The effects of multi-walled carbon nanotube exposure on soil organisms

by

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

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Abstract

With the rapid proliferation of carbon nanotube technologies and consumer products comes a need to research the toxicological and ecotoxicological effects of these materials. This research attempted to develop a baseline knowledge of the effects of bulk, unmodified multi-walled carbon nanotubes on commonly studied soil toxicology test organisms: earthworms, springtails, and agricultural plants. In order to minimize confounding factors in the study, a slurry composed of bulk multi-walled carbon nanotubes, silica sand, and water was used to amend test soil without the use of surfactants or functionalization.

Analysis of data produced by these experiments showed no significant trends resulting from the exposure of the test organisms to artificial soil amended by the multi-walled carbon nanotube slurry. It was observed, however that carbon nanotubes accumulated in the gut of the earthworm *Eisenia andrei* and were expelled as castings in the test soil.

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List of Abbreviations

CB	Carbon black
CBNM	Carbon-based nanomaterial(s)
CBNP	Carbon-based nanoparticle(s)
CDNP	Combustion-derived nanoparticle(s)
CNF	Carbon nanofibre(s)
CNT	Carbon nanotube(s)
DWNT	Double-walled nanotube(s)
ENM	Engineered nanomaterial(s)
ENP	Engineered nanoparticle(s)
LCA	Life cycle assessment(s)
MBNP	Metal based nanoparticle(s)
MWCNT	Multi-walled carbon nanotube(s)
NM	Nanomaterial(s)
NOM	Natural organic matter
NP	Nanoparticle(s)
PM	Particulate matter
PSF	Phagosomal stimulant fluid
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
SWCNT	Single-walled carbon nanotube(s)
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis

“Somewhere, something incredible is waiting to be known.”

- Carl Sagan

1.0 Introduction

1.1 General Introduction

Through the majority of the approximately 200 000 years of human innovation, technological advances have generally come in the form of the large scale, the grandiose. With the advent of the computer age, this trend began to slow, and then to reverse. Instead of "bigger is better", high technology is becoming smaller and smarter. Today nanomaterials – objects that are often mere molecules in size – are not the future of technological innovation; they are an aspect of our everyday lives.

This nanotechnology is the making and manipulating of materials on the nanometer scale. The fundamental principle is that materials within the nano size range have novel and exploitable properties.

In science fiction, from Arthur C. Clarke's 1956 short story *The Next Tenants* to Michael Crichton's 2002 novel *Prey*, nanotechnology has been a subject of caution and apprehension. While we are not (yet) under threat from marauding nanomachines, the introduction rate of nanotechnology into human affairs has increased tremendously. This has been unaccompanied by knowledge of their impact on human and environmental health. As a result, consumer advocacy groups focused on nanoparticles are becoming more common. For example, PEN, the Project on Emerging Nanotechnologies is an online database containing lists of consumer products containing (or claiming to contain) nanomaterials (http://www.nanotechproject.org/inventories/consumer/).

Clearly there is a need to know how nanotechnology is impacting all aspects of the environment, but any discussion of this requires a clarification of terminology. Yet

even terminology can be controversial (Hansen *et al*., 2007; Lovestam *et al*., 2010; Maynard, 2011; Stamm, 2011). According to Hansen *et al*. (2007), for something to be considered nanotechnology, two criteria must be met: the technology must have some structure that has at least one dimension in the approximate range of 1-100 nm, and the nanostructure must provide the system with properties different from the bulk properties. Nanomaterials possess different properties to bulk materials of the same type due to their size and increased surface area, often even expressing different colouring than bulk material. For example, gold nanoparticles appear to be red (Tiede *et al.,* 2008). Differences in physicochemical properties have generally been attributed to the influence of atomic forces such as Van der Waals forces that would not be seen in bulk form materials, as well as the dramatic increase in surface to volume ratio (Kennedy *et al.,* 2008; Tiede *et al.,* 2008).

Part of the terminology debate revolves around the distinction between nanomaterials and nanoparticles, which in this document will be referred to as NM and NP, respectively. Most agree that both have at least one dimension in the 1-100 nm range and NP should be considered a subset of NM. Klaine *et al.* (2008) adopted this distinction. Any substance with one dimension measuring less than 100 nm is a NM. Substances that possess two or more dimensions between 1 and 100 nm are NP. For example, a nanomaterial, nanowire (composed of Ni, Si, SiO_2, or one of several other possible materials) could have a diameter of 1-2 nm, while having a length more than 1000 times longer. Conversely, a buckminsterfullerene, as a spheroid with diameter less than 1 nm, would be considered a NP. In this case a NP has a dimension less than 1 nm, emphasizing that 1 nm is not always an absolute cut off. Hansen *et al.* (2007) suggested

that NM should be categorized on "the location of the nanoscale structure in the system". This led to three main categories: materials were either nanostructured in the bulk, have nanostructure on the surface, or contain nanostructured particles (NP). The NP in turn could be surface bound, suspended in liquids, suspended in solids or airborne. However, in the literature the terms NM and NP are not applied consistently, but despite this, for the purpose of this thesis, the terminology used in the papers being cited will be maintained. Because the research in this thesis is on carbon nanotubes, which can conform to the descriptor NP, NP will be used most often. The final classification of NM and NP revolves around their origins. NP and NM can arise from natural processes, as inadvertent or incidental by-products of industrial processes, or through intentional preparations. The latter is referred to as engineered NP or engineered NM.

1.1.1 Natural nanoparticles

There have always been NP and NM present in the environment, albeit historically at exceedingly low concentrations. Carbon nanotubes (from forest fires and volcanic activity), silver, gold, and iron oxides, organic colloids (suspended aggregates ranging in diameter from 1 nm to 1 μm), biogenic magnetite (produced by bacteria, mollusks, arthropods, birds, the brains of humans and presumably other mammals), and viruses are all naturally occurring nanoparticles (Nowack & Bucheli, 2007; Oberdörster *et al.*, 2005). Biological NP are released into the environment directly by the organisms in the form of exudates, or through the degradation of organic matter (Handy *et al.*, 2008). NP of non-biological origins are typically the result of the weathering of rock materials or precipitates (e.g. clay minerals, iron oxyhydroxides found in soils or in

aquatic environments), or through volcanic eruptions or meteorite impacts (e.g. carbon nanoparticles) (Handy *et al.*, 2008).

1.1.2 Nanoparticles as byproducts

NP are also inadvertently produced as the byproducts of industry and the combustion of fossil fuels (Nowack & Bucheli, 2007). NP produced and released in this manner are sometimes referred to as combustion-derived nanoparticles (CDNP). Examples and sources include: diesel exhaust particles from combustion of diesel oil, welding fume from welding processes, and fly ash from combustion of coal or oil (Donaldson *et al.,* 2005). In the combustion of gas and diesel fuels in engines, CDNP are produced from diesel (Donaldson *et al.,* 2005). It has been estimated that up to 36% of all NM produced (as of 2008 – this number is likely much lower today) are the byproduct of gasoline combustion (Klaine *et al.,* 2008). NM produced and released in this manner are not likely to be as serious a toxicological or ecotoxicological risk due to the low levels of production. Greater concern is placed upon the accidental release of purposefully engineered NP from consumer goods.

1.1.3 Engineered nanoparticles

The intentional engineering and production of NP and NM has quickly become a widespread and profitable industry, as well as a major area of research interest. In 2008, it was estimated that nanotechnology would become a $3 trillion industry by 2014 (Wardack *et al.*, 2008). The commercial uses of NP and NM include cosmetics, sunscreen, electronics, construction, aerospace, sporting goods, textiles (clothing), energy storage, and medical applications (Theng & Yuan, 2008; Marchant *et al.,* 2010; Oberdörster *et al.*, 2005). The engineered NM can be divided into two main classes based

on their chemical composition: metal-based nanoparticles (MBNP) and carbon-based nanoparticles (CBNP).

1.1.4 Toxicology and ecotoxicology of nanoparticles and nanomaterials

NP from all three sources have toxicological and/or ecotoxicological implications. Most naturally occurring NM are normal components of the environment and regularly interact with the biota, or are in some cases part of the biota (Pan & Xing, 2012). Some of these naturally occurring NM are a concern to human and environmental health; bismuth oxide and cristobalite are found in volcanic dust and ash, and have been shown to cause lymph node granulomas and lung inflammation to rats exposed via inhalation (Lee & Richards, 2003).

