Squares analysis was used to analyse the categorical avoidance data gathered (providing the X^2-value, here and in all other pertinent tables) (Table 27). Additonally, the directionality biassays was analysed using a paired t-test. *Lumbricus terrestris* was not impacted by these biosolids since there was neither a negative or positive impact observed (Table 27). The overall value at the bottom of the table is the summation of all six trials and again no significant difference was seen (X^2 =0.370, p=0.719).

Table 27: Results of *Lumbricus terrestris* Avoidance Bioassay using Kitchener biosolids (8 tonnes/ha) analysed using Chi-Square Analysis (NSD = No Significant Difference)

Analysis	Unit	Ref Mean	BS Mean	X^2 value	p-value	Impact of Biosolids
Trial #1	#	5.5	4.5	0.112	0.752	NSD
Trial #2	#	5	4	0.111	0.739	NSD
Trial #3	#	6	4	0.400	0.527	NSD
Trial #4	#	5	5	0.000	1.000	NSD
Trial #5	#	4	5	0.111	0.739	NSD
Trial #6	#	4	6	0.400	0.527	NSD
				t-value		
OVERALL	#	4.9±0.8	4.8±0.8	0.370	0.719	NSD

Concurrently, directionality experiments were also carried out by repeating the above avoidance bioassay using the same earthworm, except this time rotating the vessel so that instead of the reference being on the left as in the initial experiment, it was now on the right. This was to determine if shape of the vessel played a role in the final location of the earthworm (Yeardley *et al.* 1996). In Table 28, right/left indicate on which side of the rectangular vessel the earthworm was found, as opposed to being in either the reference or treatment side. That information can be gained from Table #27. The shape of the avoidance chamber did not have an effect on the bioassays. Thus the rectangular shaped chamber can replace the circular vessel as used in government protocols and by other researchers.

Table 28: Results of *Lumbricus terrestris* Directionality Bioassay using Kitchener biosolids (8 tonnes/ha) analysed using a Paired t-test (NSD = No Significant Difference)

Analysis	Unit	Right	Right S.D.	Left	Left S.D.	t- value	p-value	Impact of Biosolids
Trial #1	#	9.5	0.5	9.5	0.5	0.000	1.000	NSD
Trial #2	#	11	0.5	9	0.5	0.490	0.629	NSD
Trial #3	#	10	0.5	9	0.5	0.224	0.826	NSD

3.2.2 Plant Bioassays

For these bioassays, four different plant genera were used; *Zea mays* (corn), *Glycine max (*soya bean), *Phaseolus vulgaris* (common bean), and *Brassica rapa* (field mustard).

3.2.2.1 Summary of Results Obtained for Bioassays with Green Planters using Kitchener Biosolids

Zea mays (corn)

- Percent germination
- Rate of Germination
- Mean plant height (Day 7, 41)
- Mean leaf length (Day7)
- Mean number of leaves (Day 41)
- Shoot weight
- Root weight

Glycine max (soya bean)

- Percent germination
- Rate of germination
- Mean plant height (Day 7,15)
- Mean leaf length (Day7, 15)
- Mean number of leaves (Day 28)
- Number of pods
- Number of seeds
- Shoot weight
- Root weight

***Phaseolus vulgaris* (common bean)**

- Percent germination
- Rate of germination
- Mean plant height (Day 7, 15)
- Mean total leaf area (Day 7, 15)
- Mean number of buds (Day 28)
- Number of pods
- Number of seeds
- Shoot weight
- Root weight

***Brassica rapa* (field mustard)**

- Percent germination
- Rate of germination
- Mean plant height (Day 7, 15)
- Mean total leaf area (Day 7, 15)
- Mean number of buds (Day 15)
- Number of pods
- Number of seeds
- Shoot weight
- Root weight

For these initial bioassays, five green planter boxes containing approximately 1L of reference soil and five green planter boxes with approximately 1L treatment soil for each of the four plant genera *Zea mays, Glycine max, Phaseolus vulgaris* and *Brassica rapa* were used. Each

green planter box was planted with two seeds, except in the case of *Brassica rapa*, where there were four seeds used instead, due to the overall smaller size of this plant in comparison with the other 3 crop plants.

Percent germination refers to how many seeds germinated, while rate of germination refers to how long the seeds took to germinate (measured in days). Since there was more than one plant in each vessel, the mean value (e.g. plant height, leaf area) was determined and this was used in the statistical analysis. Shoot weight refers to all plant biomass above the ground and root weight refers to all plant biomass below the ground. There is a large deviation in the area of the leaves. This is to be expected since the leaves are at different stages of development and thus they should be different sizes. The values here represent the mean leaf area or length depending on the variable measured. Figure 58 below illustrates how these measurements were made. Since their leaves are not square, the longest and widest point was used when making measurements of their dimensions.

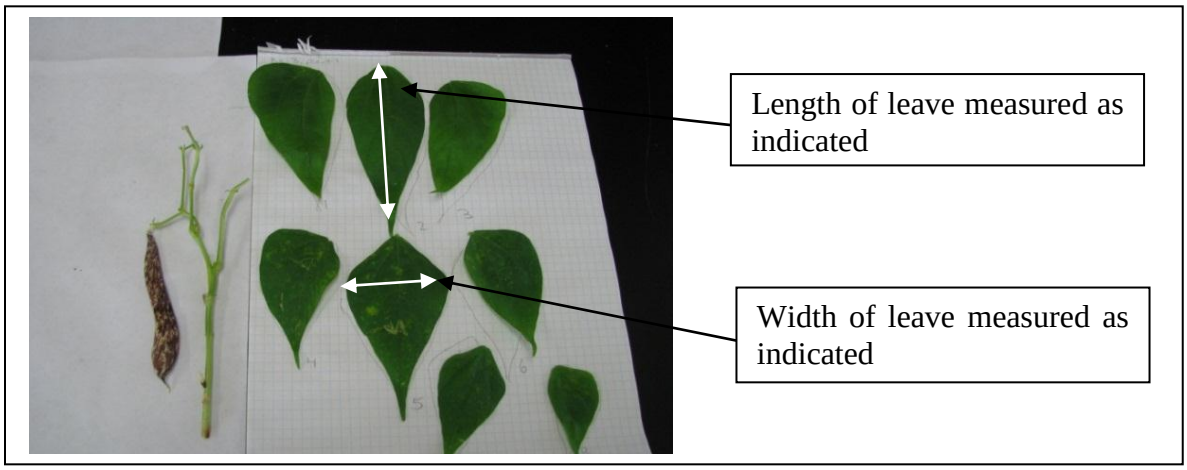

Figure 58: Illustration of *Phaseolus* used to measure leaf dimensions

Since the green planter boxes were the initial plant bioassays carried out, protocol development was still underway. Therefore, they were only used with Kitchener biosolids and the whole suite of measurements used in later bioassays was not performed here. The main function of using the green planter boxes was to ensure that the construction material of

the crop troughs did not interfere with the life-cycle bioassays of the plants that were to be performed in them.

Overall, little impact observed was with the biosolids with the plants grown in the green planters. Where an impact was seen, a positive impact of biosolids indicates that the plant parameter measured was significantly enhanced in the presence of biosolids. A negative impact of biosolids indicates the opposite: the plant did better in the reference soil.

As seen in Table 29 for *Zea mays*, there was a significant difference in the number of leaves later in the development of the plants (H=4.900, p=0.027) which led to a significant increase in plant biomass for the treatment plants (t=4.132, p=0.003). This observation indicates that the biosolids did have a positive impact on these plants producing more leaves with more biomass.

Table 29: Results of *Zea mays* bioassays grown in Green Planters with Kitchener Biosolids analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H-value	p-value	Impact of Biosolids
% Germination	%	100	0.0	100	0.0		0.000	1.000	NSD
Rate of Germination	days	5.5	1.6	5.1	0.3		0.800	0.371	NSD
Height Day 7	mm	24.63	2.8	23.46	2.8	0.667		0.523	NSD
Height Day 41	mm	161.71	17.5	159.33	10.5	0.261		0.800	NSD
Leaf Length Day 7	mm	80.73	7.6	77.7	10.5	0.522		0.616	NSD
# of Leaves Day 41	#	6.2	1.2	8.4	1.2		4.900	**0.027**	positive
Shoot Weight	g	1.9109	0.27	2.7339	0.36	4.132		**0.003**	positive
Root Weight	g	1.7991	0.33	1.4710	0.40	1.410		0.196	NSD

The results summarized in Table 30 indicate no impact on *Glycine max* when grown in Kitchener biosolids. Upon closer examination of the data, the germination rate for the plants in the treatment were generally slower with a wider spread between the first and last plants, indicated by the standard deviation. This in turn, led to shorter plants (but not significantly so) and the larger deviation in the leaf length by Day 15 when compared to the reference

plants (Table 30). These differences did not affect the overall plant biomass (as indicated by shoot weights) at the termination of the bioassay. At this time, the treatment plants were similar to the reference plants, possibly due to the extra nutrients provided to these plants from the biosolids. Root nodules were not observed on either the reference or treatment plants. This could be due to the fact that the soil was sifted prior to use and in doing so, was dehydrated. Thereby, any *Rhizobium* bacteria that would have been naturally present in the soil to cause the root nodule formation was present or active.

Table 30: Results of *Glycine max* bioassays grown in Green Planters with Kitchener Biosolids analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H-value	p-value	Impact of Biosolids
% Germination	%	100	0.0	80	0.4		1.000	0.317	NSD
Rate of Germination	days	5.5	1.0	7.5	2.3		1.980	0.159	NSD
Height Day 7	mm	92.21	17.0	63.41	53.4	1.167		0.287	NSD
Height Day 15	mm	167.32	9.6	148.97	24.5	1.400		0.204	NSD
Leaf Length Day 7	mm	20.36	1.2	18.13	1.9	2.007		0.101	NSD
Leaf Length Day 15	mm	37.14	1.8	32.22	10.0	0.096		0.371	NSD
# of Leaves Day 28	#	16.0	5.6	15.4	4.9		0.048	0.827	NSD
# Pods	#	4.8	0.16	4.8	1.8		0.045	0.831	NSD
# Seeds	#	8.0	1.7	7.2	2.3		0.562	0.454	NSD
Shoot Weight	g	2.2356	0.29	2.3485	0.44	0.478		0.645	NSD
Root Weight	g	0.6355	0.13	0.4630	0.17	1.819		0.106	NSD

When examining the results of *Phaseolus vulgaris* grown in Kitchener biosolids (Table 31) again there is little impact on the growth of these plants in the presence of this biosolid. The only impact was a significant increase in shoot biomass for those plants grown in the biosolids (t=3.191, p=0.015). This was expected since biosolids offer many nutrients that the plants can utilize for growth. When looking closer at the data in this case, the reference plants have more variability in height by Day 15 as seen by their standard deviation when compared to the treatment plants. And the treatment plants have more variability in leaf area by Day 15. Using a Leven's test, p=0.306 when compairing the height of the reference plants to the

treatment plants on Day 15, and p=0.064 for leaf area for the same conditions. Although neither result is significant, they are interesting to note. Again, root nodules were not observed on either the reference or treatment plants.

Table 31: Results of *Phaseolus vulgaris* bioassays grown in Green Planters with Kitchener Biosolids analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H-value	p-value	Impact of Biosolids
% Germination	%	100	0.0	100	0.0		0.000	1.000	NSD
Rate of Germination	days	5.0	0.0	5.2	0.4		1.000	0.317	NSD
Height Day 7	mm	90.89	10.1	80.53	12.8	1.149		0.194	NSD
Height Day 15	mm	94.03	21.7	99.96	12.4	0.531		0.510	NSD
Leaf Area Day 7	mm^2	3055.43	1274.2	3178.78	720.8	0.188		0.855	NSD
Leaf Area Day 15	mm^2	11363.38	886.1	13550.9	2551.9	1.811		0.108	NSD
# of Buds Day 28	#	3.2	0.8	3.0	2.4		0.935	0.334	NSD
# Pods	#	3.0	1.0	2.4	0.5		1.041	0.307	NSD
# Seeds	#	4.2	0.8	5.0	2.4		0.108	0.742	NSD
Shoot Weight	g	2.4997	0.31	5.4539	2.08	3.191		**0.015**	positive
Root Weight	g	0.6863	0.12	0.8120	0.25	1.003		0.349	NSD

Table 32 shows that *Brassica rapa* was negatively impacted by the presence of Kitchener biosolids in terms of the plant height at Day 15 (t=2.615, p= 0.035) as well as the number of buds on the same day (H=6.222, p=0.013). This was due to the variability in germination rate; taking as much as 10 days for the treatment (see Appendix IV for raw data) and only a maximum of 5 days for the reference. This observation is possibly due to the impact of the biosolids-soil matrix on the small seed size of the *Brassica* plant, making it difficult, but not impossible (as seen by there being no significant difference between the percent germination of the reference and treatment) for the seed to germinate and the hypocotyl to make its way up through the soil.

This difference did not translate into an impact in the number of seeds produced (H=0.011, p=0.917) or shoot biomass (t=1.047, p=0.325). Had government protocols been followed and

plants only examined for the first 14 or 21 days of their life cycle, this difference would not have been observed and the overall erroneous interpretation would have been that biosolids had a detrimental effect on these plants. By continuing the bioassays to include a full life-cycle of the plant, the true picture can be seen, namely biosolids do not have a negative impact on the overall growth. Therefore, there is no overall impact, and in the case of root biomass, there is a positive impact (t=3.691, p=0.006) was seen.

Table 32: Results of *Brassica rapa* bioassays grown in Green Planters with Kitchener Biosolids analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H-value	p-value	Impact of Biosolids
% Germination	%	85	0.4	75	0.4		0.429	0.513	NSD
Rate of Germination	days	3.4	1.5	5.4	3.1		2.378	0.123	NSD
Height Day 7	mm	37.72	6.0	31.39	4.9	1.824		0.105	NSD
Height Day 15	mm	72.38	5.1	54.99	12.4	2.615		**0.035**	negative
Leaf Area Day 7	mm^2	216.51	19.4	206.25	118.2	0.191		0.853	NSD
Leaf Area Day 15	mm^2	1361.29	339.4	1197.33	546.6	0.521		0.618	NSD
# of Buds Day 15	#	3.5	0.6	1.6	0.9		6.222	**0.013**	negative
# Pods	#	15.8	7.6	14.2	7.9		0.101	0.750	NSD
# Seeds	#	65.8	24.0	66.6	17.6		0.011	0.917	NSD
Shoot Weight	g	0.6389	0.08	0.7453	0.21	1.047		0.325	NSD
Root Weight	g	0.0379	0.01	0.0598	0.01	3.691		**0.006**	positive

3.2.2.2 Summary of Results Obtained for Bioassays with Crop Troughs using Kitchener Biosolids

Zea mays (corn)

- Percent germination
- Rate of germination
- Plant height (Day 7, 13, 39)
- Plant height to tassle at termination (Day 74)
- Tassel length (Day 74)
- Mean leaf lenght(Day 13, 39)
- Mean number of leaves (Day 7, 39)
- Mean number of leaves at termination (Day 74)
- Total number of ears
- Weight of ears
- Total number of seeds
- Weight of seeds
- Shoot weight

Glycine max (soya bean)

- Percent germination
- Rate of germination
- Plant height (Day 13, 39, 64)
- Mean leaf length (Day 13, 39
- Leaf area (Day 60)
- Mean number of leaves (Day 13, 39, 64)
- Total number of leaves (Day 64)
- Number of nodes (Day 32)
- Number of buds (Day 32)
- Total number of pods
- Weight of pods
- Total number of seeds
- Weight of seeds
- Shoot weight

Phaseolus vulgaris (common bean)

- Percent germination
- Plant height (Day 7, 22, 46)
- Leaf area (Day 7, 22, 39)
- Number of leaves (Day 7, 39)
- Height to first node (Day 36)
- Days to flower
- Total number of pods
- Weight of pods
- Total number of seeds
- Weight of seeds
- Shoot weight

Brassica rapa (field mustard)

- Percent germination
- Rate of germination
- Plant height (Day 3, 5, 42)
- Mean leaf area (Day 5, 42)
- Number of leaves (Day 5, 42)
- Day to flower
- Total number of pods
- Mean Weight of pods
- Total number of seeds
- Mean Weight of seeds
- Shoot weight

Once preliminary results from the green planter bioassays were obtained, the next set of bioassays to be undertaken were those using the crop troughs. Due to space limitations in the

laboratory, the initial crop trough bioassays using Kitchener bioassays for *Zea mays*, *Glycine max*, and *Phaseolus vulgaris* consisted of one crop trough of reference soil and one crop trough of treatment (biosolids amended) soil each, all containing five plants (Figure 59, left). Although this was not ideal in terms of replication, it did allow for different genera to be tested simultaneously. Due to their smaller overall size, the bioassays for *Brassica rapa* were carried out in much smaller rectangular vessels (34.2 cm x 20.9 cm x 11.8cm) (Figure 59, right), again one for reference and one for treatment soil but this time being planted with ten seeds.