Combustion-derived nanoparticles (CDNP) have been a subject of study by respiratory toxicologists for some time (Donaldson *et al*., 2005; Stone *et al*., 2007). In the air, NP are often referred to as ultrafine particles, and measurements are made for particles of a certain size rather than for particles of specific types. Two much-studied fractions are particulate matter 10 (PM10) and particulate matter 2.5 (PM 2.5). PM10 contains particles up to 10 μm in size; PM2.5, particles up 2.5 μm in size. They both will contain CDNP. PM10, PM2.5, and CDNP are a hazard to the lungs where they can cause oxidative stress, inflammation and cancer (Donaldson *et al*., 2005). Epidemiological studies suggest that PM10 can even have effects on the cardiovascular system (Donaldson *et al*., 2005).

Research into the environmental and health implications of engineered NP has increased dramatically in recent years, undoubtedly in an attempt to catch up to the incredible rate of the technologies' proliferation. Like other sources, engineered NP have

an increased potential for pulmonary damage due to the increased surface-to-volume ratio compared to bulk forms of comparable materials (Oberdörster *et al.*, 2005; Tiede *et al.*, 2008; Smart *et al.*, 2006). The risk of toxicological effects are also greatly increased due to this effect, as well as the increased likelihood of imperfections on the surface of the materials, which become active sites (Tiede *et al.,* 2008).

The toxicology and ecotoxicology of engineered NP is just beginning, but is important to investigate and understand for several reasons. First, it is of the upmost importance to protect both the general population and the environment from dangerous levels of exposure to potentially damaging substances. Second, it must be determined how to safely and responsibly continue the potentially world-changing technological innovations provided by pursuing nanotechnology. However, studying the toxicology and ecotoxicology of NP is quite difficult and complex. One reason for this is the wide variety of engineered nanomaterials. Another is the uniqueness of the problem. As a whole, NP are defined as a different class of substance with different properties and behavior than the corresponding bulk materials (Forloni, 2012). This means that conventional toxicological exposure regimens and methods of deriving dose-effect relationships might not apply (Forloni, 2012). Some researchers have estimated that toxicity testing on existing NP and NM in the United States would take up to 54 years and cost up to one billion dollars (Choi *et al.*, 2009).

Some thoughts have been expressed on how NPs might generally exert toxic effects (Pan & Xing, 2012). NP might generate reactive oxygen species (ROS), which could result in oxidative stress in organisms. NP might also disrupt cell membranes by puncturing them. They might interfere with the flow of electrons in energy metabolism,

and can interact directly with proteins (and other biomolecules) and change their conformation, possibly leading to the disruption of cell signaling pathways.

Engineered NP can potentially be released into air, water, and soil. Materials of particular interest have been TiO_2, Ag, Cu, Al_2O_3, and CNT due to their widespread use in consumer products (McShane *et al.,* 2011; Petersen & Henry, 2011; Lin & Xing, 2007). The focus of this thesis is on engineered NP in soil, which might be the most complex environment.

1.2 Soil

Soil is the outermost layer of the planet earth. The upper limit is the air/soil boundary; the lower limit is often set arbitrarily at 2 m. Soil is characterized by having layers (horizons) and/or the ability to support plants (Coleman, 1994). Three main components make up soil: minerals, organic matter, and living organisms. Minerals come from rocks below or nearby. Organic matter (humus) arises from the decay of microbes, plants and animals that use the soils. The living organisms in soil are very diverse: from bacteria to mammals. Innumerable aggregates of matter interspersed with innumerable number of small pores gives soil enormous surface area and stable habitats for microorganisms. Three large particle size classes are clays (0.1 -2 μm in diameter), silts (2-25 μm in diameter), and sands (0.05-2 mm diameter), but soil also contains NP and NM.

1.2.1 Natural nanoparticles in soil

With the dynamic nature of soil ecosystems, and the diversity of potential components, it is unsurprising that there is a wide variety of naturally occurring NM present in a given system. Table 1 lists a number of commonly occurring natural (and a

few manufactured or byproduct) NP and NM that could be found in a typical soil sample. Nanoclays and metal oxides and hydroxides are common, as they are products of the natural weathering of soil components (Nowack & Bucheli, 2007; Theng & Yuan, 2008). Biological soil NP include viruses, enzymes and the byproducts of microbial decomposition of organic matter (Handy *et al.*, 2008). Although they would be rare and depend on geographical location, carbon NP and NM from meteorite impacts or volcanic activity could also be present (Handy *et al.*, 2008).

Table 1. List of engineered and naturally occurring nanoparticles in soil (Nowack & Bucheli, 2007; Theng & Yuan, 2008).

General Particle Description	**Formation**	**Class**	**Examples**
Nano clay	Abiotic weathering processes of rock	Planar Hydrous Phyllosilicates	Saponite, hectorite, monomorillonite
Metal (hydr)oxides	Abiotic weathering	Al-oxides, iron-oxides, Mn-oxides	Biggsite, boehmite, goethite, hematite, birnessite, vernadite
Humic substances (biologically derived molecules)	Aggregates bound by weak dispersive forces and forming micelles in solution	Organic, carbon-containing, some inorganic	Bacterial enzymes, biogenic magnetite
Viruses	Released from infected prokaryotic and eukaryotic organisms	Bacteriophages, animal and plant viruses	T4, MS2*
Mobile colloids	Aggregation of above particle types	Varies	Biopolymers, metal oxide aggregates
Combustion by-products	By-product	Carbon-containing	CNT, fullerenes, carbon black
Oxides	Engineered	Inorganic	TiO_2, SiO_2
Metals	Engineered	Inorganic	Silver, gold
Salts	Engineered	Inorganic	Metal phosphates

*T4 and MS2 are bacteriophages: viruses that infect bacteria.

1.2.2 Engineered nanoparticles in soil ecosystems

As detailed in Table 1, there are a number of engineered NP and NM found in soil ecosystems. The release of bulk, raw-form engineered NM into the environment is likely to be an extremely rare event, with industrial spill incidents being the most common. Carbon NP and NM could be found in low concentrations, as the byproducts of fossil fuel combustion and industrial processes (Nowack & Bucheli, 2007). The most likely and most problematic pathway for NP and NM into the soil ecosystem is through waste treatment biosolids, as components of medications, cosmetic products, and clothing (Turco *et al.*, 2011). The other major form of NP and NM contamination in soil is believed to be that of nanocomposites from the degradation and recycling of consumer products (Turco *et al.*, 2011).

1.2.3 The soil ecosystem

Soils contain a wide variety of microhabitats that support a very diverse biota (Coleman, 1994). The biota is the combined flora and fauna of a region, and soil biota can be classified in several ways, a traditional one being size. The microflora are less than 0.1 mm in size, and viruses, archaea, bacteria, fungi, and algae can be organized into this class. The macroflora would include mosses and the roots of vascular plants, which are continuously growing and dying in soil, supplying food to soil organisms. The fauna is divided into micro-, meso-, and macro-fauna. The microfauna is less than 0.1 mm in size and includes protozoa and nematodes, although nematodes sometimes can be considered to transition into the next size class. Mesofauna are 0.1 to 2 mm in size and the most abundant members are Collembola (springtails) and mites. Mesofauna use existing pore spaces, cavities, or channels to move about the soil, whereas the next size

class has the ability to reshape the soil. Macrofauna are larger than 2 mm in size and include earthworms, spiders, termites, and ants. A few mammals such as voles would also be put in this class.

The physical and chemical components of soil are as complex and diverse as the biological component. The composition of soil varies based on parent materials, climate, topography, and the biological components (Kilham, 1996).

1.2.4 Soil ecotoxicology

Soils often become the recipients of a wide range of hazardous or potentially hazardous materials generated by human activities. Soil ecotoxicology is the study of the toxicity of these chemical, physical or biological substances to the organisms of soil (Van Gestel, 2012). The discipline includes exploring the sources, fate (biodegradation), transport, and effects of the contaminants. Two approaches to ecotoxicological risk assessment can be distinguished: the predictive (prognosis) approach aims to forecast possible effects of new materials in order to regulate their usage or restrict their introduction into the market, while the diagnostic approach tries to assess the actual ecological harm arising from a contamination event and may give insight into remediation and management of contaminated land.

The predictive approach uses laboratory tests to derive toxicity data (empirical) that can be used to set "safe" exposure levels. Two variables in these tests are the types of soil in which the tests are done and the kinds of organisms tested. Artificial soil is often the medium of choice. Artificial soil is composed of 70% (by mass) silica sand, 20% kaolin clay, and 10% dried *Sphagnum* sp. peat, pH adjusted to 6.0 - 7.5 with calcium carbonate, and hydrated with deionized water. It is designed to approximate a natural soil

and to be easily and consistently produced in a laboratory setting (Environment Canada, 2004). A variety of soil organisms has been used in toxicity tests. Some important criteria for choosing a particular organism and test have been pointed out by Van Gestel (2012). One is how practical the maintenance and use of the organism is, another is the ecological relevance of the test.

1.2.5 Earthworms in ecotoxicology

Earthworms are ubiquitous in soil ecosystems worldwide (excepting permafrost), and can account for more than 90% of soil macrofauna biomass (Doube & Brown, 1998; Bonkowski & Schaefer, 1997). Organism density can reach up to 2000 individuals, or roughly 1 L of earthworm gut per square meter of soil (Drake & Horn, 2007). Decomposition and movement of soil organic matter, soil aeration and penetrability, nutrient movement, and soil microbial activity are all influenced by earthworm activity (Doube & Brown, 1998; Killham, 1996).

There are generally held to be approximately 19 species of earthworm in Ontario, 17 of which are considered invasive (Reynolds, 1977). All three of the commonly used test species (*Eisenia andrei, Eisenia fetida, Lumbricus terrestris*) are indigenous to Europe (Reynolds, 1998). *Eisenia andrei* and *Eisenia fetida* are the most commonly used species in toxicity testing, as their gregarious nature translates well into the contexts of reproduction tests (Environment Canada, 2004).

Earthworm endpoints for toxicity testing are adult survival, juvenile production and survival, and juvenile wet/dry mass and percent moisture content after a 63-day exposure period (Environment Canada, 2004).

1.2.6 Springtails in ecotoxicology

Springtails (members of the class Collembola) are arthropods, although they are not considered to be insects (class Insecta) based on phylogenetic data (Nardi *et al.*, 2003). Like earthworms, collembolans are extremely widespread in their distribution. They are typically found in the leaf litter layer, and consume fungi and the aforementioned leaf litter (Fountain & Hopkin, 2005). The "springtail" common name is a descriptive one, attributable to the presence of a furca, which is an organ that allows springtails to propel themselves over relatively large distances when disturbed.

Folsomia candida is a parthenogenic species of springtail that is commonly used in soil toxicology (including this research), largely because it can be easily cultured and large numbers of age-synchronized individuals can be produced for testing (Fountain & Hopkin, 2005).

Folsomia candida endpoints for toxicity testing are adult survival and progeny production and survival after a 21-day exposure (Environment Canada, 2007).

1.2.7 Seedling emergence and growth in soil ecotoxicology

Plant species are the most visible members of the soil community, and the most directly vital to human civilization. A wide variety of plant species are commonly used in soil ecotoxicology testing. The species recommended in experimental procedures are generally agronomic, garden, and grassland species; typically species that are commonly found in the area of concern, and are of ecological or agricultural importance (Environment Canada, 2005). It is common to test both a monocotyledonous (single seed-leaf, or grass-like) and dicotyledonous (two-leaf seed) in order to account for differences among these two major groups of flowering plants (Environment Canada, 2005).

Plant exposure endpoints (after 14 or 21-day exposures, depending on the species) are seedling emergence, root length and dry weight, and shoot length and dry weight (Environment Canada, 2005).

1.2.8 Soil ecotoxicology studies on nanoparticles and nanomaterials

Soil nanotoxicology as a field of study is relatively new, but growing quickly. Pan and Xing (2012) have recently stressed the need for more information on the behavior of NP in soil and the impact on the soil ecosystem. The entry of NP into soil will likely depend on the product. One example is personal care products, such as cosmetics and dietary ingredients. In these kinds of products, NP are well dispersed and enter the environment mostly via sewage treatment plants. NP could accumulate in biosolids and the sludge could be applied to the soil as an organic amendment. Most work on toxicity to soil organisms has been done with earthworms and the NP have been metal-based (Tourinho *et al*., 2012). MBNPs have been a focus because they are in a wide range of consumer products already in circulation. The metals include aluminum oxide, copper, gold, silver, titanium oxide, and zinc oxide. Copper, silver and titanium oxide have received perhaps the most attention.

Eisenia fetida exposed to copper nanoparticles in artificial soil did not exhibit any sub chronic effects, however nano-sized copper particles were found to accumulate in the tissues of the earthworms (Tourinho *et al*., 2012; Unrine *et al.,* 2010). A significant decrease in reproduction was seen in the same species when exposed to copper nanoparticles in sandy loam, or soil with a low organic content (Tourinho *et al*., 2012; Shoults-Wilson *et al*., 2011). Nano-sized copper has also been found to inhibit the growth and function of various plant species; however, the toxicity has not been found to be

significantly different from that of bulk copper, which is itself highly phytotoxic (Musante & White, 2010).

Silver nanoparticles have been studied extensively because they are used as bactericides in clothing and other products. They have been found to accumulate in and inhibit the reproduction of the earthworm *Eisenia fetida* at concentrations of 1000 mg/kg in artificial soil (Tourinho *et al.*, 2012; Heckmann *et al.*, 2011).

Titanium dioxide NM are commonly found as additives in cosmetics (make-up, skin lotions, sunscreen) and food products, particularly sweets (Weir *et al*., 2012). *Eisenia andrei* and *Eisenia fetida* were exposed to nano-sized titanium dioxide particles, resulting in the inhibition of reproduction at 1000 mg/kg in sandy loam (Heckmann *et al.*, 2011). In the same study, micro-sized titanium dioxide did not show the same inhibitory effects (Heckmann *et al*., 2011). A similar set of experiments conducted in an artificial soil medium did not result in any effect on reproduction, survival, or growth (McShane *et al*., 2011). This conflicting data present in the literature indicates that standardized materials and test conditions be utilized in the future.

In reviewing the literature on MBNPs in soil, Tourinho *et al.* (2012) conclude that overall the toxicity of MBNPs in soil is something to be concerned about, but the subject needs more study. This is because the results often conflict. Some of this has been attributed to the use of different types of soils. Another set of variables is the characteristics of the metal NM. They can differ in size, shape and coating. The endpoints in the toxicity tests and the experimental design of the toxicity tests have also been variable. All of this makes drawing conclusions about the toxicity of metal NM challenging. Tourinho *et al.* (2012) recommend that more standardized testing be done.

1.3 Carbon Nanotubes

Carbon nanotubes (CNT) are allotropes of carbon and members of the fullerene structural family. Their discovery is often attributed to Iijima in 1991 (Iijima, 1991), but carefully perusal of the scientific literature, especially the Russian literature of 1950s, suggests that CNT were first described sixty years earlier and had been identified sporadically in the intervening years (Monthioux & Kuznetsov, 2006). The importance of the 1991 article by Iijima appears to have been in bringing CNT to a broader audience that was ready to consider nanotechnology. The Iijima study was the first to show the laboratory synthesis of CNT without the need for any catalysts. CNT can to be synthesized during natural events such as volcanic activity, and their presence identified in a 10,000 -year old ice core (Murr *et al.,* 2004). CNT levels in the environment from natural processes are extremely low (Pan & Xing, 2012). The ability to synthesize CNT has led to considerable knowledge of their properties, the development of applications for them, and concerns about their environmental impacts.

Discovered in 1991 by Japanese C60 fullerene researchers, carbon nanotubes (CNT) are essentially graphene sheets formed into a tube; carbon atoms arranged into fused benzene rings (Iijima, 1991). The two main types of CNT are single-walled CNT (SWCNT) and multi-walled CNT (MWCNT). SWCNT generally have a diameter ranging between 0.5 and 2.5 nm, with lengths that are varied, but can be engineered to theoretically any conceivable size. MWCNT, combining between 2 or more (sometimes up to 30) concentric layers of SWCNT, are far more common, and can have diameters up to several hundred nm, depending on the number of layers (Sellers *et al.*, 2009; Petersen

& Henry, 2011). It is also possible, but less common, for MWCNT to consist of one long, rolled sheet of graphene, resembling rolled parchment.

Properties common to all unaltered species of CNT include insolubility in water and solvents, an extremely high strength-to-weight ratio (460x that of steel), flexibility, light weight, and unique electrical and thermal conductivity (Sellers *et al.*, 2009; Klaine *et al.*, 2008). Aqueous suspensions of CNT can be achieved through various methods, including sonication, the addition of natural organic matter, use of surfactants such as sodium dodecyl sulfate (SDS), and polymers including polyvinylpyrrolidone, polyethyleneimine (Petersen & Henry, 2011).