Figure 59: Crop troughs used for plant bioassays, shown here on the left with *Zea mays* and the smaller vessels used for *Brassica rapa* (due to its much smaller size) as shown on the right.

When looking at the results in the following tables, percent germination refers to how many plants germinated while rate of germination refers to how long the plants took to germinate expressed as a mean value. The plant height is the mean of the five plants in a trough (or ten in the case of *Brassica*) and is measured from the soil surface to the shoot apical meristem. The number of leaves indicates how many leaves were present on the day the measurement was taken, again, expressed as a mean. This parameter becomes difficult to make use of as the plants aged, since the leaves dry and fall off more so when being handled while making

measurements. This is particularly true in the case of the corn plants with the bottom leaves. Leaf length and area are mean values and measured as long as possible. Once the plants started to senescence, only the living, green leaves were measured and used in evaluations. Even though five plants were measured in each of the reference or treatment crop troughs, this could be considered pseudo-replication since there was only one crop trough for each (i.e. n=1). Albeit in nature, crops plants are planted in close proximity thus being exposed to the same environment including soil, bacteria, and in this case biosolids. As a result, for these bioassays, stastical analysis was not performed, only qualantative analysis comparing the mean and standard deviation between the reference and treatment crop troughs for each of the genuses was performed.

Table 33 shows the summary of results for *Zea mays* grown in crop troughs. There was no impact of biosolids on this crop. Although the reference plants had the same number of leaves, they had more leaf length (and thus area) on Day 39 (but not significantly so), when compared to the treatment plants. This did not translate to more biomass at the termination of the plants life-cycle as seen by the difference in the shoot biomass. The low sample size with this bioassay was addressed in future bioassays. Statistical analysis could not be performed on weight of the ears, number of ears, number of seeds, nor the shoot biomass since, for each trough at the termination of the bioassay, all five plants were pooled together as one. By looking at the average values for these parameters, it could be suggested that the biosolids had a positive impact if any on this crop due to the almost double in biomass of ears and seeds which in turn relates to more seeds and thus more offspring being produced.

Table 33: Results of *Zea mays* bioassays grown in crop troughs with Kitchener Biosolids

Analysis	units	Reference mean	Reference S.D.	Biosolids mean	Biosollids S.D.
% Germination	%	100	0	100	0
Rate of Germination	days	5	0	5	0
Height Day 7	cm	25.50	9.7	18.43	4.5
Height Day 13	cm	54.98	7.2	64.23	8.6
Height Day 39	cm	88.68	34.8	80.92	21.7
leaf length Day 13	mm	106.00	11.7	110.70	17.9
leaf length Day 39	mm	303.74	52.3	295.05	22.8
# leaves Day 13	#	4	0	4	0
# leaves Day 39	#	8	0.7	8	0.7
# leaves Day 74	#	14.8	1.1	15.2	1.5
Ht to tassel Day 74	cm	75.48	21.4	70.32	27.4
Tassel length Day 74	cm	20.9	3.3	15.9	3.1
# ears	#	12	-	11	-
Wt of ears	g	14.5890	-	28.3606	-
# Seeds	#	34	-	54	-
Wt of seeds	g	8.3900	-	18.4777	-
Shoot Weight	g	57.8622	-	70.9511	-

The results of *Glycine max* summarized in Table 34 indicate that there could be a negative impact of the biosolids on plant height for the later time points. The reference plants at Day 39 and at termination were much taller. However the plant biomass was not significantly different as indicated by the shoot mass at termination or the total number of leaves on Day 64. Neither leaf area nor number of leaves were impacted. The treatment plants at termination, had larger and more leaves. However this is of biological importance since photosynthesis occurs in the leaves. The treatment plants, overall, were shorter and bushier and produced more buds, possibly due to the higher nutrients supplied in the biosolids, unlike the reference plants that were growing taller in search of nutrients elsewhere. Therefore this impact in height maybe should not be deemed a negative impact and it can be concluded that overall these plants were not detrimentally impacted by the biosolids.

Again statistical analysis could not be performed on the last few parameters since for each trough all five plants were pooled together at the termination of the bioassay. By looking at the averages, it could be suggested that again there was no impact of biosolids on these plants. Root nodules were not observed on either the reference or treatment plants in the crop troughs either. Again, this could be due to the fact that the soil was sifted prior to use, thus any *Rhizobium* bacteria that would naturally have been present in the soil to cause the root nodules were not able to survive these desiccated conditions brought about by the sifting of the soil.

Table 34: Results of *Glycine max* bioassays grown in crop troughs with Kitchener Biosolids

Analysis	units	Reference mean	Reference S.D.	Biosolids mean	Biosolids S.D.
% Germination	%	100	0	100	0
Rate of Germination	days	6	2.2	6.6	2.1
Height Day 13	mm	181.51	26.1	147.73	24.2
Height Day 39	mm	191.94	15.3	148.00	20.5
Height Day 64	mm	167.89	11.8	127.05	11.6
leaf area Day 60	mm^2	1923.09	223.2	1538.5	390.9
leaf length Day 13	mm	45.17	8.82	33.88	10.53
leaf length Day 39	mm	47.74	3.6	49.53	7.9
# leaves Day 13	#	2	0	2	0
# leaves Day 39	#	15.6	3.6	12.6	2.9
# leaves Day 64	#	10.6	2.7	15.2	5.17
total # leaves Day 64	#	53		76	
# nodes Day 32	#	3.8	0.5	3.0	0.7
# buds Day 32	#	60.0	0.6	80.0	0.5
# pods	#	23	-	23	-
Wt of pods	g	1.7185	-	1.877	-
# Seeds	#	43	-	41	-
Wt of seeds	g	5.8319	-	6.1062	-
Shoot Weight	g	3.8408	-	4.5978	-

For *Phaseolus vulgaris*, leaf area Day 7 was negatively impacted as seen in Table 35, but as the plants grew, all plants ended with similar leaf area and leaf number and thus potentially

simular photosynthetic capabilities. Had government protocols been followed, it would have been erroneously concluded that biosolids negatively impacted these plants since those bioassays look at initial plant biomass after a short duration and would have been terminated after 14 days.

The variability seen in leaf area is between all the leaves, at different growth stages, on the five plants in either the reference or treatment crop troughs. For example for Day 22, when looking at the raw data (see Appendix IV), there are actually 52 leaves in the reference trough and 37 in the treatment trough. This accounts for the variability between the reference and treatment, but again it was not significant.

Like corn and soya beans, statistical analysis could not be performed on the last few parameters since for each trough all five plants were averaged together. By looking at the averages it could be suggested that again there was no impact from biosolids seen on these plants. And also like soya beans, root nodules were not observed on either the reference or treatment *Phaseolus* plants in the crop troughs.

Table 35: Results of *Phaseolus vulgaris* bioassays grown in crop troughs with Kitchener Biosolids

Analysis	units	Reference mean	Reference S.D.	Biosolids mean	Biosolids S.D.
% Germination	%	100	0	100	0
Rate of germination	days	6	0	6.2	0.4
Height Day 7	cm	8.23	1.9	7.62	0.5
Height Day 22	cm	92.39	19.4	89.22	8.6
Height Day 46	cm	93.55	17.8	88.48	8.1
Leaf area Day 7	mm^2	3466.23	596	2028.33	583.3
Leaf area Day 22	mm^2	7411.88	1263.7	6777.95	259.8
Leaf area Day 39	mm^2	9756.83	328.9	10203.5	718.6
mean # leaves Day 7	#	2.0	0.0	2.0	0.0
mean # leaves Day 39	#	21.4	3.9	14.0	8.5
Height to first node Day 36	cm	69.98	15.3	66.30	6.4
Days to flower	day	34.4	3.5	32.8	4.4
# pods	#	18	-	20	-
Wt of pods	g	3.386	-	4.2529	-
# Seeds	#	41	-	45	-
Wt of seeds	g	12.4584	-	17.3555	-
Shoot Weight	g	13.4851	-	16.8345	-

Brassica rapa are a ruderal plant, which grow where natural vegetation has been disturbed. Like the crop plants with the larger seed size, *Brassica rapa*, with a small seed size, is also not impacted by the biosolids except in the case of plant height on Day 3 (Table 36). Once again, by plant termination, the height difference was no longer significant. If this bioassay was terminated after 7 days (as called for in government protocols) instead of taking it to the completion of the plant's life-cycle, it too would have been concluded that biosolids had a negative impact on *Brassica rapa* since the plant height was significantly shorter at Day 3 and only 50% of the seeds germinated. The lower germination, (as also seen with the green planter box biassays), could possibly be due to the small seed size and the biosolids-soil matrix, making it harder, but not impossible, for the hypocotyl to physically push its way through.

Again like the crop plants, the number and weight of the pods and seeds could not be statistically analysed since all remaining plants in each vessel were pooled together. But by looking at the averages, it could be suggested that the plants grown with the biosolids seemed to produce more and larger seeds from plants with more biomass.

Table 36: Results of *Brassica rapa* bioassays grown in small vessels with Kitchener Biosolids

Analysis	units	Reference mean	Reference S.D.	Biosolids mean	Biosolids S.D.
% Germination	%	70	0.05	50	0.5
Rate of germination	day	3.1	0.4	3.0	0.0
Height Day 3	cm	1.09	0.3	0.44	0.4
Height Day 5	cm	1.06	0.6	0.96	0.4
Height Day 42	cm	18.61	3.6	17.22	6.5
leaf area Day 5	mm^2	42.64	10.5	23.1	9.0
leaf area Day 42	mm^2	139.57	86.0	129.00	96.5
# leaves Day 5	#	2	0	2	0
# leaves Day 42	#	5.2	1.6	5.8	1.6
Day to flower	day	22.7	1.6	26.8	5.5
# pods	#	5	-	13	-
Wt of pods	g	0.0249	-	0.0659	-
# Seeds	#	10	-	19	-
Wt of seeds	g	0.0069	-	0.0223	-
Shoot Weight	g	0.7955	-	1.6117	-

3.2.2.3 Assessing Impact on Seeds from Parent Plant (F_0)

One of the primary functions of an organism is to produce offspring so that the genetic information can be passed along. Thus, another criterion that can be examined to determine if biosolids have an impact on these plants (the F_0 generation) is to look at the plant's ability to a) produce offspring (seeds) and b) the viability of those offspring (F_1), thus assessing multigenerational impact. The original plants (F_0) grown in either reference or treatment soil produced seeds (the F_0 generation). These seeds were planted in reference soil only, and another lifecycle of the plants produced (the F1 generation) were monitored.

3.2.2.4 Summary of Results Obtained for Bioassays from F_0 seed (parent plants) grown in Kitchener biosolids

Zea mays (corn)

- Percent germination
- Rate of germination
- Plant height (Day 14, 70)
- Stem width (Day 48)

Glycine max (soya bean)

- Percent germination
- Rate of germination
- Plant height (Day 14, 70)
- Days to flower
- Stem width (Day 48)
- Number of pods
- Weight of pods
- Number of seeds
- Weight of seeds

Phaseolus vulgaris (common bean)

- Percent germination
- Rate of germination
- Plant height (Day 14, 70)
- Days to flower
- Stem width (Day 48)
- Number of pods (Day 47)

Brassica rapa (field mustard)

- Percent germination
- Rate of germination
- Stem width (Day 48)
- Days to flower
- # pods
- Average weight of pods
- # seeds
- Average weight of seeds

In the case of the F_0 generation, all seeds produced from the parent plants of both the reference or treatment soils (using Kitchener biosolids) were planted in green planter boxes containing reference soil only. For each of the four plant genera, seeds were collected from all of parent plants from the crop trough bioassays. Each green planter box was planted with two of these randomly selected seeds, except in the case of the smaller plants of *Brassica rapa* where there were four seeds used. In the following tables, when it refers to reference or biosolids, it is referring to the source of the seed and where the parent plant (F_0) was grown.

No impact was seen in F_1 *Zea mays* plants, from seeds originally grown in treatment soil as can be seen when comparing their growth parameters to that of the reference in Table 37. *Zea mays* plants are pollinated by the wind and in the case of hybrid plants, if cross-pollinated with themselves they produce offspring that are smaller with fewer kernels produce. (Pers. Comm. Jeff Robinson, Woodrill Farms). In practice, farmers plant different varieties in close proximity to increase yield. Additionally hybrid plants should be infertile, (about 3% of the seeds are fertile) (Pers. Comm Michael Payne, OMAFRA meeting, Ryerson University March 2010). Thus in this case since there was germination of the F_0 seeds, the seeds must have been fertile.

Table 37: Results of *Zea mays* F_0 seed bioassays grown in green planters to produce F_1 plants analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H-value	p-value	Impact of Biosolids
% Germination	%	100	0	100	0		0.000	1.000	NSD
Rate of germination	day	5.4	0.4	5.3	0.8		0.057	0.811	NSD
Height Day 14	cm	3.0	0.5	3.2	0.6	0.577		0.580	NSD
Height Day 70	cm	15.3	1.4	17.2	1.9	1.805		0.109	NSD
Stem width Day 48	cm	4.01	0.4	4.33	0.5	1.212		0.206	NSD

Table 38 provide the results of the F_1 plants for *Glycine max.* Again, no impact was seen from biosolids on *Glycine max* plants for any of the parameters measured. Thus it can be concluded that the biosolids did not impact the parents' plants ability to produce viable offspring.

Table 38: Results of *Glycine max* F_0 seed bioassays grown in green planters to produce F_1 plants analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H-value	p-value	Impact of Biosolids
% Germination	%	100	0	100	0		0.000	1.000	NSD
Rate of germination	day	2.8	1	2.8	1		0.014	0.905	NSD
Height Day 14	cm	7.8	1.1	8.5	0.6	0.998		0.347	NSD
Height Day 70	cm	15.8	1.3	17.1	0.9	1.789		0.111	NSD
Days-to-flower	day	30.5	0.8	29.8	0.6		2.157	0.142	NSD
Stem width Day 48	cm	2.53	0.2	2.58	0.4	0.557		0.593	NSD
# pods	#	2.1	0.4	1.8	0.3		2.250	0.134	NSD
wt pods	g	0.3946	0.50	0.1810	0.01	0.954		0.368	NSD
# seeds	#	3.1	0.7	3.0	0.0		0.417	0.519	NSD
Wt seeds	g	0.5222	0.06	0.5141	0.04	0.250		0.809	NSD

Table 39 provide the results for the *Phaseolus vulgaris* F_0 seeds and again there is no negative impact from biosolids on the offspring of these parent plants and very little variability among the seeds as seen by the small standard deviations of the different parameters measured. The only impact seen is the broader stems produced by seeds from parents grown in the biosolids (t=3.771, p=0.005) and thus possibly a stronger plant.