Solubility in water can be attained through the addition of functional groups to the surface wall of CNT. These are molecules or polymers, referred to as chemical functions, bound to surface imperfections, often covalently (Petersen *et al*., 2011a). A wide variety of chemical functions are commonly employed, including polyethyleneimine (PEI) ammonium, acetamido fluorescein isothiocyanate, methotrexate, and amphotericin groups (Kostarelos *et al.*, 2007). The ability to be functionalized is a key factor in the usefulness (and potential danger) of CNT. The addition of these chemical functions completely alters the surface chemistry of the particle, resulting in a new set of chemical properties (Kostarelos *et al.*, 2007).

Compared to SWCNT, the multi-walled forms tend to be more chemically active, due to an increased tendency for imperfections in the outer wall structure. These discrepancies include pentagonally arranged C (as opposed to the usual hexagonal arrangement), sp^3 hybridization (instead of sp^2), and lattice vacancies (Petersen *et al*., 2011b; Köhler *et al.,* 2008).

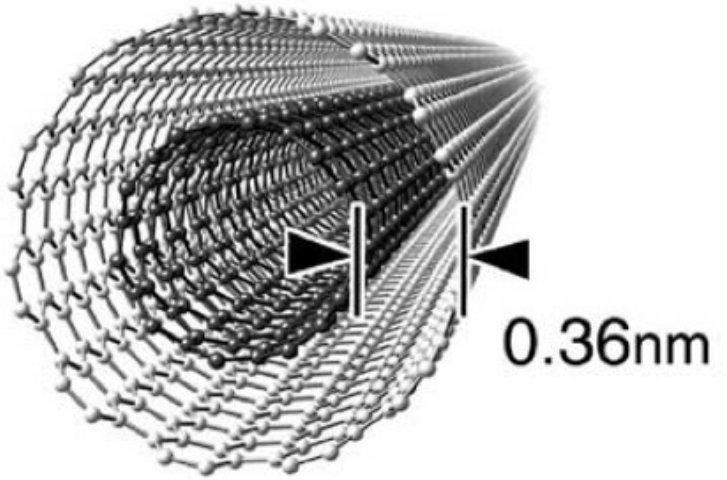

Fig. 1.1 Artist's rendering of the structure of a multi-walled carbon nanotube. Note the hexagonally arranged carbon, and concentric tubes of graphene sheets (Iijima, 2002).

CNT have special properties that have led to worldwide interest in their scientific and technological applications. These properties include high electrical conductivity, rapid heat transport, large surface/mass ratios, and great strength. Compared to other fiber materials, CNT have a unique combination of stiffness, strength, and tenacity and at the same time being very lightweight. The properties of CNT can vary with the method of fabrication and whether the CNT are chemically functionalized, long or short, open or closed at the ends, and single-, or multi-walled.

1.3.1 Commercial applications of carbon nanotubes

CNT are being produced commercially and are found in a growing list of products. The worldwide production has been estimated at between 350 and 500 tons per year (Mueller & Nowack, 2008). Their unique set of properties has resulted in a wide range of commercial applications, from drug delivery, water purification, chemical sensors, molecular computing, super capacitors and energy storage (fuel cell electrodes and batteries), to structural components of high-end tennis racquets and bicycles (Klaine

et al., 2008). Undoubtedly, these products will eventually enter the soil, either through landfill or industrial byproducts.

CNT are often used in the production of composite materials. Composites are made from two or more natural or engineered components, often a strong fiber and a surrounding matrix. CNT have been incorporated into various resins because as well as their strength and lightness, CNT are very pliable and have elasticity. The resins are used in hockey sticks, tennis rackets, baseball bats, skis, and vehicle parts. The breakdown of these composites is expected to be a major source of CNT contamination in the environment.

1.3.2 Ecotoxicology of carbon nanotubes

Although beneficial, CNT are being scrutinized for their potential impacts on both human and ecosystem health. This is being looked at from two very different perspectives. One consideration focuses on the manufacturing and cradle-to-grave life cycle assessments (LCA) (Upadhyayula *et al.,* 2012). The life cycle of CNT products can be considered to have four interconnected phases: the acquisition of raw material, manufacturing, use, and disposal. Several conclusions were made from a recent LCA. The first is that manufacturing can dominate the environmental impact because of the energy-intensive processes required for CNT production (Upadhyayula *et al.,* 2012). The second consideration is the toxicity/ecotoxicity of CNT released into the environment through the course of manufacturing, use, and disposal. This approach needs more data on the toxicity/ecotoxicity of specific CNT and is the focus of this thesis.

Little information is available on the release of CNT from the use and disposal of CNT products, but speculation on possible routes has begun (Petersen *et al.,* 2011c). A

frequently used example is a CNT/polymer composite framed tennis racket. Whenever a racket frame is scratched on the court, some CNT might be released. In a landfill, the composite could undergo hydrolytic degradation. Washing and rain falling onto nanocomposite surfaces might release loosely bound CNT. Incineration of CNT products might release carbon nanofibers (CNF). CNF are larger than CNT, with diameters of 50 to 150 nm and lengths of up to 100 μm.

A wide range of organisms has shown sensitivity to the effects of CNT exposure. Both functionalized and non-functionalized single and MWCNT have been shown to be internalized by a wide variety of cells, including mammalian fibroblasts, protozoa, yeast, fungi, and bacteria (Ghafari *et al*., 2008; Kostarelos *et al.*, 2007). Of particular concern is the evidence of carcinogenic effects when CNT are introduced to the lungs of mammals (Smart *et al.*, 2006). Cellular damage caused by CNT is thought to be due to micro abrasion, similar to the effect seen with asbestos, and through oxidative stress caused by free radicals (Smart *et al.*, 2006).

In ecotoxicology testing with aquatic organisms, carbon nanotubes have been found to accumulate on and cause damage to the gill surface of exposed rainbow trout (Handy *et al.*, 2008). *Daphnia magna* (an ecotoxicological mainstay known as the water flea) were immobilized and killed when CNT accumulated in their gut (Zhu *et al.*, 2009; Eddington *et al.,* 2010). Conversely, when the fruit fly *Drosophila melanogaster* was given food spiked with SWCNT, no effects on health or reproduction were noted (Leeuw *et al.*, 2007).

1.4 Carbon nanotubes in soil toxicology and ecotoxicology

Consumer products containing CNT are still relatively rare, consisting mainly of sports equipment. There will soon be an influx of CNT waste material and contamination in soil ecosystems. CNT soil contamination will most likely come in the form of polymers and CNT nanocomposites (Petersen *et al*., 2011b). This composite contamination will be the result of degradation during the usage and disposal of CNT-containing products, due to ultraviolet radiation, biological decomposition, abrasion/physical damage, incineration, hydrolytic and thermodegradation (Petersen *et al.*, 2011b). There will also be a certain amount of "raw" carbon nanotube contamination due to industrial production, manufacturing, and spills.

Carbon nanotubes have not yet been found to be toxic in a soil medium (Petersen *et al.*, 2008; Scott-Fordsmand *et al.*, 2008). In a reproductive test in which the earthworm *Eisenia veneta* was given food spiked with double-walled carbon nanotubes, the production of cocoons was significantly decreased at concentrations of 495 mg/kg food dry weight and up (Scott-Fordsmand *et al.*, 2008).

Petersen and his colleagues have focused on the effects of carbon nanotubes on earthworms in soil media (Petersen *et al.*, 2008; Petersen *et al.,* 2011 a & b). After a 28-day exposure to MWCNTs, *Eisenia fetida* had mass lower than expected (Petersen *et al.*, 2008). A 2011 study examining the accumulation of carbon nanotubes in oligochaete tissues showed no significant accumulation in *Lumbriculus variegatus* tissues (Petersen & Henry, 2011).

Studies involving crop and garden plant species are more common than those involving invertebrates, largely due to the health and economic implications of

contaminated food crops. Root growth inhibition was noted in lettuce and tomato seedlings exposed to both SWCNT and MWCNT (Cañas *et al.,* 2008). In contrast, this same study found that this exposure actually stimulated root growth in cucumber and onion seedlings (Cañas *et al.*, 2008).