Table 39: Results of *Phaseolus vulgaris* F_0 seed bioassays grown in green planters to produce F_1 plants analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H-value	p-value	Impact of Biosolids
% Germination	%	90.0	0.2	100.0	0.0		1.000	0.317	NSD
Rate of germination	day	4.4	0.4	4.3	0.3		0.000	1.000	NSD
Height Day 14	cm	6.1	0.6	6.3	0.3	0.818		0.437	NSD
Height Day 70	cm	16.5	2.2	17.2	0.7	0.715		0.495	NSD
Days to flower	day	33.0	0.8	31.7	1.2		3.032	0.082	NSD
Stem width Day 48	cm	3.8	0.1	4.1	0.1	3.771		**0.005**	positive
# pods	#	1.4	0.4	1.3	0.3		0.000	1.000	NSD

In Table 40, the only impact that can be seen of the seeds from parents grown in biosolids is the day-to-flowering. For these seeds, reference plants flowered sooner (H=5.463, p=0.019). When looking at the protocol for *Brassica rapa* from the University of Wisconsin (where they have been extensively studied) (Williams 1990), the reference plants are more in keeping with where the plant should be in its life cycle at that point. By Day 25, as is the case for the biosolids plants, these plants should have developed seed pods. Looking at the raw data (See Appendix IV) and the similar standard deviations, this later date held for all biosolids plants; it wasn't a case of a few outliers. The same number of plants germinated over a similar time frame, so a reason for the difference in flowering times is hard to establish.

Table 40: Results of *Brassica rapa* F_0 seed bioassays grown in green planters to produce F_1 plants analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H-value	p-value	Impact of Biosolids
% Germination	%	80	0.3	80	0.3		0.000	1.000	NSD
Rate of germination	day	3.5	2.8	3.3	2.0		0.591	0.442	NSD
Stem width Day 48	mm	2.11	0.4	2.42	0.2	1.155		0.300	NSD
Days to flower	day	15.4	3.32	25.3	0.96		5.463	**0.019**	negative
# pods	#	8.38	2.5	7.13	3.5		1.729	0.189	NSD
Wt of pods	g	0.1712	0.21	0.0728	0.04	0.886		0.410	NSD
# Seeds	#	22.88	11.7	17.75	9.0		1.333	0.248	NSD
Wt of seeds	g	0.1548	0.24	0.0382	0.01	0.982		0.364	NSD

3.3 Summary of Protocol Development with Kitchener Biosolids

After running the avoidance bioassays with the springtails, the 100-minute bioassay was no longer used after this initial trial with Kitchener biosolids because it was felt that the longer duration avoidance bioassays in the Mason jars were a better representation of a normal exposure of springtails to biosolids. Although the 100-minute bioassay was useful as a quick indicator of avoidance, and gave the same results as the longer duration bioassays (no negative impact), the 3-, 7-, 14-day would be more environmentally relevant due to the longer exposure and were used for future research (i.e. for studies of Guelph biosolids)

without the inclusion of the 100-minute bioassay. If these protocols were to be implemented into routine analysis at a WWTP, the 100-minute bioassay may be too labour intensive to set up and monitor, therefore the longer duration bioassays may be more practical since they need less attention.

When comparing the results of the plants grown in the green planter boxes with those of grown in the crop troughs, the percent germination and rate of germination were similar for both setups thus indicating that the construction material of the crop troughs did not hinder the bioassays and could thus be used for further research. Plant biomass could not be directly compared between the two methods (green planters and crop troughs) because due to the small size of the green planter boxes, they were not expected to produce large crop plants without restricting the growth of these plants. They did however, provide a guideline of expectations for the crop troughs (for example: dates to flowering, pod development, pods and plant biomass, etc.).

3.4 Guelph Biosolids

The biosolids from Guelph WWTP were the second type of biosolids tested. These biosolids were processed by anaerobic digestion and dewatered by mechanical means followed by further processing using the Lystek method described in the Introduction. After the initial bioassays were run using the Kitchener biosolids, the methods were further fine-tuned where needed and re-run using Guelph biosolids. Again, these bioassays were carried out using a worst-case scenario application rate of 22 tonnes/ha of biosolids on a dry weight basis (unless otherwise specified). This amount was used at the suggestion of OMAFRA and MOE, the funding bodies for this research, as this represents the total amount of biosolids that can be applied over a five-year period

3.4.1 Animal Bioassays

For these bioassays, two different genera were used. They include *Folsomia candida* (springtails) and *Lumbricus terrestris* (earthworms) which will each be discussed in turn.

3.4.1.1 Summary of Results Obtained for Avoidance Bioassays with Folsomia candida using Guelph biosolids

***Folsomia candida* (springtails)**

• Avoidance Behaviour:
  • 3-day
  • 7-day
  • 14-day (8 and 22 tonnes/ha)

Avoidance bioassays were performed in 125ml Mason jars with 10-day old age-synchronized springtails. Each of the five jars containing fifteen springtails were divided in half; one side contained reference soil and the other side contained treatment soil. Trials #1, #2, and #3 for the avoidance bioassays were from the first batch of Guelph biosolids with bioassays carried out consecutively, while Trials #4 and #5 were from a second batch obtained a year later. For chemical and biological analysis if the first batch of Guelph biosolids, see Appendix III.

As previously explained with the Kitchener biosolids, there was a significance difference found in the reproduction of springtails at 22 tonnes/ha therefore, this time the method was adjusted to include bioassays with an application rate of 8 tonnes/ha biosolids added on a dry weight basis

Looking at Table 41, it can be seen that there was a significant difference in favour of biosolids for the 3-day avoidance bioassay in Trial #1 ($H=5.600$, $p=0.018$), while in Trial #2, there is an avoidance of the biosolids ($H=5.333$, $p=0.021$). Conversely, the third trial shows no significant difference at all at this time point ($H=0.702$, $p=0.402$). As time goes on, as can be seen by the 14-day avoidance bioassays results for Trial # 1, 2, and, 3, this difference no longer exists. Additionally, there is no significant difference between the reference and treatment vessels at Day-14 at either of application rates, or, for either batch of biosolids

(Trials #3, 4, and 5). As mentioned previously, this could be due to the activated charcoal absorbing volatile gases such as ammonia or their being released during the counting of springtails.

Table 41: Results of *Folsomia candida* Avoidance Biosolids using Guelph Biosolids *(22 tonnes/ha unless otherwise indicated)* analysed using the non-parametric Kruskal-Wallis test (NSD = No Significant Difference)

Analysis	Ref Mean (%)	Ref S.D.	BS Mean (%)	BS S.D.	H- value	p-value	Impact of Biosolids
3 day avoid trial #1	30	12	70	12	5.600	**0.018**	positive
3 day avoid trial #2	67	12	33	12	5.333	**0.021**	negative
3 day avoid trial #3	55	13	46	13	0.702	0.402	NSD
7 day avoid trial #1	30	26	70	26	3.073	0.080	NSD
7 day avoid trial #2	75	10	25	10	5.333	**0.021**	negative
7 day avoid trial #3	51	11	49	11	0.044	0.833	NSD
14 day avoid trial #1	50	38	50	38	0.000	1.000	NSD
14 day avoid trial #2	58	20	42	20	0.527	0.468	NSD
14 day avoid trial #3	51	17	49	17	0.011	0.916	NSD
14 day avoid trial #4	62	20	38	20	1.866	0.172	NSD
14 day avoid trial #5 **8 tonnes/ha**	38	22	62	22	1.336	0.249	NSD

3.4.1.2 Summary of Results Obtained for Reproduction Bioassays with Folsomia candida using Guelph biosolids

***Folsomia candida* (springtails)**

- Reproduction (8 and 22 tonnes/ha)

Reproduction bioassays were carried out in 125ml Mason jars with 10 day old aged synchronized springtails. There were five reference jars and five treatment jars each with ten

springtails. At the end of the 39-day bioassay, the number of organisms present was counted without distinguishing age. Trials #1, #2, for the reproduction bioassays were from the first batch of biosolids while Trials #3 and #4 were from a later batch.

As can been seen in Table 42, there is a significant difference with reproduction of springtails at an application rate of 22 tonnes/ha (t=2.589, p=0.036, t=4.360, p=0.005, t=10.531, p=0.000, Trial # 1, 2, and 3 respectively). There was not a significance difference at the more environmentally-relevant land-application rate of 8 tonnes/ha (Trial #4). After discussing the characteristics of this biosolids with experts from OMAFRA, it was discovered that these biosolids (from a pilot Lystek facility) should not have been as malodorous as the batch we obtained (Pers. Comm. Michael Payne, OMAFRA). Thus possibly the facility was not functioning properly at the time the sample was obtained. This improperly processed biosolids could have impacted the result, both in the previous avoidance bioassays and in these reproduction bioassays. Once the Lystek process is functioning properly, future studies should be carried out using those biosolids to determine if indeed an impact is seen. It is worth noting that the biosolids obtained for this study would not have been land-applied due to their odour but would have been instead sent to a landfill site or incinerated.

Table 42: Results of *Folsomia candida* Reproduction Bioassays using Guelph Biosolids *(22 tonnes/ha unless otherwise indicated)* analysed using a t-test (NSD = No Significant Difference)

Analysis	Ref # present	Ref S.D.	BS # present	BS S.D.	t-value	p-value	Impact of Biosolids
reproduction trial #1	1776	1046	365	288	2.589	**0.036**	negative
reproduction trial #2	2105	309	1185	287	4.360	**0.005**	negative
reproduction trial #3	859	173	42	16	10.531	**0.000**	negative
reproduction trial #4 **8 tonnes/ha**	836	156	834	541	0.008	0.994	NSD

3.4.1.3 Summary of Results Obtained for Avoidance Bioassays with Lumbricus terrestris using Guelph biosolids

***Lumbricus terrestris* (earthworms)**
• Avoidance Behaviiour   • 3-day

Avoidance bioassays for *Lumbricus terrestris* were performed using either Method #1 (18.9L buckets following Environment Canada's Kaushik chambers) or Method #2 (8L rectangle vessels) using adult earthworms. In Method #1, alternating segments of the avoidance chamber were filled with either reference soil or treatment soil containing 22 tonnes/ha of biosolids and the earthworms were placed in the center chamber. Eight earthworms were used in each. These bioassays were not successful since the design of the avoidance chamber by Environment Canada, even though they do specify that *Lumbricus* is to be used, was not conducive to the size and burrowing behaviour of these worms. Therefore, this method was discarded. After further experimentation and improvements, Method #2 (the rectangular vessels) was established and was used as the avoidance chamber in all further avoidance behaviour research with earthworms. In Method #2, each of the five vessels was divided in half; one side contained reference soil and the other side contained treatment soil (again at 22 tonnes/ha). One organism was placed along the center line of each and results recorded after 3 days of exposure.

For the avoidance bioassays, the results are represented as the number of earthworms found in either the reference or treatment half of the vessel and a Chi Square test was used to analyze the categorical results. In Table 43 the results of this test show that the earthworms did avoid the Guelph biosolids (X^2=5.000, p=0.025) in the first trial, but did not in the second trial since there was no significant difference in this instance (p=0.179). Although the statistics would indicate in Trial #2 that there was not a significant difference between the reference and treatment in terms of the avoidance behaviour of the earthworms, X^2 is only a

tool, and the observations of the results (4 in the reference verses 1 in the biosolids) would indicate otherwise. This could be due to the small sample size (n), of 5 in this instance. The two trials could be pooled to increase the sample size to 10 since both trials were carried out using the same vessels with the same soil and biosolids, but noting that different earthworms were used in each case and that there was a time lag of several days between the two trials. In this pooled analysis a negative impact is seen (p=0.011) again suggesting, like the springtail avoidance bioassays, that there was something wrong with this malodourous batch of improperly manufacturend Lystek biosolids.

The methodology was modified for future use to increase the sample size to 10 instead. A power analysis could have been performed to determine the appropriate sample size, but ten was chosen because that was the physical number that the laboratory space could accommodate at the time. Additionally, when the earthworms avoidance bioassay was re-run (using Kitchener biosolids as discussed earlier), an environmentally-relevant application rate of 8 tonnes/ha was used instead of the worst case scenario rate of 22 tonnes/ha, since these initial results indicated avoidance, although at the time, it was not known about the 'bad batch' of Lystek biosolids. It is suggested that this bioassay be re-run at the 22 tonnes/ha application rate when the pilot Lystek plant is running correctly with the increased sample size to check these results.

Table 43: Results of *Lumbricus terrestris* Avoidance Bioassays (Method #2) using Guelph Biosolids analysed using Chi-Square Test (NSD = No Significant Difference)

Analysis	Unit	Ref Mean	BS Mean	X^2 value	p-value	Impact of Biosolids
Trial #1	#	5	0	5.000	**0.025**	negative
Trial #2	#	4	1	1.800	0.179	NSD
Pooled	#	9	1	6.400	0.011	negative

3.4.1.4 Summary of Results Obtained for Long-term Bioassays with Lumbricus terrestris using Guelph biosolids

***Lumbricus terrestris* (earthworms)**

- **Long-Term Bioassays**
  - **Buckets:**
    - Total number alive (Day 7, 28, 90)
    - Intitial weight (total, average)
    - Combined weight (Day 28, 90)
    - Average weight (Day 28, 90)
  - **Evan's boxes**:
    - Total number alive (Day 7, 28, 90)
    - Initial weight (total, average, per box)
    - Combined weight (Day 28)
    - Average weight (Day 28)
    - Combined weight Day 90)
    - Average weight (Day 90)

Reproduction or long-term bioassays for *Lumbricus terrestris* did not exist in government protocols and therefore needed to be developed. They were first carried out in 18.9 L buckets (Method #1) with ten earthworms in each of the buckets. The subsequent bioassays, using Method #2 (the Evans boxes), five earthworms were used instead to prevent overcrowding. In both cases, there were five reference buckets or Evans boxes and five treatment containers which contained 22 tonnes/ha of biosolids layered over the reference soil. At the end of the bioassay, the number of live organisms present was determined and, depending on the particular bioassay, the weight was also found for those earthworms still present.

In these results shown in Table 44, the figures represent the total number of organisms found per vessel. In the case of the earthworms when determining weight, a combined weight per vessel was used since it would be impossible to distinguish individual earthworms after they have been freely moving about in the vessel. Average weights were then also determined to

compare against initial weights to see, if overall, the worms were maintaining a constant weight. Additionally, in Table 44, Total Weight means the total biomass in the all five of the reference and biosolids vessels respectively. Combined Weight indicates the combined biomass of the earthworms in *each* of the vessels. Mean Weight refers to the mean weight per earthworm per vessel and is thus calculated by dividing the number of live earthworms present by the combined weight per vessel. When comparing the initial and subsequent weights of the earthworms throughout the duration of the bioassay using a paired t-test (Table 44), there was no significant difference in weight change between the reference and treatment earthworms when comparing all three time points ($t_{(2)}$=2.210, p=0.158) or just the final time point ($t_{(2)}$=5.000, p=0.126).