No significant effects on germination or root elongation were seen in zuccini seeds exposed hydroponically to non-functionalized MWCNT dispersed with SDS (Stampoulis *et al.*, 2009). Similarly, rapeseed, radish, lettuce, corn, and cucumber seeds exposed hydroponically to non-functionalized MWCNT sprouted normally, with no effect on root growth (Lin & Xing, 2007). A study in which tomato seeds were grown in MWCNT-spiked agar medium showed a significant decrease in seedling emergence time, suggesting that the nanotubes may have penetrated the seed coat resulting in imbibation (Khodakovskaya *et al.*, 2011). Further study found MWCNT in tomato roots, leaves, and fruit of plants grown in a simlar medium, as well as those grown in spiked commercial soil mix (Khodakovskaya *et al*., 2011). This suggests the possibility for uptake and translocation of CNT by plants.

1.5 The Carbon Nanotube Slurry

A method of delivering non-functionalized nanotubes without the use of a surfactant is a great challenge. The propensity for the nanotubes to aggregate together, and their insoluble nature required that a novel procedure be developed for amending soils with nanotubes. A "slurry" of MWCNT was used. This slurry was composed of silica sand, de-ionized water, and at one point ethanol. The silica sand was from the same stock used to produce the artificial soil. Its purpose was to provide a physical matrix for the nanotube dispersal without reacting with the tubes, and without the tubes

adsorbing strongly to their surface. Ethanol was originally used in order to act as a solvent, but was excluded from the definitive tests.

1.6 Research Objectives

1. To develop a method of amending artificial soil with non-functionalized carbon nanotubes.
2. To determine the effects of carbon nanotube-amended artificial soil to the survival and reproduction of the soil invertebrates *Eisenia andrei* and *Folsomia candida*
3. To determine the effects of carbon nanotube-amended artificial soil to seedling emergence and growth of the crop plants *Hordeum vulgare* and *Medicago sativa*

In order to achieve these objectives, a series of range-finding and definitive (chronic) toxicity tests were conducted on two plant species (*H. vulgare* and *M. sativa*) and two invertebrate species (*E. andrei* and *F. candida*). A method was developed to amend soil using a MWCNT slurry (Fig. A.1, Appendix). The test organisms were exposed to artificial soil amended with the MWCNT slurry. The results of these toxicity tests and the method of development of the slurry are the focus of this thesis.

2.0 Chapter 2: Material and Methods

2.1 Production of Artificial Soil

Artificial soil was formulated in the Stantec laboratory in Guelph, ON using procedures that closely follow those outlined in "*Biological Test Method: Tests for Toxicity of Contaminated Soil to Earthworms"* (Environment Canada, 2004). While wearing a p95 facemask to avoid inhalation of particles, 1 kg of dry *Sphagnum* sp. peat (obtained by weighing a sample of peat that was dried for 48 hours in a drying oven at 105 ºC, then determining the % moisture content) was added to 2 kg pulverized EPK kaolinite clay in a large Rubbermaid container and mixed inside a fume hood. When this mixture was homogeneous (by visual inspection), 7 kg of silica sand was added, and the mixture was once again mixed until homogeneous. Two liters (2 L) of deionized H_2O was then added (to achieve an approximate moisture content of 20%). The hydrated substrate was then homogenized using a hand blender. Approximately 160 mL of sieved $CaCO_3$ was added to the water-soil mixture in order to adjust the soil pH to 6.0-7.5 and mixed thoroughly. The amount of $CaCO_3$ tended to vary depending on the peat being used, and generally varied between batches of soil. The container was then labeled with the batch number, preparation date, name of the preparer, and the amount of added $CaCO_3$. The soil was allowed to sit for at least 3 days before being tested for pH once again.

2.2 Production of multi-walled carbon nanotube slurry

Non-functionalized multi-walled carbon nanotubes were obtained from Cheap Tubes Inc. (Brattleboro, VT). Energy dispersive X-ray spectroscopy was used to determine a purity of 95% by weight, with impurities consisting of approximately 1.5% carbon ash, 0.21% Cl, 0.56% Fe, 1.87% Ni, and 0.02% S, of total weight. The outer diameters of the tubes ranged in length from 30-50 nm; the inner diameters ranged from 5-15 nm. Nanotube lengths were stated to be between 10 and 20 um by the supplier.

1 kg of MWCNT (all from the same batch) was packaged in a large zip-lock bag. For health and safety reasons, they were stored within a fume hood at all times, and the package was opened to accommodate a scoopula. Future studies should utilize either pre-solublized tubes or request that the tubes be aliquoted into smaller, more easily handled packages.

When planning for screening tests involving *E. andrei* and *F. candida*, it was believed that the addition of a small amount of ethanol in the slurry would improve the emulsification of the slurry, while not significantly affecting toxicity. In order to produce 250 g of the required slurry, inside a fume hood, 11.89 g of dry MWCNT was weighed with an analytical balance and transferred to a sealable volumetric flask. Ethanol (2.5 mL of 98%) was added to the flask followed by 51.70 g of silica sand and approximately 184 mL of deionized H_2O. The slurry was then agitated, physically mixed, and sealed.

The creation of the slurry for the definitive tests was completed in the same manner; however, the ethanol component was omitted because toxic effects were observed in the screening tests and attributable to ethanol (Fig. 2 – 5, Appendix). Therefore, for the definitive tests, 49.16 g of dry MWCNT, 211.93 g of silica, and 52.98

mL of diH_2O were used in the formulation of the slurry. The soils were prepared concurrently for the earthworm and springtail tests; however, those used for the plant tests were prepared separately (Fig. A.1).

2.3 Culture of the Test Organisms

2.3.1 *Eisenia andrei*

Eisenia andrei cultures were developed and maintained at the Stantec soil laboratory in Guelph, ON according to requirements detailed by Environment Canada (Environment Canada, 2004). Genetically verified cultures of worms are grown in culture bins (Rubbermaid or similar bins ranging in volume from 10-50 L with perforated lids to allow for air exchange) containing a substrate consisting of approximately 43% potting soil and 57% *Sphagnum* sp. peat moss. This substrate is kept at a consistent moisture level (e.g., 70% of water-holding capacity). The substrate pH was adjusted with $CaCO_3$ to approximately pH 6. New substrate was allowed to stabilize for three days (at which point the moisture content and pH are confirmed) before worms were introduced. Culture bins were kept in a temperature (20+-2^oC) and humidity controlled culture room, with a regulated photoperiod (12h light, 12h dark) of at mean light intensity of 568 lux (Environment Canada, 2004).

The worms in culture were fed weekly a mixture of hydrated and cooked quick oats prepared with deionized water and allowed to cool for at least two hours before adding to the culture substrate. A supplement of decomposing lunchroom compost (fruits/vegetables) was also added to each culture monthly.

Cultures were inspected on a regular basis (usually done during weekly feeding) and checked for excess moisture, mold, dead worms, and worm density. Environment Canada protocols dictate a maximum density of 0.03 g wet wt/cm^3 in each culture. Population reduction was achieved through the splitting of a densely populated culture bin into two bins with fresh substrate. Culture age, condition, and feeding records were recorded and kept in a logbook for future reference.

Sexually mature, clitellate earthworms (250-600 mg wet weight) were used in the tests (see Fig. 2.1).

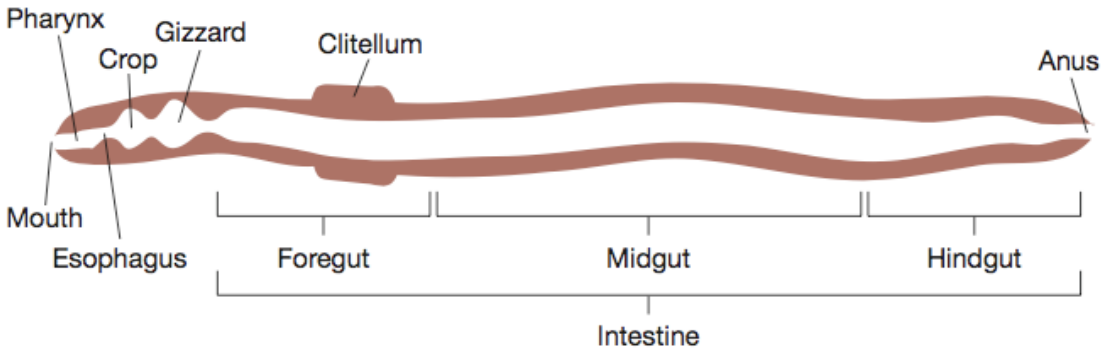

Fig. 2.1. Basic anatomy with alimentary canal of a standard oligochaete (Drake & Horn, 2007).

2.3.2 *Folsomia candida*

Folsomia candida (Fig. 2.2) cultures were developed and maintained at the Stantec soil laboratory in Guelph, ON according to requirements detailed by Environment Canada (Environment Canada, 2007). Genetically verified springtail cultures were kept in culture bins (1-6 L capacity translucent plastic Rubbermaid or similar containers with manually perforated lids for aeration) with a substrate mixture of 8:1 Plaster of Paris: activated charcoal (375 μm mesh) at a depth of approximately 1 cm. Springtail culture

bins were kept in culture rooms along with earthworm cultures at the same temperature, humidity, and lighting cycle. Cultures were monitored for population density and quality, and moisture every week.

Folsomia candida were fed commercially purchased activated dry yeast. The yeast was placed directly onto the surface of the Plaster of Paris/charcoal substrate weekly and then hydrated with a small amount of deionized water sprayed onto the surface.

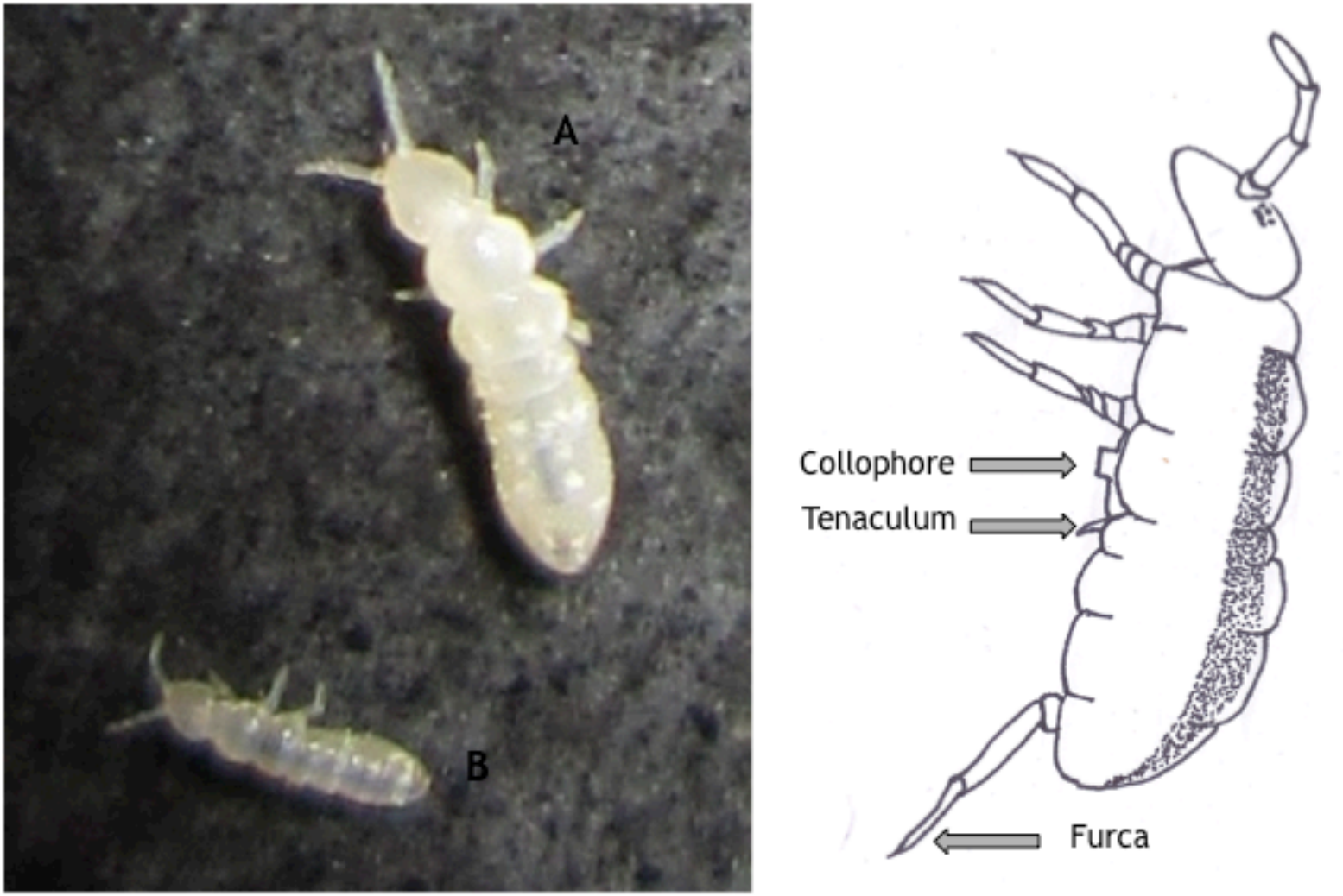

Fig. 2.2. Adult *Folsomia candida* (A) and juvenile *Folsomia candida* (B). Illustration denotes basic anatomy including "springing" appendages (furca and tenaculum) and the osmoregulation and excretion structure (collophore or ventral tube).

2.4 Earthworm Survival, Growth and Reproduction test

Test units for the chronic (63-d) earthworm test with *Eisenia andrei* were 500 mL glass, wide-mouth, mason jars. Ten (10) replicate jars were required for each treatment. The jars were labeled with the date, species, identifying name of the test, treatment, and treatment replicate. Soil used for the test was the artificial soil. A treatment series of exposure concentrations (100, 180, 320, 560, 1000, 1800, 3200, 5600, and 10 000 mg/kg multiwalled carbon nanotubes) was created using the stock slurry of MWCNT, silica sand, and diH_2O (described above). Experimental control treatments were unaltered artificial soil, a 1000 mg/kg activated carbon treatment, as well as a treatment of sand and water matching that of the 1000 mg/kg MWCNT treatment (without nanotubes added). The amended soil was added to each test unit (270 g in each); test units were then covered with lids. The units were then left to sit overnight to allow settling of the soil.

On the day the exposure was to begin, adult *Eisenia andrei* were isolated from the Stantec stock cultures. All organisms were sexually mature, each with a clitellum, and between 250 and 600 mg (wet weight). Worms were transferred using gloved hands and rounded forceps, taking care not to drop or roughly handle them. Any dropped or damaged worms were discarded. Two adult worms per unit, 10 units per treatment, and 12 treatments, 240 earthworms were required, so approximately 260 worms were collected from the cultures.

E. andrei collected for the test were transferred from the cultures into plastic bins lined with moist paper towel, after being cleaned in diH_2O. A sampling of 20 worms was weighed to ensure compliance with mean minimum weight requirements.

The test units were randomized, and a small amount (about 5 mL) of cooked oatmeal was added to each test unit (a small hole was hollowed out, then the oatmeal added, and then covered over). Two of the collected worms were then added to each test unit, and were observed until they had burrowed into the test soil (failure to enter the soil would suggest an acute avoidance response behavior). Units were then hydrated with diH_2O applied with a spray bottle. Each unit was then covered with aluminum foil, which was perforated 5 times to allow for some air exchange. The aluminum foil was secured with the ring portion of the mason jar lid. Units were then transferred to the test chambers, which were set at 20 ± 1^oC, 400-800 lux on a 16 h light, 8 h dark cycle, and constant humidity.

After 14 days of exposure, the units were removed from the chambers, and feeding and hydration were repeated. Units were randomized and returned to the chambers. This process was repeated on day 28.

On day 35, adult removal occurred. Test units were removed from the chambers, and reorganized according to treatment. Working from lowest concentration to highest concentration (sand and carbon controls were completed separately on clean aluminum foil to avoid cross contamination), the contents of each test unit are placed onto a sorting tray, one at a time, and the two adults were carefully removed, washed, weighed, and placed into the drying oven at 90 ºC. The condition of the adults, the presence of cocoons (egg packets), juveniles, and springtails or mites (common co-inhabitants of the worm cultures) were noted. The soil was carefully returned to the test unit, which was then hydrated with diH_2O from a spray bottle. After this process was completed in every test unit, they were randomized and returned to the test chambers. After 48 hours in the

drying oven, the dry weight of the adults was obtained. Feeding was completed again on days 42 and 56.

Processing of offspring occurred on day 63. Procedures for the processing were similar to those for the removal of adults. The soil was carefully separated using a spoon and forceps, into the smallest pieces possible to ensure that no juveniles were missed. The number of hatched (hollow and translucent) and unhatched (turgid and opaque) cocoons was also recorded. Juveniles were washed, dried with blotting paper, placed into labeled and pre-weighed aluminum pans, weighed, and placed into the drying oven for 48 hours, after which a dry weight was obtained.