Although there was no significant difference between reference and treatment in terms of the number of earthworms still alive at 90 days (H=0.653, p=0.419) or their combined weight (t=1.499, p=0.231) (Table 44) or any of the other parameters measured thus far (except mean weight of the earthworms after 90 days (t=7.566, p= 0.005), the *actual* number that is still alive is concerning; 5 or 2 respectively out of an initial 50 in each. Thus, it was concluded that there was a design flaw in these chambers. It was felt to be a compaction issue of the soil at the bottom of the buckets due in part to the soil settling when watering. When the opaque buckets were dismantled to make observations this compaction was noticed. Biosolids are known to hold moisture (Pers. Comm. Michael Payne, OMAFRA) so this additional weight too could have added to the impact seen. This was a contributing factor that led to this method being discarded, and the change to the Evans Boxes (Method #2) for the long term bioassays. The other was the lack of the non-invasive ability to observe the earthworms.

Table 44: Results of *Lumbricus terrestris* Long-term bioassays (Method #1) using Guelph Biosolids analysed using either a t-test or the Kruskal-Wallis Test where appropriate (NSD = No Significant Difference)

Analysis	Unit	Ref Mean	Ref S.D.	BS Mean	BS S.D.	t-value	H- value	p-value	Impact of Biosolids
Initial wt (total)	g	186.95	2.31	179.74	0.87	1.308		0.227	NSD
Initial wt/bucket	g	37.39	2.31	35.95	0.87	1.308		0.227	NSD
Int. mean. wt/worm	g	3.74	0.23	3.60	0.09	1.297		0.231	NSD
# alive Day 7	#	46.0	1.1	42.0	1.5		1.041	0.307	NSD
# alive Day 28	#	36.0	3.0	31.0	3.3		0.158	0.667	NSD
Total wt Day 28	g	98.14	11.43	79.06	9.99	0.562		0.589	NSD
Combined wt Day 28	g	19.63	11.4	15.81	10.0	0.562		0.589	NSD
Mean. wt/worm Day 28	g	2.61	0.75	2.45	0.4	0.422		0.684	NSD
# alive Day 90	#	5.0	0.8	2.0	0.5		0.653	0.419	NSD
Total wt Day 90	g	10.25	1.39	3.72	0.06	1.499		0.231	NSD
Combined wt Day 90	g	3.42	1.4	1.86	0.1	1.499		0.231	NSD
Mean. wt/worm Day 90	g	2.58	0.12	1.86	0.06	7.566		**0.005**	negative
Paired t-test of weights (all 3 time points)						2.210		0.158	NSD
Paired t-test of weights (1st and 3rd time points)						5.000		0.126	NSD

Table 45 provides a summary of the data for the long-term bioassays of the earthworms in the Evans boxes (Method #2). This setup consisted of five reference vessels and five treatment vessels, each with five earthworms. When looking at acute (7-Day) and chronic (28-Day) bioassays, there was no impact seen of the biosolids on *Lumbricus terrestris* at an application rate of 22 tonnes/ha on a dry weight basis; nor was impact seen with the longer 90-Day duration bioassays. Here the mean weight per earthworm increased (4.62g from 4.07g for reference and 5.10g from 4.26g for treatment), a biologically significant result. Thus the Evans boxes with their better drainage, transparent sides for non-invasive viewing and much larger depth were used in all future *Lumbricus terrestris* long-term bioassays.

Table 45: Results of *Lumbricus terrestris* Long-term bioassays (Method #2) using Guelph Biosolids analysed using either a t-test or the Kruskal-Wallis Test where appropriate (NSD = No Significant Difference)

Analysis	Unit	Ref Mean	Ref S.D.	BS Mean	BS S.D.	t-value	H- value	p-value	Impact of Biosolids
Initial wt (total)	g	101.82	0.92	106.57	1.46	1.230		0.254	NSD
Initial wt/ box	g	20.36	0.92	21.31	1.46	1.230		0.254	NSD
Mean wt/worm	g	4.07	0.18	4.26	0.29	1.241		0.250	NSD
# alive Day 7	#	24.0	0.4	24.0	0.4		0.000	1.000	NSD
# alive Day 28	#	23.0	0.5	24.0	0.4		0.429	0.513	NSD
# alive Day 90	#	20.0	1.0	17.0	1.3		0.569	0.451	NSD
Total wt Day 90	g	92.99	5.54	87.23	7.33	0.280		0.786	NSD
Wt (combined) Day 90	g	18.6	5.5	17.45	7.3	0.280		0.786	NSD
Mean wt/worm Day 90	g	4.62	0.67	5.10	0.32	1.444		0.187	NSD

3.4.2 Plant Bioassays

For these bioassays, four different plant genera were used; *Zea mays* (corn), *Glycine max* (soya bean), *Phaseolus vulgaris* (common bean), and *Brassica rapa* (field mustard).

3.4.2.1 Summary of Results Obtained for Bioassays of Parent (F_0) Generation plants grown in Crop Troughs using Guelph biosolids

Zea mays (corn)

- Percent germination
- Rate of germination
- Plant height (Day 20, 82)
- Leaf area (Day 20, 82)
- Number of leaves (Day 7, 22, 82, 132)
- Number of ears (Day 117)
- Number of ears
- Weight of cobs (ears and kernals)
- Shoot mass
- Plant height over time

Glycine max (soya bean)

- Percent germination
- Rate of germination
- Days-to-flowering
- Days to senescencing
- Plant height (Day 14, 65)
- Leaf length (Day 14, 69)
- Number of leaves (Day 14, 69)
- Stem width (Day 79)
- Number of pods (Day 69)
- Weight of pods (Day 120)
- Number of seeds (Day 120)
- Weight of seeds (Day 120)
- Plant height over time

Phaseolus vulgaris (common bean)

- Percent germination
- Rate of germination
- Days-to-flowering
- Plant height (Day 25, 73)
- Leaf length combined (Day 25, 60, 73)
- Number of pods
- Weight of pods
- Number of seeds
- Weight of seeds
- Shoot mass
- Root length at termination (Day 80)
- Plant height over time

Brassica rapa (field mustard)

- Percent germination
- Rate of germination
- Plant height (Day 11, 28, 47)
- Days-to-flowering
- Days-to-pod development
- Number of pods (total, average)
- Weight of pods (total, average)
- Number of seeds (total, average)
- Weight of seeds (total, average)
- Shoot mass
- Root mass
- Root length
- Plant height over time

By the time the bioassays for *Zea mays, Glycine max, Phaseolus vulgaris* and *Brassica rapa* were repeated using Guelph biosolids, more physical laboratory space was available. Therefore, for this set of bioassays, a larger sample size (n) of three could be used. Consequently, each genus consisted of three troughs containing reference soil and three troughs of treatment soil, each containing five plants. The bioassays for *Brassica rapa* were carried out in the smaller vessels, again with three vessels for reference soil and three for treatment soil but this time being planted with ten seeds due to the small plant size.

The mean for all parameters of the five plants in each trough was determined then these three values were averaged together to determine an overall (grand) mean for each parameter measured. When looking at the results, percent germination refers to how many plants germinated while rate of germination refers to how long the plants took to germinate expressed in days. The plant height is measured from the soil surface to the shoot apical meristem and is cumulative over time. The value on a specific day is the total height, not the difference (or new growth) since previous measurement. The number of leaves indicates how many leaves are present on the day indicated. This parameter becomes difficult to use because as the plants senescence (age), the leaves dry and fall off (in the case of the corn plants, just the bottom leaves) especially when being handled while making measurements. Leaf length and area are average values and measured for as long as possible. Once the plants start to senescence, only the living, green leaves were measured. As mentioned previously, leaf area was determined by measuring the widest part of the leaf and multiplying by the longest part of the leaf. Although this does not give an exact area since leaves are not square, the same method was used consistently so any error would be consistent and thus immaterial.

With these cobs, the seeds were difficult to count so instead they were weighed with the cob. Therefore, when looking at pod biomass for the corn plants, biomass included the husk and cob. As seen in Table 46, there is no impact from the Guelph biosolids on *Zea mays* except for the number of leaves on Day 22 where there was a negative impact ($H=4.355$, $p=0.037$). On Day 82 the reverse is the case with the biosolids plants having more leaves ($H=3.857$,

p=0.050). Since at the end of the plant's lifecycle there was no significant difference in leaf number (H=1.00, p=0.317) nor was there a difference in the leaf area on Day 20 (when there was a difference in number) (t=1.194, p=0.298) or the seeds produced, it can be concluded that overall there was no impact on *Zea mays* from the biosolids.

Table 46: Results of *Zea mays* bioassays grown in Crop Troughs with Guelph Biosolids analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H- value	p-value	Impact of Biosolids
% Germination	%	100	0	100	0		0.000	1.000	NSD
Rate of germination	day	6	0	6	0		0.000	1.000	NSD
Height Day 20	cm	7.1	0.1	7.6	0.6	1.263		0.275	NSD
Height Day 82	cm	51.8	8.9	56.8	12.3	0.570		0.599	NSD
leaf area Day 20	mm^2	16.6	2.2	14.6	1.9	1.194		0.298	NSD
leaf area Day 82	mm^2	240.4	16.9	281.3	21.3	2.597		0.060	NSD
# leaves Day 7	#	5.0	0.0	4.7	0.6		1.000	0.317	NSD
# leaves Day 22	#	20	0	18	1		4.355	**0.037**	negative
# leaves Day 82	#	48	1	53	3		3.857	**0.050**	positive
# leaves Day 132	#	4.6	0.5	4.3	1		1.000	0.317	NSD
# cobs	#	2.9	0.3	3.2	0.5		0.484	0.487	NSD
Wt of cob	g	2.3043	3.0868	0.4684	0.0163	1.030		0.361	NSD
Shoot Weight	g	16.59	5.465	23.52	2.56	1.989		0.118	NSD

Figure 60 is the graph for plant height of *Zea mays* from germination until it was terminated on Day 82 indicating there is very little difference between the reference and the treatment plants through the entire lifecycle. This can also be seen by the plant heights on various days in Table 46. Therefore, it can be seen that the plants did not grow at different rates over time and it can be concluded using a time x treatment effect from a rmANOVA analysis on log-tranformed data that there was no overall impact of biosolids on the cumulative growth of corn.

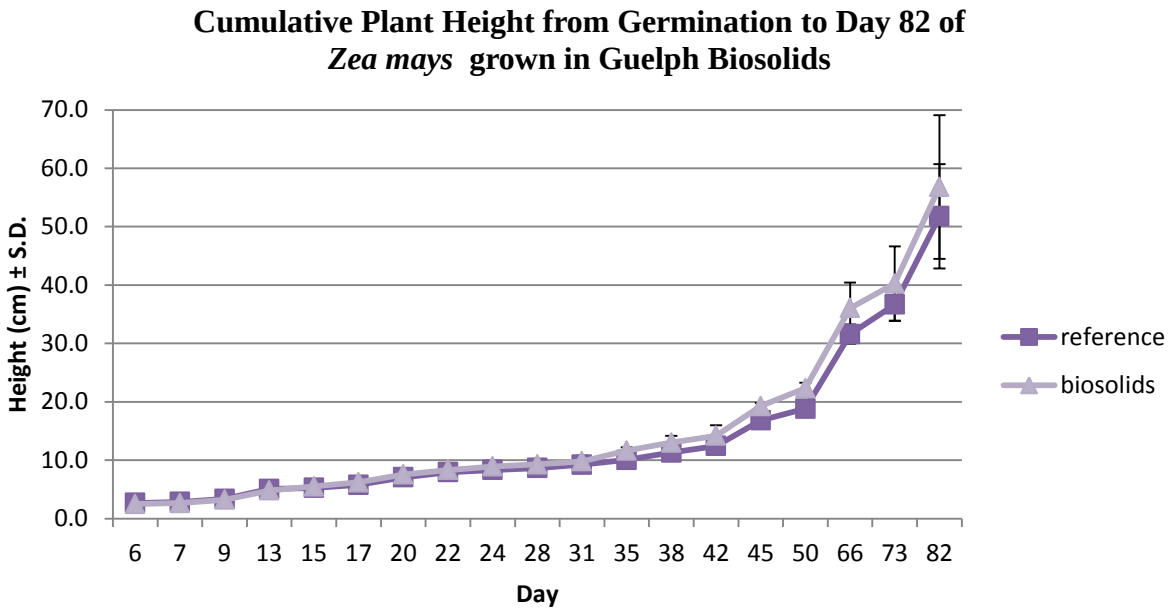

Figure 60: Cumulative Height of *Zea mays* grown in Guelph Biosolids

Table 47 provides the results for *Glycine max*. As can be seen, there is a significant difference in leaf length on Day 14 and on Day 69 in favour of the reference soil (t=2.989, p=0.041 and t=4.319, p=0.012 respectively). When looking at leaf area on Day 20 (t=9.222, p=0.005) there is again a significant difference but this time in favour of the biosolids plants. Similar observations were made for the plants on Day 33 (t=3.537, p=0.026). Again, as seen with the Kitchener biosolids, *Glycine max* produced shorter, bushier plants when grown in the biosolids possibly due to the extra nutrients provided by the biosolids. Thus overall, these plants had more surface area available for photosynthesis. When the plants were mature and became too dry and brittle to continue to measure, indicated by turning yellow, was also observed. For *Glycine max*, this was significantly different between the treatments (H=4.500, p=0.034). The plants grown in the biosolids amended soil matured sooner. When looking at the Days to flower and seed production, there was no impact. Therefore, overall the Guelph biosolids did not have a negative impact on soya beans.

Although there was not a significant difference in plant height on Day 14, there is a large variation in the reference plants. When looking at the raw data (see Appendix IV) there are two plants (in different troughs and in different positions in the troughs) that are much shorter than all the others. Howerver by Day 65, these same two individual are the same height or taller than other plants in the same troughs. It was not a factor of germination, and therefore maybe a genetic anomaly with the seed, a localized nutrient deficiency in the soil, or some other unconsidered factor.

Root nodules were not observed on either the reference or treatment *Glycine max* plants in the crop troughs, although they are a legume. Again, this could be due to the fact that the soil was sifted prior to use and any *Rhizobium* bacteria that would have been naturally present in the soil to cause the root nodules was no longer present due to the moisture loss caused by the sifting process.

Table 47: Results of *Glycine max* bioassays grown in Crop Troughs with Guelph Biosolids analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H-value	p-value	Impact of Biosolids
% Germination	%	100	0	93.3	11.6		1.000	0.317	NSD
Rate of germination	day	10.4	1.04	11.9	1.03		1.225	0.268	NSD
Height Day 14	mm	146.95	29.1	124.74	4.61	1.305		0.262	NSD
Height Day 65	mm	272.03	15.3	242.22	25	1.760		0.153	NSD
Leaf length Day 14	mm	27.00	3.26	20.57	1.86	2.968		**0.041**	negative
Leaf length Day 69	mm	31.12	1.76	22.17	3.13	4.319		**0.012**	negative
Leaf Area Day 20	mm^2	112.1	68.6	1151.9	182.8	9.222		**0.005**	positive
Leaf Area Day 33	mm^2	412.91	93.9	661.8	77.6	3.537		**0.026**	positive
# leaves Day 14	#	3.7	1.15	4.3	0.58		0.833	0.361	NSD
# leaves Day 69	#	64.7	31.3	95.3	36.4		1.190	0.275	NSD
Stem Width Day 79	mm	2.08	0.24	2.02	0.23	0.328		0.759	NSD
Days to flower	day	40.9	0.83	42.1	2.48		0.048	0.827	NSD
Days to sensenses	day	69	0	67.8	0.19		4.500	**0.034**	positive
# pods	g	3.4	1.22	3.4	1.07		0.048	0.827	NSD
Wt pods	g	0.3015	0.07	0.3242	0.12	0.280		0.793	NSD
# seeds	#	5.8	1.73	4.85	1.88		0.049	0.825	NSD
Wt seeds	g	0.9377	0.25	0.8414	0.45	0.328		0.759	NSD

There was no impact on *Phaseolus vulgaris* from Guelph biosolids for the parameters measured (Table 48). When examining the biomass parameters at termination, the seeds were slightly bigger, but not significantly so, as seen by there being fewer seeds, but with a larger weight. The plants grown in biosolids amended soil were slightly shorter than the reference plants, but overall this did not impact the plant's ability to produce offspring (i.e. seeds). Root nodules were not observed in this bioassay either.

Table 48: Results of *Phaseolus vulgaris* bioassays grown in Crop Troughs with Guelph Biosolids analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H-value	p-value	Impact of Biosolids
% Germination	%	100	0	86.7	11.6		2.500	0.114	NSD
Rate of germination	day	6.2	1.06	7.4	0.69		2.634	0.105	NSD
Height Day 21	cm	15	1.9	13	2.8	1.003		0.372	NSD
Height Day 73	cm	25	0.3	21.4	4.3	1.463		0.217	NSD
Leaf length Day 25	mm	253.54	10.4	187.87	46.7	2.376		0.076	NSD
Leaf length Day 60	mm	503.69	37.2	456.4	123.3	0.636		0.559	NSD
Days to flower	day	30.2	0.35	32.3	1.84		2.402	0.121	NSD
Stem Width	mm	3.42	0.5	3.2	0.32	0.655		0.548	NSD
# pods	g	2.4	0.2	2.4	1.3		0.048	0.827	NSD
Wt pods	g	0.9031	0.024	0.7241	0.43	0.726		0.508	NSD
# seeds	#	7.7	0.95	6.1	2.63		0.196	0.658	NSD
Wt seeds	g	2.3885	0.29	2.5813	1.51	0.217		0.839	NSD
Shoot weight	g	5.439	1.3	5.4464	3.2	0.005		0.996	NSD
Root length	cm	8.5	1.6	10.4	3.9	0.769		0.485	NSD

The results in Table 49 show that initially there was a negative impact of Guelph biosolids on *Brassica rapa*. These plants took longer to germinate (H=4.091, p=0.043) as well as fewer germinated (H=4.091, p=0.043). This could be a factor of seed size and the compaction issue of the biosolids, making it harder for the hypocotyl to get through the soil and into the light. Observation of negative impact continued to Day 7, as indicated by the stunted growth of the biosolids plants (t=5.588, p=0.005). However, once the plants emerged and started growing, the additional nutrients provided in the biosolids aided these *Brassica rapa* plants. When the plants were terminated, they were taller (t=2.329, p=0.080) and produced significantly bigger pods and more seeds (t=2.688, p=0.055 and H=3.857, p=0.050 respectively). Also observed was more plant biomass (t=3.963, p=0.017 and t=5.545, p=0.005). Thus again, overall the biosolids had a positive impact on *Brassica rapa*. Had government protocols been followed (which examine germination and emergence) the erroneous conclusion would have been made that biosolids had a detrimental impact on these plants. In actual fact, when the

complete life-cycle of the plant was observed, the opposite was the case: the plants grown in the biosolids-amended soil were significantly larger and produced more offspring. When looking at the results in the Table 49, for example with seed number, average is the number of seeds per vessel, where total seed number is how many seeds were produced on all the plant, in all three vessels combined, for either the reference or treatment plants. Grand total was used for stastical analysis and used the individual number of seeds per plant in the calculation instead of the mean number of seeds (as used with the calculation for 'total' above). While this grand total number is biologically significant (it is almost double for the number of seeds present), it is not statistically significant (p=0.282) since it is the vessel that is the replicate, and not the individual plants within the vessel.

Table 49: Results of *Brassica rapa* bioassays grown in rectangle vessels with Guelph Biosolids analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H- value	p-value	Impact of Biosolids
% Germination	%	93	12	53	6		4.091	**0.043**	negative
Rate of germination	day	3.1	0.1	4.5	0.9		4.091	**0.043**	negative
Height Day 7	mm	33.7	4.2	17.9	2.5	5.588		**0.005**	negative
Height Day 47	cm	211.5	13.3	261.6	34.8	2.329		0.080	NSD
# pods (avg)	#	3	0.7	6.8	3.2		3.137	0.077	NSD
# pods (total)	#	105	2.5	155	14		0.429	0.513	NSD
Wt pods	g	0.065	0.008	0.1724	0.067	2.688		**0.055**	positive
# seeds (avg)	#	16.4	4.1	45.5	26.9		3.857	**0.050**	positive
# seeds (total)	#	578	23.7	1071	125.5		2.330	0.127	NSD
Wt seeds (avg)	g	0.0379	0.009	0.0817	0.04	1.842		0.139	NSD
# pods (grand total)	#	105		155			1.484	0.224	NSD
# seeds (grand total)	#	578		1071			1.159	0.282	NSD
Shoot weight	g	0.2187	0.04	0.6344	0.18	3.963		**0.017**	positive
Root length	mm	31.73	4.0	55.81	8.2	4.573		**0.010**	positive
Root weight	g	0.0129	0.001	0.0388	0.008	5.545		**0.005**	positive

Figure 61 is the cumulative height of *Brassica rapa* plants grown in Guelph biosolids for the whole life cycle of these plants. As can been seen from the graph, the biosolids plants started

off smaller with, as mentioned above, a significant difference between the heights on Day 7, but as time elapsed, these plants overtook the reference plants in terms of plant height (as well as other biological markers such as number of offspring (seeds) produced) and by the termination of the bioassay, the plants grown in biosolids were taller. Overall, as seen in Figure 61, there is a significant difference in the final height of *Brassica rapa* grown in the different treatments but again there is no overall negative impact of biosolids on this terrestrial biota. Instead the biosolids had a positive impact on the height of these plants and again as seen in Table 49 this resulted in significantly more biomass being produced in the plants grown in the biosolids amended soil.

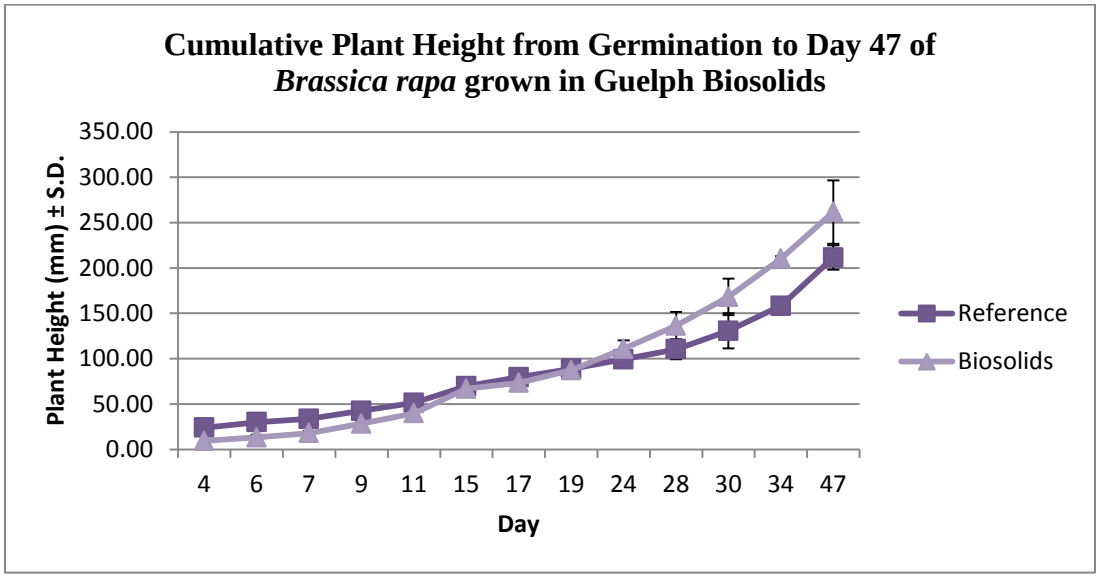

Figure 61: Cumulative Height of *Brassica rapa* grown in Guelph Biosolids

3.4.2.2 Summary of Results Obtained for Bioassays of Offspring (F_1) Generation of Parent (F_0) seeds grown in Crop Trough using Guelph biosolids

Glycine max (soya bean)	*Phaseolus vulgaris* (common bean)	*Brassica rapa* (field mustard)
• Percent germination • Rate of germination • Plant height (Day 68) • Number trifolates (Day 68) • Number of pods (Day 68)	• Percent germination • Rate of germination • Plant height (Day 47) • Number of leaves (Day 47) • Number of pods (Day 47)	• Percent germination • Rate of germination

Instead of using the green planter boxes to grow and observe the F_1 plants, as was the case for the Kitchener biosolids bioassays (which limited the full growth of the plants), the protocols were further developed once more troughs and space was available, to allow these bioassays to take place in crop troughs, which is a more environmentally-relevant setup. All parent seeds (F_0) were planted in crop troughs using Environment Canada artificial soil without the addition of biosolids. There were three crop troughs for each species to plant the F_0 seeds from reference plants and F_0 seeds from treatment plants. All seeds from the parent plants were pooled together (keeping each treatment separate) and then randomly selected and planted. Each trough was planted with five seeds.

Environment Canada artificial soil was used this time because all government protocols recommend its use. It is nutrient-poor, consisting of sand, clay and peat moss and no other additionally nutrients.

Since the initial seeds used for *Zea mays* were hybrids, they did not, as expected, produce F_0 seeds that were viable.

As Table 50 shows, there was no impact of biosolids on the offspring of the soya bean plants throughout the duration of the bioassay for this generation. Although not statically significant, the number of pods produced by Day 68 was biologically significant in the treatment plants as it was almost double that amount present in the reference plants.

Table 50: Results of *Glycine max* bioassays with F_0 seeds from parents grown in Guelph biosolids analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H- value	p-value	Impact of Biosolids
% Germination	%	93.3	11.6	93.3	11.6		0.000	1.000	NSD
Rate of germination	day	7.78	1.8	6.1	0.4		1.765	0.184	NSD
Height Day 68	cm	24.11	7.00	23.75	4.89	0.075		0.944	NSD
# Trifoliate Day 68	#	59	5.13	51	10.39		0.441	0.507	NSD
# pods Day 68	#	34	3.21	61	8.08		2.402	0.121	NSD

Table 51 again shows that there was no statistically significant difference between reference and treatment of the offspring of the *Phaseolus vulgaris* plants grown in Guelph biosolids throughout the duration of the bioassay.

Table 51: Results of *Phaseolus vulgaris* bioassays with F_0 seeds from parents grown in Guelph biosolids analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H- value	p-value	Impact of Biosolids
% Germination	%	100	0	100	0		0.000	1.000	NSD
Rate of germination	day	8.07	1.0	9.07	0.9		1.190	0.275	NSD
Height Day 47	cm	25.07	6.5	22.81	4.4	0.500		0.643	NSD
# Trifoliate Day 47	#	6.1	1.6	5.4	1.4		0.196	0.658	NSD
# pods Day 47	#	4.8	1.3	4.3	0.7		0.048	0.827	NSD

The Environment Canada artificial soil was quite hard (defined as offering moderate resistance to pressure) (SSSA 2012), resulting in poor germination with the small seed of the *Brassica rapa*. Since the required 80% germination was not achieved even in the reference,

this bioassay was not carried further. Nevertheless, as can be seen in Table 52, there was no significant difference between the reference and treatment plants. Thus, the biosolids did not impact the offspring of this genus either.

Table 52: Results of *Brassica rapa* bioassays with F_0 seeds from parents grown in Guelph biosolids analysed using Kruskal-Wallis test (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H- value	p-value	Impact of Biosolids
% Germination	%	50	0.5	50	0.5		0.000	1.000	NSD
Rate of germination	day	5	0	6	2.2		1.000	0.317	NSD

3.4.3 Further Investigation of Land-applying Biosolids using Artificial Soil and Rhizobium japonicum

The reference soil used in the all bioassays was obtained from Stratford, Ontario and consists of Perth Clay Loam, which is Grey Brown Podzolic and was nutrient rich (Pers. Comm. Michael Payne, OMAFRA). For the chemical analysis of this soil, refer to Appendix III. This could potentially be the reason why there was not an overall significant different seen in the growth of the plants grown in the biosolids (as would be expected since one of the beneficial uses of biosolids is as a fertilizer). Therefore to assess this, a further bioassay was conducted, this time using artificial soil. This soil is nutrirent poor and was chosen here because, although it provides enough nutrients for the plant to grow, with all 'extra' nutrients removed, a truer picture of the potential nutrient value of the biosolids could be observed. This bioassay was carried out using only *Glycine max* since, even though *Phaseolus vulgaris* is also a legume like *Glycine max*, *G. max* ia a more important crop in Southern Ontario and one to which biosolids would normally be applied.

Additionally, due to the fact that no root nodules were seen in any of the previous bioassays with the legume plants (either *Glycine max* or *Phaseolus vulgaris*) possibly due to the bacterium being desiccated during the soil preparation stage, *Rhizobium japonicum*, a soil

bacterium responsible for the formation of root nodules in legumes, was added to the artificial soil in this bioassay.

3.4.3.1 Summary of Results Obtained for Bioassays with Glycine max and Rhizobium japonicum planted in Artificial Soil using Guelph biosolids

***Glycine max* (soya bean)**
• Percent germination • Rate of Germination • Plant height (Day 26,60) • Trifolate Leaf length (Day 32) • Trifolate Leaf width (Day 32) • Trifolate Leaf area (Day 32) • Number of trifolates (Day 26,60) • Number of trifolates > 5.8 cm (Day 41) • Stem width (Day73) • Number of pods (Day 73) • Weight of pods and seeds (Day 73) • Number of seeds (Day 73) • Number of root nodules at termination (Day 73) • Shoot weight (Day 73) • Root weight (Day 73)

For these bioassays, three crop troughs containing Environment Canada soil and three with Environment Canada soil amended with Guelph biosolids at a rate of 22 tonnes/ha were prepared. Each of these troughs was planted with five *Glycine max* seeds treated with *Rhizobium japconium* bacterium.

Due to the difficulty in measuring all the parameters later in the growth cycle of the plants due to their drying, more qualitative measurements were made instead of actual measurements since it was the root that were of more interest in this bioassay. For instance, the numbers of trifoliates (a grouping of leaves) that were greater than 5.8 cm was determined instead of actually measuring each leaf length and width each time as was the case in previous bioassays.

The benefit of using artificial soil is that minimal nutrients are provided and thus the impact of the biosolids in a laboratory setting can be more closely examined. As can be seen in Figure 62 below, the artificial soil provides enough nutrients for plant growth, indicated by the reference plants on the left, but not the additional nutrients as provided by the biosolids as seen by the plants on the right. Additionally, in Figure 63, it can be seen that the plants were able to grow to maturity in this artificial soil as indicated by the reference plants in the image on the left and the plants grown in biosolids amended soil on the right.

Figure 62: *Glycine max* growing in reference Environment Canada artificial soil with *Rhizobium japonicum* on the right and biosolids amended soil on the left.

Figure 63: *Glycine max* plant at termination of bioassay. Reference plants are on the left and plants grown in biosolids amended soil are on the right

To remove the plants from the soil in the crop troughs, it would be impossible to extract all the roots. Instead a 2.5 cm diameter section of soil was removed around each plant and the roots collected from there.