All dried juvenile *Eisenia andrei* were retained and frozen, in order to be used for thermogravimetric analysis, Raman spectroscopy analysis, and possible SDS analysis at a future date.

2.5 Springtail Survival and Reproduction test

2.5.1 Age synchronization of *Folsomia candida*

In order to reduce variability in survival and reproductive ability during test procedures, *Folsomia candida* to be used for testing were age-synchronized. Paper strips were dipped into wet springtail culture media and allowed to dry. Small culture containers were also created in Petri dishes using the same method for large-scale culturing. When synchronization begins, the Petri cultures were hydrated with deionized water to the point of saturation, and then allowed to drain. Culture strips (usually 2) were then added to the Petri cultures.

Eggs from the main culture bins were carefully collected with a damp fine paintbrush and transferred to culture strips. For these experiments, 420 springtails

between the ages of 10 and 12 days were required. Culture strips were then placed into a Petri culture dish that had been hydrated with deionized water. The synchronization cultures were monitored daily, using a moist paintbrush method to transfer offspring found each day into a new culture dish. Egg strips were temporarily removed from the Petri culture, and a small amount of deionized water was used to wash any remaining offspring from the main dish into the new culture dish. The parent culture dish was then inspected once more for springtails. Applying pressure to them with the paintbrush destroyed all individuals found in this way. A small amount of yeast was added and the hydration of the new culture was assessed and adjusted. The new culture was then labeled with the date, and the number of offspring recorded.

This process was repeated each day, using a new recipient Petri culture dish, until the required number of *Folsomia candida* (in this case 420) between the ages of 10 and 12 days were obtained. During the synchronization process, cultures of cohorts in Petri dishes were labeled and stored within an empty Rubbermaid or a similar container with translucent sides, inside the culture room. Synchronized cohorts became the test organisms for the 28-day definitive test.

2.5.2 Procedures for Springtail test

Fifty-four (54) clean, 100-mL, wide-mouth, mason jars served as the test units for the test. Six (6) units were needed for each of the three (3) control treatments (5 replicates and one blank – a unit containing soil but no organisms), while four (4) units were required for each of the nine (9) carbon nanotube-amended treatment (3 replicates and one blank). Thirty (30) grams of each amended soil were weighed and placed into the appropriately labeled jars that corresponded to each treatment. A 25-g sample of soil

from each treatment was also collected and the pH and conductivity were determined. Ten (10) age-synchronized *F. candida* were then added to each unit (except the blanks, which contain no organisms). Springtails were placed onto black construction paper for ease of counting and transfer to the units. A small amount of dried yeast was added to each unit containing organisms. The units were then hydrated with a small amount of diH_2O applied with a spray bottle, and the lids tightened lightly to allow air exchange. Test units were randomized and placed into test chambers, which were set at 20^o C, 400-800 lux on a 16 h light, 8 h dark cycle, with constant humidity.

On day 7 (7 days after the addition of organisms), the test units were removed from the chamber, and the lids removed to allow aeration. The contents in the test units were hydrated, as required. Test units were randomized and returned to the environmental chamber. On day 14, the units were once again removed, and a small amount of dry active yeast was added to each unit, which was then hydrated if required. Units were again randomized and replaced into the chambers. On day 21, the aeration procedure completed on day 7 was repeated.

Twenty-eight (28) days after the addition of the test organisms, the test units were removed, organized by treatment, and processed. The number of surviving adults and the number of progeny produced in each test unit were determined by manually counting individuals in each unit. This was achieved by adding a small amount of water to the test unit, which was then transferred into an empty Petri dish. Due to the hydrophobic nature of the springtail's waxy epicuticle, the *F. candida* would float to the surface, where they were counted using a lighted magnifying glass. Individuals were considered to be alive if movement was present. This process was repeated until there was no substrate remaining

in the test unit. The "Blank" test units with substrate but no tubes were used to measure the pH and conductivity of each treatment.

2.6 Plant Tests: Seedling emergence and growth tests

2.6.1 *Hordeum vulgare* - Barley

Five hundred grams (500 g) of amended or un-amended test soil was added to each 500-mL food-grade plastic container: 6 test units for each of 3 control treatments, 4 units for the 100, 180, 320, 560 mg/kg treatments, and 3 units for the 1000, 1800, 3200, 5600, and 10000 mg/kg treatments. Barley seeds were added to the soils the same day that the soils were prepared. Using forceps and working from lowest to highest concentrations of CNT amendment, 5 barley seeds were carefully planted in a pattern with a uniform distribution in the soil of each unit. Each seed was inspected for quality before it was chosen for the experiment. Seeds were planted at a depth of approximately 2x their width within the test soil, pushed down and covered with soil using a glass rod. Planted test vessels were then hydrated with nutrient solution using a spray bottle. The lids were applied, and the units were transferred to the plant growth chambers located at the University of Guelph. Units were randomized and placed within the chambers, which were set to a regular light cycle of 16h light, 8h dark**,** with constant temperature (22 ± 2^{o}C) and humidity.

Every 48 hours, the test vessels were checked for hydration. If required, the surface was saturated with either de-chlorinated tap water (tap water left to sit for at least 48 hours) or nutrient solution. Water and nutrient solution were used on alternating watering events. After each watering, the lids were replaced and the units randomized once again.

After 7 days, the lids were removed from the units to allow for shoot growth beyond the crest of the test unit. After the initial 7-day period, watering and randomization was completed daily because water evaporation occurred more quickly. Again, plants were watered with de-chlorinated tap water, and on alternating days, with a weak nutrient solution.

On the 14th day following the planting of the *Hordeum vulgare* seeds, the units were returned to the laboratory at Stantec (Guelph, ON) for processing. First, the number of emerged seedlings was counted and recorded. Any growth 3 mm or more above the soil surface was counted as "emerged". The seedlings were then carefully liberated from the soil, with care taken to ensure that all soil was removed from the roots without damage to their structure. A spray bottle, and occasionally a spray extension from the tap were used to dislodge soil particles. Once the seedling roots were separated from the soil (See Fig. 2.3), the health of the plants was noted. The root and shoot were then separated by cutting through the seedling at the point between root and shoot. Root and shoot length were then measured with a ruler, and the roots and shoots of each unit were placed into separate previously labeled and weighed weigh boats, and placed into a drying oven at 90 ºC for 48 hours. These were weighed after the drying period.