Since it was the roots that were of interest here, these bioassays were terminated at Day 73 which did not allow the seeds to mature. Consequently, no weight was obtained for the seeds alone. As seen in Table 53, the biosolids had a positive impact on many growth parameters. There were more leaves longer than 5.8 cm on Day 41 (H= 4.091, p= 0.043) thus more photosynthetic area available, as well as more trifoliates on Day 60 (H= 3.971, 0.046) again a benefit to these plants. At the termination of this bioassay, the stems were thicker on the plants grown in the biosolids amended soil (t= 8.279, p= 0.001) as well as significantly more pods and seeds were produced (H= 3.857, p= 0.050 and H= 3.971, p= 0.046 respectively). Another interesting fact is that the reference plants produced significantly more root nodules (H- 3.857, p= 0.050). The assumption is that the biosolids provide the plants with enough nitrogen in a useable form that these plants did not need to rely on a symbiotic relationship with the soil bacterium *Rhizobium japconium* and to manufacture the root nodules to produce the needed nitrogen for plant growth. Figure 64 is an image of the root nodules found in both the reference and biosolids plants while Figure 65 provides an enlargement of these images. The root nodules in the reference plants were larger and mostly close to the main root while those in the biosolids were smaller and found further out along the root hairs.

Table 53: Results of *Glycine max* bioassays grown in Environment Canada Soil with *Rhizobium japonicum* and Guelph biosolids analysed using a t-test or Kruskal-Wallis test as appropriate (NSD = No Significant Difference)

Analysis	units	Ref mean	Ref S.D.	BS mean	BS S.D.	t-value	H- value	p-value	Impact of Biosolids
% Germination	%	80	20	93.3	11.6		0.889	0.346	NSD
Rate of germination	day	7	0	7	0		0.000	1.000	NSD
Height Day 26	cm	14.1	1.8	15.0	0.8	0.877		0.452	NSD
Height Day 60	cm	29.16	10.65	51.85	10.42	2.636		0.058	NSD
Leaf length Day 32	cm	5.6	0.1	6.3	0.5	2.540		0.064	NSD
Leaf width Day 32	cm	4.0	0.6	4.4	0.1	0.931		0.405	NSD
Leaf area Day 32	cm^2	22.8	3.8	27.9	2.3	1.999		0.131	NSD
# leaves > Day 41	#	0.4	0.8	4.3	0.6		4.091	**0.043**	positive
# trifoliate Day 26	#	2.0	0.1	2.0	0.1		1.667	0.197	NSD
# trifoliate Day 60	#	5.0	0.5	12.0	2.9		3.971	**0.046**	positive
Stem width Day 73	mm	2.49	0.08	3.00	0.08	8.279		**0.001**	positive
Wt pods & seeds	g	2.66	0.28	7.02	1.64	4.524		**0.011**	positive
# pods	#	5.7	2	14.6	3.6		3.857	**0.050**	positive
# seeds	#	9.4	0.5	23.3	6.6		3.971	**0.046**	positive
# root nodules	#	19.14	2.1	4.17	1.4		3.857	**0.050**	negative
Shoot wt	g	3.35	1.24	9.15	4.66	2.087		0.105	NSD
Root Weight	g	0.3	0.05	0.45	0.08	2.639		0.058	NSD

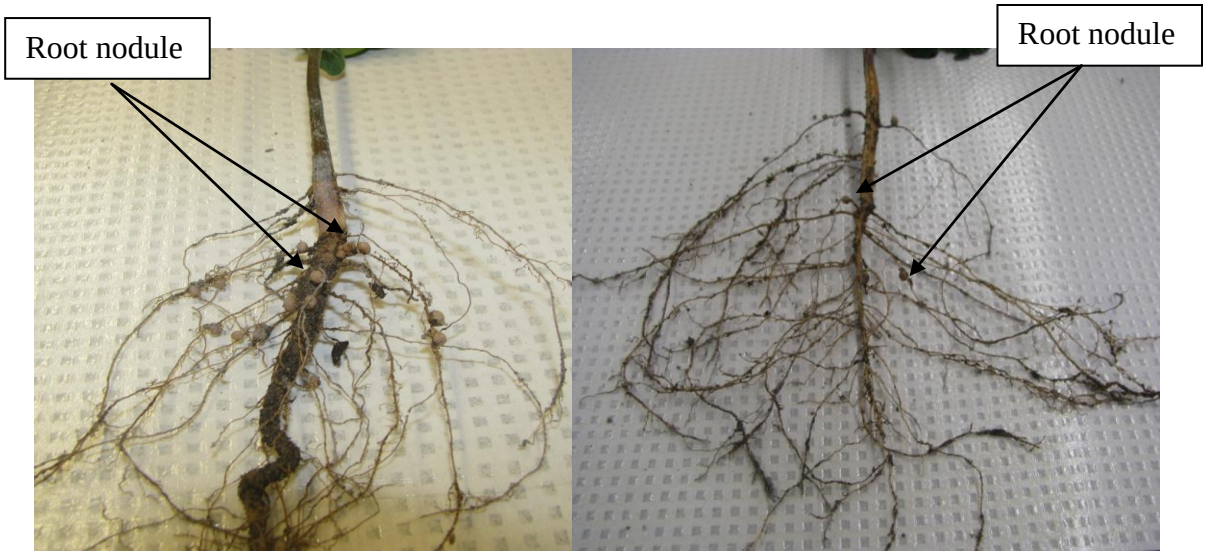

Figure 64: Location of root nodules. On the reference plants (left) the nodules are larger and closer to the main root while on the biosolids plants (right) they are fewer, smaller and further along the root hairs.

Figure 65: Close up of the root nodules. Right reference plant, left biosolids plant

3.5 Summary of Protocol Development with Guelph Biosolids

Further protocol development took place with the subsequent running of more bioassays with Guelph biosolids. For example, more concentrations were used with the *Folsomia candida* bioassays; 8 tonnes/ha application rate were used alongside the 22 tonnes/ha of biosolids. The Evans boxes were developed instead of using the buckets for the long-term bioassays with *Lumbricus terrestris*. Dittbrenner *et al.* (2011) in their research looked at the burrow volume of earthworms using X-ray technology. Using the transparent Evan's boxes, this could be something to consider in future work. For the plant bioassays, with more physical space available, more troughs and light banks were constructed to use with each plant genus to increase the sample number to three, thereby providing a larger number of replicates and more statistical power to the analyses. Although different sources of biosolids were used in the repeated bioassays, similar results were observed, i.e. little impact was seen. It is of interest to note that results from one trough were similar to those obtained from the three troughs. Therefore, if these protocols were to be implemented at a WWTP to assess the biosolids, and if space was limited, one crop trough for reference and one for treatment might be adequate to obtain information in regards to its impact on these crops. The addition of the soil bacterium *Rhizobium japonicum*, in further bioassays using artificial soil and *Glycine max* aided in the production of root nodules.

4. **Conclusion**

The main objective of this research was to determine if the land-application of biosolids to agricultural biomes was a sustainable practice and not detrimental to the existing terrestrial biota. To address this objective, the research needed to be accomplished in two steps. Since the existing government protocols of the time were not adequate for the task, new protocols needed to be developed for the environmentally-relevant biota used. Once developed, these protocols then needed to be tested using the appropriate organisms. Incorporating a holistic approach is extremely important when assessing the potential impact of land-application of biosolids. To accomplish this, the behavioural and reproduction end-points of two different animal species as well as on the development and reproduction of several plant species were examined. In this work, *Folsomia candida* and *Lumbricus terrestris,* two important indigenous members of terrestrial biomes where biosolids are applied were used along with *Zea mays* and *Glycine max* two crops of global importance, and *Phaseolus vulgaris* and *Brassca rapa*.

4.1 Protocol Development

4.1.1 Folsomia candida and Lumbricus terrestris Protocols

While the existing protocols for *Folsomia candida* were adequate, slight changes were made in this work. Changes included extending the duration of the bioassays to allow offspring to be older, and thus larger, at the termination of the bioassays. Their larger size made counting easier and therefore more accurate and reliable. Additionally, bioassays were performed in the dark so as not to hinder the reproduction of this organism since it has been found that bright lights reduce the number of eggs laid. In the case of the earthworm, Environment Canada's protocol on the use of the Kaushik chamber for avoidance behaviour was determined. Extensive testing in our laboratory indicated limitations to this design. An environmentally-relevant chamber which allowed the earthworm more natural movement was developed. For assessing impact on chronic and reproductive behaviours, Evan's Boxes that were first documented in 1947 (Evans 1947), were utilized with alterations. These transparent chambers with a vertical depth of 100 cm allowed a more natural and less

stressful environment for these organisms and for observing their burrowing behaviours than the 500 mL Mason jars prescribed in government protocols. Secondly, a modification to the top of these Evan's Boxes was added to allow space for *L. terrestris* to reproduce above ground (their natural mating behaviour).

Development and modifications to protocols were ongoing throughout the duration of this thesis until satisfactory protocols were achieved. From a chronological standpoint, after running the avoidance bioassays with the *Folsomia candida*, the 100-minute bioassay was no longer used after the initial trial with Kitchener biosolids because it was felt that the longer duration avoidance bioassays in the Mason jars were a better representation of a normal exposure of springtails to biosolids. Although the 100-minute bioassay was useful as a quick indicator of avoidance, they were too labour-intensive and time-consuming and essentially gave the same results as the longer duration bioassays. The 3-, 7-, 14-day bioassays are more environmentally-relevant due to the longer exposure times and are thus suggested for future research only. From the results gained from the reproduction bioassays with Kitchener biosolids, further protocol development took place with the subsequent running of more bioassays with Guelph biosolids. This time, more concentrations were used with the *Folsomia candida* bioassays; 8 tonnes/ha application rate was used alongside the 22 tonnes/ha of biosolids.

When examining the protocol development of *Lumbricus terrestris*, avoidance Method #1 using the Kaushik chambers, following Environment Canada's construction and Guelph biosolids was discarded due to the inadequacy of this setup to accommodate the size and burrowing behaviour of this organism. Instead, Method #2 was developed using rectangle vessels and used in subsequent bioassays with Kitchener biosolids. From what was learned from the *Folsomia candida* bioassays, environmentally-relevant application rates of 8 tonnes/ha were used. Secondly, Method #2 (Evan's boxes), were developed instead of using the buckets of Method # 1 for the long-term bioassays with *Lumbricus terrestris* which solved the compaction issue and allowed for greater burrowing depth for these vertical

dwellers as well as a non-invasive method of viewing the organisms throughout the bioassays.

4.1.2 Plant Protocols

Plant species used in conventional government protocols recommended, among others, *Daucus carot, Cucumis sativus* and *Lactuca sativa*. These food crops were inadequate for this study since they would not have biosolids applied to them and thus are environmentally-irrelevant. Thus, plants that were relevant were incorporated in this work. Government protocols prescribe seeds to be grown in 1L containers with 500 mL soil. Additionally, since whole lifecycles were being examined here, newly designed "crop troughs" containing up to 110 L of soil were utilized for the growing of plants. While traditional plant protocols primarily measure germination rates, root and shoot lengths, and biomass at the completion of a 14- or 21-Day bioassay, the entire lifecycle of the plants as well as their progeny was examined in this work. Along with the traditional observations just mentioned, number and area of leaves were constantly measured throughout these bioassays along with pod development, number of seeds produced, and biomass of the pods and seeds at termination. Lastly, the percent germination and germination rates of the seeds produced by the F_0 plants were also assessed in transgenerational bioassays. With these extra measurements made throughout the entire lifecycle (120 days in some cases), a more comprehensive assessment could be made between reference plants and those grown in biosolids-amended soil to ascertain if any impact was present and at specific developmental stages.

After the initial bioassays were run using the Kitchener biosolids, which included the green planter boxes and single crop troughs, methods were further fine-tuned as to what growth parameters to measure. The results from these bioassays indicated that the construction material of the crop troughs did not hinder the growth of the plants and therefore the crop troughs are recommended for use in further research. When the repeat analyses of plant bioassays were to take place, this time, using Guelph biosolids, more physical space was available. Therefore, more troughs and light banks were constructed to use with each plant

genera to increase the sample number to three, thereby provide a larger number of replicates and more statistical validity to the results. The addition of the bacterium *Rhizobium japonicum* to the soil aided in the production of root nodules in final bioassays with *Glycine max*.

4.2 Impact

The findings showed that biosolids had little negative impact on the terrestrial biota examined and as a general rule, there was no impact observed. Where effects were observed, the majority of instances were positive. In the few instances where there was negative impact observed, for example in the initial growth stages of the plant bioassays, with further development of the organism, there was no longer a significant difference between the reference and treatment plants. Additionally, if only existing government protocols had been followed (which only look at germination and root and shoot size of the seedlings), these anomalies would not have been noticed. For example, by observing later in the life cycle of the plants, an erroneous conclusion would have been avoided regarding the biosolids having a detrimental impact on the terrestrial biota. Where impact was observed, overall, biosolids had a significant positive effect on the growth and development of the terrestrial biota.

Based on the results of this research for two wastewater treatment plants tested, the land-application of biosolids appears to be a sustainable practice. The local farmers benefit from an inexpensive source of organic fertilizer and the municipality (and in turn everyone through less cost and taxes) benefit by having a sustainable method of disposing of the biosolids beside the costly and not environmentally-friendly methods of land-filling or incinerating. Also, the environment benefits by the reduced use of inorganic artificially-made fertilizers that are still heavily in use today.

4.2.1 Kitchener Biosolids

4.2.1.1 Folsomia candida and Lumbricus terrestris

When examining the individual organisms exposed to biosolids produced using anaerobic digestion such as at the Kitchener WWTP, *Folsomia candida* did not show a significant difference in the short-term avoidance bioassays, but rather a preference for the biosolids-amended soils. Nor was there an impact seen with the reproduction bioassays. In the longer duration avoidance bioassays, *F. candida* did exhibit a significant difference in avoidance behaviour with one trial of biosolids (H=8.534, p=0.003) but not with the other two trials (H=0.101, p=0.751 and H=0.544, p=0.461). It was concluded that it was a storage issue with that particular batch of biosolids and not an issue of the biosolids per se. In the avoidance bioassays using *Lumbricus terrestris*, there was no significance difference seen.

4.2.1.2 Plants

When examining the various plant bioassays, there were only a few observations of negative impact of the biosolids, but as the plants developed, this was no longer the case. As seen in green planter bioassays, the plant height of *Brassica rapa* at Day 15 (t-2.615, p= 0.035) and the number of buds on the same day (H=6.222, p=0.013) showed a significant difference but this did not translate into the number of seeds produced (H=0.011, p=0.917) or the root biomass where there was actually a significant difference in favour of the plants grown in biosolids-amended plants (t=3.691, p= 0.006). Again, the same can be seen in the crop trough bioassays. *Glycine max* plants had a difference in plant height on Day 64 but not with the number of leaves or the size of the leaves (measured on Day 60) or the number of nodes measured Day 39. Thus, the plants grown in the biosolids-amended soil were shorter, but bushier. When examining the offspring of the parent plants, there was no significant difference seen when the seeds of these plants grown in the biosolids-amended soil were grown in reference soil.

4.2.2 Guelph Biosolids

4.2.2.1 Folsomia candida and Lumbricus terrestris

When examining the individual organisms exposed to biosolids by the Lystek method (Guelph Waste Water Treatment Plant), *Folsomia candida* did not show a significant difference in avoidance behaviour at 14 days but did show a significant difference during reproduction. When exposed to biosolids at an application rate of 22 tonnes/ha on a dry weight basis, a significant difference in reproduction was seen (t=2.589, p=0.036) but when exposed to an environmentally-relevant application rate of 8 tonnes/ha on a dry weight bases there was no significant difference observed (t=0.008, p=0.994). When examining *Lumbricus terrestris* there was no significant difference in the long-term bioassays.

4.2.2.2 Plants

When examining the plant bioassays, there was a significant difference with *Zea mays* with regards to the number of leaves on Day 22 (H=4.355, p=0.037) but not at the termination on Day 132, nor was there any significant difference in plant height throughout the life cycle of these plants. *Glycine max* showed a significant difference in leaf length on Day 14 (t=2.968, p=0.041) but not in leaf area on Day 20 where in fact there was a significant difference in favour of the plants grown in biosolids-amended soil (t=9.222, p= 0.005). *Brassica rapa* showed a negative impact from the biosolids in terms of germination and plant height Day 7 (H=4.091, p=0.043 and t=5.588, p=0.005 respectively) but had a positive impact in terms of number of seeds, shoot weight, and root weight at termination (H=3.857, p=0.050; t=3.963, p=0.017; t=5.545, p=0.005 respectively). Therefore, had government protocols only been followed, an erroneous conclusion would have been drawn that biosolids negatively impacted these plants and thus were not a sustainable practice while in fact the opposite is true; biosolids had a positive impact. Again, when examining the offspring of the parent plants, there was no significant difference seen when the seeds of these plants grown in the biosolids-amended soil were grown in reference soil.

Looking at *Glycine max* grown in artificial soil and in the presence of the nitrifying bacteria *Rhizobium japconium*, there is a positive significant difference all around in plants grown in the biosolids amended soil. For example, the number of leaves on Day 41 ($H=4.061$, $p=0.043$), stem width Day 73 ($t=8.279$, $p=0.001$), weight of seeds and pods ($t=4.524$, $p=0.001$), and the number of root nodules ($H=3.857$, $p=0.050$) indicated more biomass and thus healthier, more productive plants. Therefore these results illustrate the beneficial use of biosolids as a fertilizer.

4.3 Summary

In closing, had government protocols only been followed, an erroneous conclusion with regards to the sustainable practice of land-application of biosolids would have been drawn. To accurately assess the use of biosolids as a means of organic fertilizer for agriculture crops in the southern Ontario area, a holistic approach needs to be taken using several environmentally-relevant organisms and assessing their whole lifecycles. As a note, impact needs to be determined on a case-by-case basis, i.e. each different WWTP needs to be assessed individually. This could be done using simple avoidance bioassays. As the need for a more holistic approach becomes necessary to accurately assess potential contaminant impact, it is recommend that the methodology developed in this thesis be incorporated into existing protocols for further assessing the potential impact of land-applying other sources of biosolids.

5. **Future Work**

There are many directions this project could go. One would be to repeat the bioassays as prescribed in this research using different sources of biosolids. Another direction to follow would be to expand from a laboratory-based setting to a larger scale field-study. To analyse the earthworms or springtails, a sample plot could be dug (at least a meter deep in the case of the earthworms) and a count of the natural abundance of these organisms determined. Next, physically separated to prevent cross-contamination, but identical reference and treatment plots need to be established. After a predetermined timeframe (e.g. several months), the plots could be re-examined and population counts for the springtails or earthworms performed again to determine if there was any impact on these organisms owing to the land-application of biosolids. Due to the extremely small size of the springtails, this might prove difficult. The crop plants would be much easier to assess. Again, separated plots for reference and biosolids-treated land could be planted with the different genera and the measurements of growth as laid out in this research could be collected and analysed to determine if any impact from the land-application of biosolids existed on this much larger scale.

The scope of this thesis was to examine the impact, if any, of biosolids on the terrestrial biota. Another logical step would be to determine if biosolids have any impact on the aquatic biota. While preliminary work was undertaken in this research, a comprehensive assessment of the runoff of land-applied biosolids into the aquatic environment must be conducted. A study was initiated by Gebert (2010) that must be built upon. While biosolids are regulated to prevent their introduction into the aquatic environment, situations could occur for this to happen. For example, a spill while transporting the biosolids to their destination could potentially lead to the direct run-off into surface water. Another possibility, a once-in-a-hundred-year thunderstorm could potentially wash biosolids from their site of land application into receiving waters or cause their leaching through the soil to ground or tile water. Thus, biosolids should be evaluated for their impact, if any, on the aquatic environment. Before an organism can be used as a biological monitor to evaluate in this case, the aquatic environment, their behaviour must be understood under normal reference

conditions so that when a stressor is applied, any abnormal behaviour can be noted. Once this is successfully accomplished, an assessment on the impact of a stressor, as in this case, biosolids runoff from an agricultural field into a receiving body of water, can be ascertained. From the work of Giesy and Hoke (1989) and many other researchers including McCarthy (2003), Gebert (2010), and Brose (2011), two such organisms were identified: *Daphnia magna* and *Hyalella azteca*. *Daphnia magna* is a cladocera that is found in the water column and thus would be exposed to any hydrophilic contaminants that could potentially be present in the water from biosolids that ran off into the receiving water and secondly, *Hyalella Azteca* is an amphipoda that lives in the benthos and thus could be negatively impacted by mainly hydrophobic contaminants that enter the surface waters from biosolids run off and settle onto the sediment.

Another direction perhaps to take would be a fast, scientifically rigorous, holistic, “miner’s canary” that incorporated both terrestrial and aquatic organisms that could be developed to utilize the protocols established here, and be implemented at all WWTP producing biosolids for land-application.

Appendix I - Calculations

6.1 Protocol for the Determination of the Amount of Biosolids Required

Note these calculation need to be performed for each new batch of biosolids

6.1.1 Materials and Equipment:

Biosolids obtained from known source (Kitchener, Guelph)

50 ml graduate cylinder

10 crucibles

Drying oven (temperature 60-80°C)

Analytical balance

6.1.2 Obtaining a Homogenous Mixture of Biosolids

6.1.2.1 Kitchener as source of Biosolids

Since the biosolids from this source have very little solid matter which easily separates into two distinct phases, they must be thoroughly mixed prior to use

1. To make a homogenous mixture from several buckets of biosolids, transfer a portion of biosolids from the first bucket into two or three clean buckets (depending on quantity to be mixed)
2. To these buckets, pour a portion of biosolids from the second source bucket
3. Repeat with all source biosolids
4. Using the now emptied source buckets, repeat the process by pouring the mixed biosolids back into theses bucket again dividing the liquid each time
5. Repeat step 4, pouring back and forth until the contents of all buckets are thoroughly mixed and eventually all of the biosolids from all buckets are contained in the original two or three buckets

6.1.2.2 Guelph as a Source of Biosolids

The biosolids from the Lystek process does not have 2 distinct phases but are rather a very thick liquid, the consistency of tar, therefore only need stirring

1. To ensure a homogenous mixture, using a long clean utensil, stir the contents of the 18L bucket of biosolids until well mixed

6.1.3 Determination of Biosolids Density

1. Using the homogenous biosolids, measure the weight of a 50 mL graduate cylinder and record the mass
2. Add a volume of biosolids to this graduate cylinder and using an analytical balance determine the weight
3. Record the mass and volume
4. Repeat step 2 and 3 for 10 different volumes (i.e. 15 ml, 20 ml, 25 ml,…, 50 ml)

   To calculate the density of the biosolids for each of the 10 trials, use the formula

   Density = mass (g)/volume (mL)
5. Calculate the mean density and standard deviation

6.1.3.1 Sample Calculation - Density of Biosolids

Mass of 40.00 mL of Kitchener biosolids = 40. 6621 grams

Density = mass (g)/volume (mL)

= 40.6621 g/ 40.00mL = 1.016g/mL (or 1.016 g/cm^3)

Therefore the density of Batch # 1 Kitchener biosolids is 1.016g/mL (g/cm^3)

The density of Batch # 1 Guelph biosolids 1.006 g/mL (g/cm^3)

6.1.4 Determination of % Biosolids

1. Thoroughly clean 10 crucibles using an alcoholic KOH bath for an hour
2. After the elapsed time, wash each crucible according to Ryerson Washing protocol
3. Label 10 crucibles (1-10), weigh and record the mass
4. Using a graduate cylinder, measure 10 mL of biosolids and transfer to a crucible

5. Measure the weight of the crucible and biosolids (wet weight) using an analytical balance. Record
6. Repeat steps 4 and 5 for each of the remaining 9 crucibles
7. Put all 10 crucibles into the drying oven set at a temperature of 60°C to 80°C for 24 hour or until constant weight is obtained
8. Remove the crucible from the oven and store in a desiccators until cool
9. When crucibles have reached room temperature, determine the weight of each crucibles (dry weight) using an analytical balance for weighing and tong for transferring. Do not touch crucible with fingers
10. Calculate the wet weight to dry weight ratio for each using the formula;

    % Biosolids = wet weight (g)/ dry weight (g) x 100%

11. Calculate the mean and standard deviation

6.1.4.1 Sample Calculations - % Biosolids

Average of wet weights of biosolids = 26.69824 grams

Average of dry weights of biosolids = 1.65632 grams

% Biosolids = wet weight (g)/ dry weight (g) x 100%

= 1.6563 g / 26.6824g x 100% = 6.2039%

Therefore the % biosolids in Batch # 1 from Kitchener is 6.2039 %

The % biosolids in Batch # 1 from Guelph is 16.27 %

6.1.5 Determining amount of Biosolids needed for Bioassays

This research used a worse case scenario application rate of 22 tonnes/ha on a dry weight basis. This needs to be scaled down to workable lab dimensions which are in units of g/cm^2. Since;

1 tonne = 1000 kg and 1 ha = 10^8 cm^2 therefore;

22 tonne/ha = <u>22 x 10^3 kg/10^8 cm^2</u> <u>or 0.22g/cm^2</u>

6.1.5.1 Sample Calculation: Folsomia candida Reproduction Bioassay

Area of the jar used in reproduction bioassay = $A = \pi r^2$ = 19.63 cm^2

For application rate of 22 tonnes/ha;

$$19.63 \text{ cm}^2 \times 22\text{x}10^3 \text{ kg}/1\text{x}10^8 \text{ cm}^2 = 0.0043197\text{kg} = 4.320 \text{ g}$$

Since such small jars were used, the application rate needed to be adjusted. Therefore 4.4 tonnes/ha (4.4 x 10^3 kg/10^8 cm2) would be the equivalent of 22tonne/ha in these smaller vessels. Thus;

$$19.63 \text{ cm}^2 \times 4.4\text{x}10^3\text{kg}/1\text{x}10^8 \text{ cm}^2 = 0.0008637\text{kg} = 0.8637 \text{ g}$$

Therefore 0.8637 g of this batch of biosolids would be needed to be added to the soil in each jar for the *Folsomia candida* reproduction bioassay

6.1.5.2 Sample Calculation: Crop Troughs for Plants

Example the area of the 90 cm Crop Trough is 2787.09 cm^2

From above, % biosolids in Batch # 1 from Guelph is 16.27 % (therefore 83.73 % water) and the density is 1.006 g/cm^3. Therefore for application rate of 22 tonnes/ha;

$$2787.09 \text{ cm}^2 \times 0.22\text{g/cm}^2 = 613.16 \text{ g}$$

Therefore 613.16 g biosolids dry wt needs to be added to each 90 cm crop trough. Since biosolids are applied wet, the wet weight needs to be determined, therefore;

$$613.16g/16.27\% \times 100\% = 3768.65 \text{ g (wet weight)}$$

Since the biosolids are in liquid form when applied to the soil, volume was used to measure the amount of biosolids needed. Therefore;

$$3768.65g \times 1.006 \text{ g/cm}^3 = 3746.18 \text{ cm}^3 = 3.746L$$

Therefore 3.746 L of Guelph biosolids are needed for a 90 cm crop trough. Similarly, 4.99 L are needed for a 120 cm crop troughs and 1.20 L for the smaller vessels used for the *Brassica rapa* bioassays. Since these vessels were not as deep as the crop troughs, the application rate was adjusted for a depth of 5 cm instead of 15 cm

Appendix II - Soil Survey

From this soil survey from 1952, we know that the type of soil used in this research was Perth Clay Loam, which is Grey Brown Podzolic

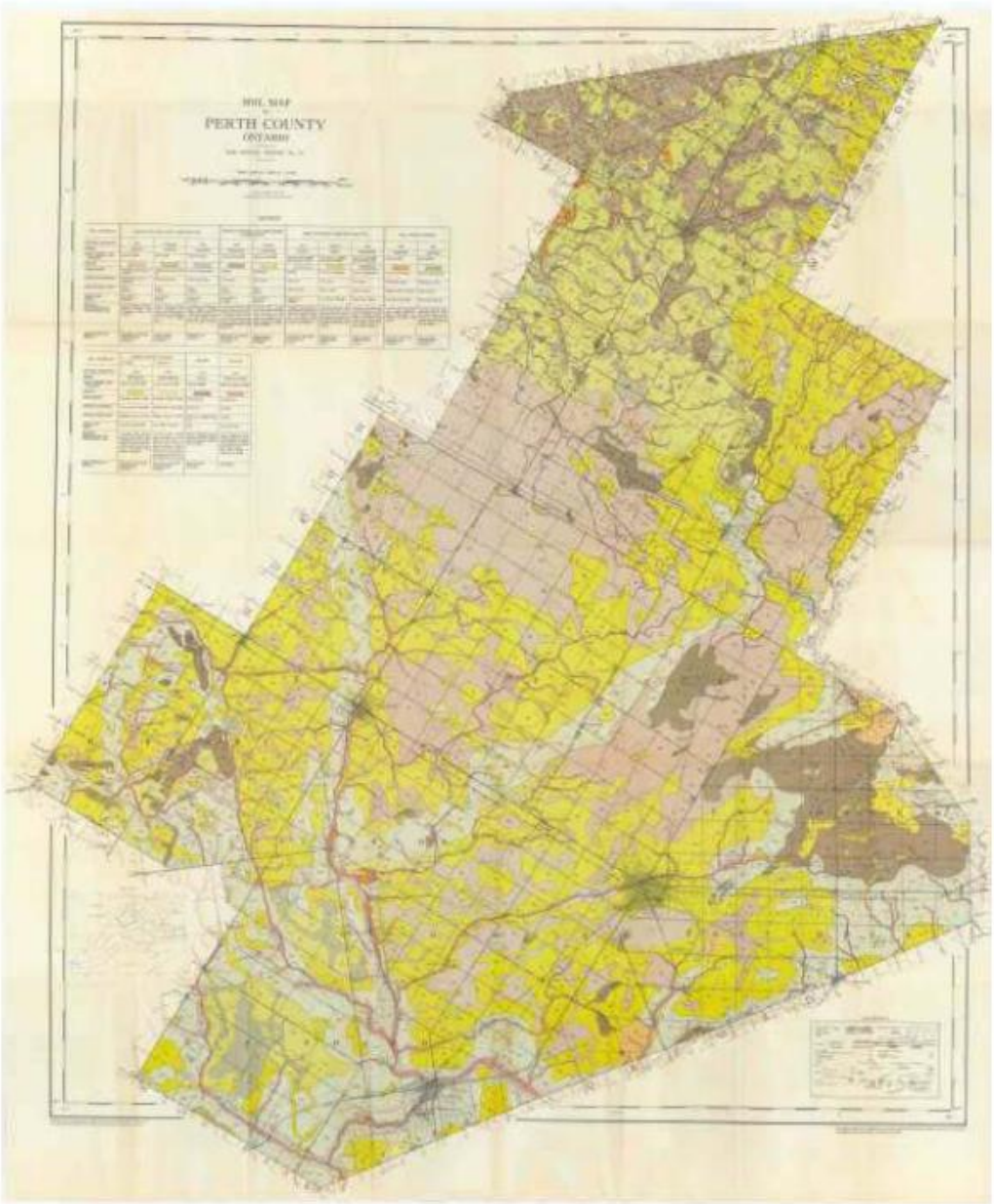

Figure 66: Soil Survey of Perth County - Report No. 15 of the Ontario Soil Survey by D.W. Hoffman Experimental Farms Service, Canada Department of Agriculture and N.R. Richards Ontario Agricultural Collage, Guelph, Ontario. April 1952.

From the Ontario Soil Survey results shown in Table 1, the results from the *Survey of Regulated and Non-regulated Metals in a Range of Ontario Agricultural Soils*, the background metal present in the soil in Ontario can be determined. The source of this information is from Crop Advances: Field Crop Report 2007. Project contact Payne, Michael OMAFRA.

Table 54: Concentration of Regulated and Non-regulated Metals in Ontario Soils

Crop Advances: Field Crop Reports

Table1: Concentration of Regulated and Non Regulated Metals in Ontario Farm Fields Compared to the Ontario Typical Range for Metals Based on MOE Standards

Site Types	Chloride	Mercury	Beryllium	Magnesium	Aluminum	Calcium	Vanadium	Chromium	Manganese	Iron	Cobalt	Nickel
	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g
1*	23.32	0.07	1.1	10000	29000	23000	62	45.1	984	35100	17.1	37.4
2	2.8	0.02	0.5	4850	15000	6800	39	23	570	20000	7.7	17
3	232.6	0.12	0.56	11040	26260	61920	53.3	40.3	870	27780	14	37.5
4	3.6	0.02	0.25	2900	8950	5450	27	14	320	13500	4.6	13
5	45	0.13	1.1	20000	30000	55000	77	58	2200	35000	16	38

Site Types	Copper	Zinc	Molybdenum	Cadmium	Barium	Lead	Strontium	Boron	Silver	Sodium	Total Potassium	Sulphur
	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g
1	33	120	3.04	1.1	197	22	56.8	28	0.2	505	5870	0.05
2	14	63	0.25	0.45	71.5	10	20	9.7	0.075	120	2000	0.025
3	309	142.6	7.07	1.03	181.2	31.9	97.1	34.5	0.11	290	4912	0.34
4	16	56	0.25	0.25	43	11	17	9.2	0.05	95	1300	0.03
5	41	120	1	0.71	160	45	64	30	0.27	660	6500	0.079

Site Types	Total Nitrogen	Total Phosphorus	Total Solids	Uranium	Arsenic	Selenium	Antimony	Fluoride	Titanium	Tin	Thallium	
	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	ug/g	% dry wt	ug/g	ug/g	
1	3.1	1.6	91.7	3.04	9.79	0.57	0.8	187	0.567	1.37	0.89	
2	1.8	0.92	50	0.65	3.5	0.2	0.2	57	0.42	0.61	0.29	
3	30.3	2.9	825.2	4.61	15.52	1.63	0.619	474	0.53	9.02	10.66	
4	1.6	1	42	0.8	4.15	0.18	0.1	78	0.33	0.96	0.598	
5	5.7	1.9	na	2.1	11	0.93	0.43	61	0.52	na	0.81	

1*	This value is the 98th percentile value from 134 Field Crop Sites from across Southern Ontario.
2	This value is the median value from 134 Field Crop Sites from across Southern Ontario.
3	This is the 98th percentile value from 44 Horticultural Field sites from across Southern Ontario.
4	This is the median value from 44 Horticultural Field sites from across Southern Ontario.
5	The Ontario Typical Range is the average value for Rural Parkland (parks, cemeteries, forests, woodlots and other undeveloped areas) as determined by a MOE survey published in 1993 (ISBN O-778-1979-1).

The 98th percentile values presented in Site Types 1 and 3 are the points where 98% of the samples have values lower than this value, and 2% have values higher...in other words, it represents the highest values you are likely to find in the data set.

While Table 2 provide the amount of regulated metals that are allowable in soils across Canada, that can have biosolids applied. If the soil analysis, that must be done before a

farmer can land-apply biosolids as part of his Certificate of Analysis, is in excess of any of the regulated metals shown here, than biosolids cannot be applied to this land.

Table 55: Standards for Allowable metal concentration in Soil in Canada (CCME 2009)

Table 5-B: Standards for Allowable metal concentrations in soil in Canada

Jurisdictions	Concentration in Soils (mg/kg DM)										
	Cd	Cr	Cu	Hg	Ni	Pb	Zn	As	Se	Mb	Co
NL	1.6	120	100	0.5	32	60	220	14	1.6	4	20
NS	1.4	64	63	0.5	32	60	200	12	1.6	4	20
PEI[4]	1.6	120	100	0.5	32	60	220	14	1.6	4	20
NB											
QC	For agricultural land application, Québec MDDEP considers bioavailable metal as better indicator of risk than total metals. Québec Ministry of agriculture has developed criteria for bio-available metals that may be used by agronomists, especially to monitor Cu and Zn build-up in soils.										
ON	1.6	120	100	0.5	32	60	220	14	1.6	4	20
MB (kg/ha)[1]	2.5	115	113	11.9	80	126	380	22	N/A	N/A	N/A
SK	1.4	64	63	6.6	50	70	200	12	1	5	40
AB - Class 1 (kg/ha)[2]	1.5	100	200	0.5	25	100	300				
AB - Class 2 (kg/ha)	1.1	75	150	0.4	19	75	200				
AB - Class 3 (kg/ha)	0.8	50	100	0.2	12	50	150				
BC	See Contaminated Sites Regulation (B.C. Reg. 375/96) for soil standards										
NWT (kg/ha)[3]	4			1	36	100	370	15	2.8	4	30
NU	Currently uses Guidelines, Regulations and Acts of NWT and the CCME guidelines										
CCME	4	210 (interim)	150 (interim)	1	36	100	370	15	2.8	4	30

1 Based upon soil bulk density of 1200 kg/m3 and soil depth 15cm
2 Class 1 soil - slope 2% or less, > 5 m to water table, soil texture CL,SiCL, SiL,Si,SiC,L,SCL,SC Class 2 - slope 2-5%, 3-5m to water table, soil texture C, HC
Class 3 - slope 5-9%, 2-3 m to water table, LS,SI all soil pH > 6.5
3 Based upon CCME Guidelines for Compost Quality & NWT Guideline for Agricultural Waste Management
4 PEI does not have requirements currently, but propose to move forward with plan consistent with the Atlantic Canada Guidelines Manual in May 2009

Appendix III – MOE Soil and Biosolids Analysis

When examining the results in Tables 5-8 from the MOE analysis of metals and nutrients of the soil and biosolids used in this research, refer to Ontario Biosolids Guidelines in Table 5, for the acceptable amounts of the various regulated metal. Also provided here are the different government guidelines on these same metals for comparison purposes.

Table 56: Metal Regulated under various Government Legislations

Metal	Ontario Biosolids Guidelines	USEPA 503.13 Rule	Canadian Fertilizer Standards
Arsenic	170	75	75
Cadmium	34	85	20
Chromium	2800	3000	N/A
Cobalt	340	N/A	150
Copper	1700	4300	N/A
Lead	1100	840	500
Mercury	11	57	5
Molybdenum	94	75*	20
Nickel	420	420	180
Selenium	34	100	14
Zinc	4200	7500	1850

All values in Table 3 are in ppm (mg/kg) measured on a dry weight basis. *Note Molybdenum was removed from the amendment to the 503 rule in 1994 pending further consideration by the USEPA. Table 4 is the legend used in interpreting the MOE results from the analysis of soil and biosolids.

Table 57: Legend of shorthand used for following MOE Results

CODE	DESCRIPTION
UAL	UNRELIABLE: SAMPLE AGE EXCEEDS NORMAL LIMIT
<=WE	NO MEASURABLE RESPONSE (DILN/CONC): <REP'D VALUE
<T	A MEASURABLE TRACE AMOUNT:INTERPRET WITH CAUTION
WST	WET SAMPLE MASS USED RESULT REPORTED AS MG/KG
RDB	CALCULATED VIA DIGESTED BLANK FROM ANOTHER RUN
<=W	NO MEASURABLE RESPONSE (ZERO): <REPORTED VALUE
NDUA	NO DATA: UNSUITABLE FOR ANALYSIS
<=>	APPROXIMATE RESULT

Table 58: MOE Chemical Analysis on First Batch of Soil

Ontario Ministry of Environment
Central Laboratory - Resources Road
FINAL REPORT(manager)
Print date Dec. 29, 2008 11:51 AM

C164101

Field ID:	REFERENCESOIL-JUN
Sample ID:	C164101-0001
MOE"LIMS ID:	2008SO44-00141
Station ID:	
Collect Date:	01 JUN 2008
Sample Location Description:	RYERSON UNIVERSITY SOIL
Sample Comments Description:	

Parmname	Value	Units	Qual	Rmk1
Mercury	0.04	ug/g dry	<T	
Beryllium	0.6	ug/g dry	<T	
Magnesium	8500	ug/g dry		
Aluminum	16000	ug/g dry		
Calcium	15000	ug/g dry		
Vanadium	36.	ug/g dry		
Chromium	21.	ug/g dry		
Manganese	600	ug/g dry		
Iron	18000	ug/g dry		
Cobalt	7.1	ug/g dry		
Nickel	17.	ug/g dry		
Copper	11.	ug/g dry		
Zinc	62.	ug/g dry		
Molybdenum	.5	ug/g dry	<=W	
Cadmium	0.7	ug/g dry	<T	
Barium	77.	ug/g dry		
Lead	12.	ug/g dry		
Strontium	20.	ug/g dry		
Nitrogen; total Kjeldahl	1.4	mg/g dry		
Phosphorus; total	0.87	mg/g dry		
pH	7.4	none		
Arsenic	3.3	ug/g dry		
Selenium	.2	ug/g dry	<=W	
Antimony	.2	ug/g dry	<=W	

Table 59: MOE Chemical Analysis on Second Batch of Soil

Ontario Ministry of Environment
Central Laboratory - Resources Road
FINAL REPORT(manager)
Print date Jan. 09, 2009 08:55 AM

C164102

Field ID:	REFERENCESOIL-OC
Sample ID:	C164102-0001
MOE*LIMS ID:	2008SO44-00142
Station ID:	
Collect Date:	20 OCT 2008
Sample Location Description:	RYERSON UNIVERSITY SOIL
Sample Comments Description:	

Parmname	Value	Units	Qual	Rmk1
Mercury	0.05	ug/g dry		
Beryllium	0.9	ug/g dry	<T	
Magnesium	4400	ug/g dry		
Aluminum	18000	ug/g dry		
Calcium	5600	ug/g dry		
Vanadium	45.	ug/g dry		
Chromium	26.	ug/g dry		
Manganese	1500	ug/g dry		
Iron	20000	ug/g dry		
Cobalt	7.6	ug/g dry		
Nickel	23.	ug/g dry		
Copper	16.	ug/g dry		
Zinc	86.	ug/g dry		
Molybdenum	.5	ug/g dry	<=W	
Cadmium	0.9	ug/g dry	<T	
Barium	130	ug/g dry		
Lead	12.	ug/g dry		
Strontium	21.	ug/g dry		
Nitrogen; total Kjeldahl	1.7	mg/g dry		
Phosphorus; total	1.4	mg/g dry		
pH	5.8	none		
Arsenic	3.3	ug/g dry		
Selenium	.2	ug/g dry	<=W	
Antimony	0.3	ug/g dry	<T	

Table 60: MOE Chemical and Biological Analysis for Kitchener Biosolids

C162166

Ontario Ministry of Environment
Central Laboratory - Resources Road
FINAL REPORT(manager)
Print date Nov. 06, 2008 04:02 PM

Field ID:	KITCHENER
Sample ID:	C162166-0001
MOE*LIMS ID:	2008SL34-00022
Station ID:	
Collect Date:	20 AUG 2008
Sample Location Description:	KITCHENER STP
Sample Comments Description:	BIOSOLIDS

Parmname	Value	Units	Qual	Rmk1
Aluminum	1210	mg/L		
Barium	77.1	mg/L		
Beryllium	.05	mg/L	<=W	
Cadmium	0.51	mg/L		
Cobalt	0.25	mg/L	<T	
Chromium	9.33	mg/L		
Copper	141.	mg/L		
Iron	5430	mg/L		
Lead	4.70	mg/L		
Magnesium	678.	mg/L		
Manganese	25.4	mg/L		
Molybdenum	1.14	mg/L		
Nickel	6.70	mg/L		
Silver	0.31	mg/L	<T	
Strontium	66.0	mg/L		
Titanium	8.82	mg/L		
Vanadium	2.05	mg/L		
Zinc	85.6	mg/L		
Calcium	5060	mg/L		
Sodium	845.	mg/L		
Potassium	330.	mg/L		
Arsenic	0.19	mg/L		UAL
Selenium	0.26	mg/L		UAL
Antimony	0.17	mg/L		UAL
Solids; total	61200	mg/L	WST	
Solids; total, ash	26400	mg/L		
Solids; total, loss on ign	34800	mg/L		
Conductivity		uS/cm	NDUA	
pH		none	NDUA	
Alkalinity; total fixed endpt		mg/L CaCO3	NDUA	
Nitrogen; nitrite	3.4	mg/L		
Nitrogen; nitrate+nitrite	5	mg/L	<=WE	
Nitrogen; ammonia+ammonium	944.	mg/L		
Phosphorus; phosphate	134.	mg/L		
Nitrogen; total Kjeldahl	4290	mg/L		
Phosphorus; total	2920	mg/L		RDB
Escherichia coli	37000	c/1 g dry	<=>	

Table 61: MOE Chemical and Biological Analysis for Guelph Biosolids

Ontario Ministry of Environment
Central Laboratory - Resources Road
FINAL REPORT(manager)
Print date Nov. 06, 2008 04:02 PM

Field ID:	GUELPH
Sample ID:	C162165-0001
MOE*LIMS ID:	2008SL34-00021
Station ID:	
Collect Date:	20 AUG 2008
Sample Location Description:	GUELP STP BIOSOLIDS
Sample Comments Description:	

Parmname	Value	Units	Qual	Rmk1
Aluminum	1260	mg/L		
Barium	110.	mg/L		
Beryllium	.05	mg/L	<=W	
Cadmium	0.39	mg/L	<T	
Cobalt	1.75	mg/L		
Chromium	31.1	mg/L		
Copper	236.	mg/L		
Iron	34800	mg/L		
Lead	10.0	mg/L		
Magnesium	1260	mg/L		
Manganese	206.	mg/L		
Molybdenum	4.56	mg/L		
Nickel	9.10	mg/L		
Silver	.1	mg/L	<=W	
Strontium	153.	mg/L		
Titanium	15.5	mg/L		
Vanadium	3.02	mg/L		
Zinc	382.	mg/L		
Calcium	9500	mg/L		
Sodium	757.	mg/L		
Potassium	13600	mg/L		
Arsenic	0.11	mg/L		UAL
Selenium	0.36	mg/L		UAL
Antimony	0.45	mg/L		UAL
Solids; total	147000	mg/L	WST	
Solids; total, ash	75300	mg/L		
Solids; total, loss on ign	71800	mg/L		
Conductivity		uS/cm	NDUA	
pH		none	NDUA	
Alkalinity; total fixed endpt		mg/L CaCO3	NDUA	
Nitrogen; nitrite	4.9	mg/L		
Nitrogen; nitrate+nitrite	5	mg/L	<=WE	
Nitrogen; ammonia+ammonium	3400	mg/L		
Phosphorus; phosphate	209.	mg/L		
Nitrogen; total Kjeldahl	3370	mg/L		
Phosphorus; total	1260	mg/L		
Escherichia coli	770	c/1 g dry	<	

Appendix IV - Data

For all the raw data pertaining to the research for this thesis, please refer to the Excel files on the accompanying CD since the files were too large to print here. For *Folsomia* and *Lumbricus*, the data for both biosolids are in a single file. For the plants, the data are first organized by species, then by biosolids source in different files.

Appendix V - Future Work with Aquatic Organisms

For the background information and protocol development for *Daphnia magna* and *Hyalella azteca* pertaining to the assessing for potential impact of biosolids on the aquatic environment, refer to the separate file entitled Future Work with Aquatic Organisms.

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