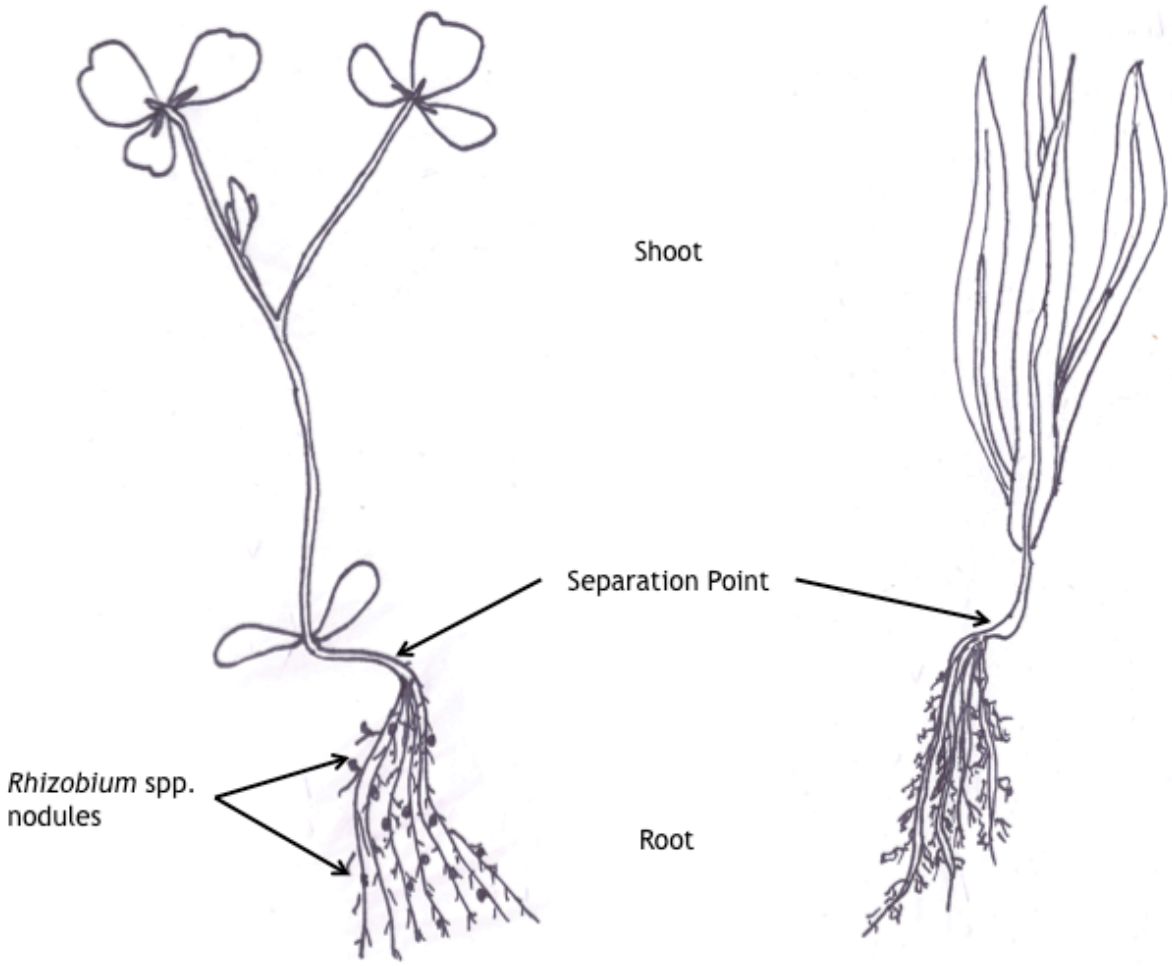

Fig. 2.3. Illustration of *Medicago sativa* (left) and *Hordeum vulgare* (right) denoting severing points between root and shoot of seedlings for measurements. Note the root nodules present on the roots of *M. sativa*.

2.6.2 *Medicago sativa* - Alfalfa

The method for the measurement of seedling emergence and growth of *Medicago sativa* in multi-walled carbon nanotube-amended soil is identical to that for *Hordeum vulgare* (previous section), with two important differences. Ten (10) alfalfa seeds were planted in each test unit, and exposure time was 21 days rather than 14 days. Soils were from the same batch as that used for the test with barley, and the experiments ran simultaneously in the same growth chamber, under the same conditions.

2.7 Statistical analysis of test results

All data processing and statistical analyses were completed using Graphpad Prism 4 (Graphpad Software, Inc, 2003). Assumptions for analysis of variance testing were tested using Bartlett's test for equal variances ($p \leq 0.05$) (homogeneity of variance), and Shapiro-Wilk normality test ($p \leq 0.05$) (normality). When found to be appropriate based on the distribution of data, one way analysis of variance (ANOVA), procedures with a significance level established at $p \leq 0.05$ was used to determine whether there were significant differences among means for each amendment and for each parameter measured. Bonferroni's multiple comparisons post-hoc test with a significance value $p \leq 0.05$ was utilized to determine where the significant variation occurred, when present. When data were not normal and variances were found to be non-homogenous, Kruskal-Wallis non-parametric test with a significance value of $p \leq 0.05$ was used. Differences among medians were identified by applying Dunn's multiple comparisons post-hoc test ($p \leq 0.05$).

3.0 Experimental Results

3.1 *Eisenia andrei*

3.1.1 Effects of 35-day exposure on adult survival

After 35-days of exposure of adult *Eisenia andrei* to artificial soil amended with varying concentrations of multi-walled carbon nanotubes, no significant ($p = 0.2503$) adult mortality was observed in any of the control treatments (artificial soil, sand, activated carbon) or the MWCNT amendments (Fig. 3.1.1). Controls of sand slurry and activated carbon were compared to each other and to the 1000 mg/kg MWCNT treatment and artificial soil control. There were no significant differences between or among treatments.

Individuals appeared healthy throughout the control treatments in terms of their vigor and colouration. Adults removed from MWCNT-amended soils were slightly less responsive and vigorous than their counterparts in the control treatments. This observation did not vary discernibly amongst MWCNT concentrations. There was mild discolouration amongst some individuals in MWCNT-amended treatments; however, this was not universal and did not trend with any significance. All units contained both cocoons and juvenile worms, and the presence of mites in the soil was observed amongst all treatments.

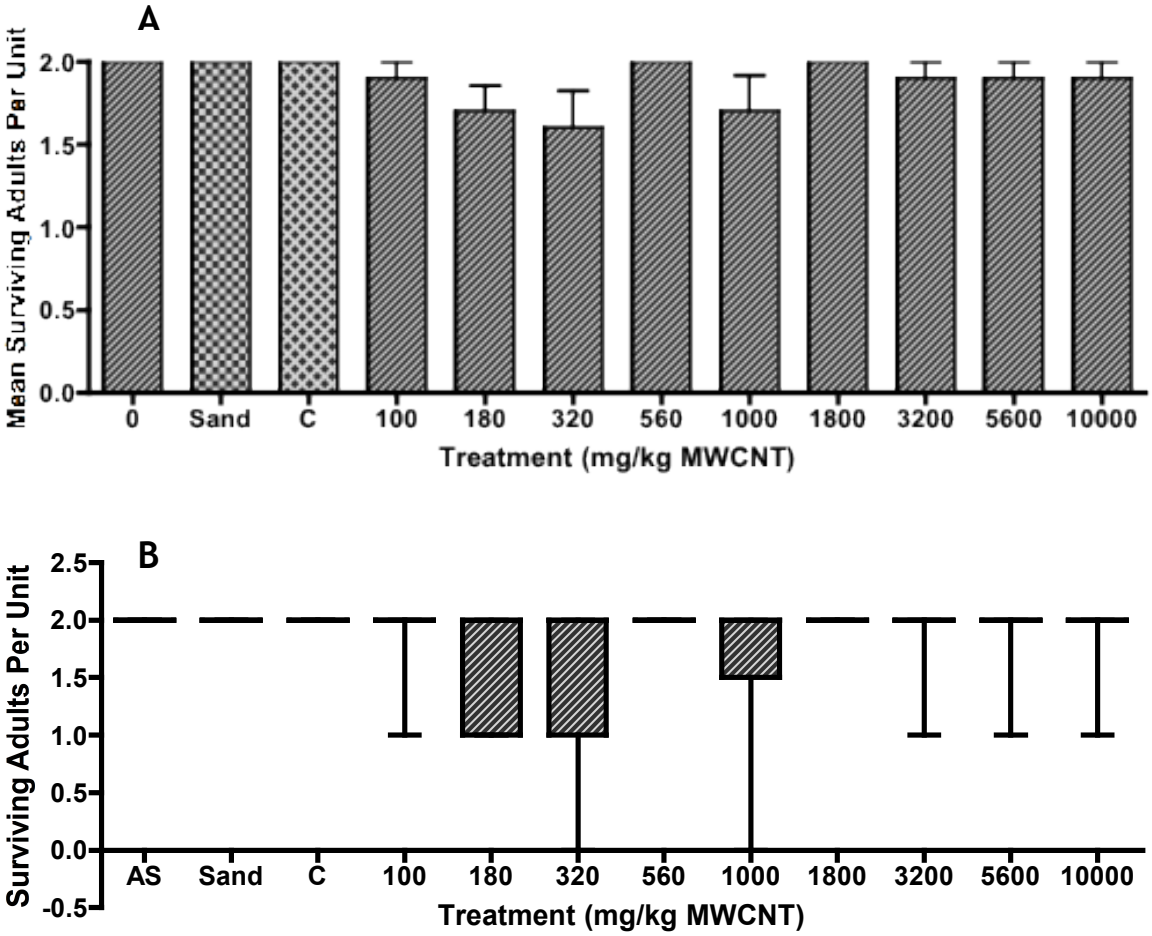

Fig. 3.1.1. A. Adult survival of *Eisenia andrei* exposed to various concentrations of MWCNT slurry. B. Box and Whisker plot showing data range for each treatment.

During the inspection of MWCNT-amended soils after removing from the test units and before breaking them apart to remove the adults, black deposits were observed throughout MWCNT-amended soils. Deposits increased in intensity and frequency as the concentration of nanotube amendment increased. Figure 3.1.2 is a collection of photographs of amended soils after being removed from the test units and before being processed. Figure 3.1.2-A is taken from test unit 560-4, 3.1.2-B is from test unit 1000-1, and 3.1.2-C is taken from 1800-8. Due to the randomization of the test units for adult removal and the unexpected discovery of this effect, the progression of this effect throughout the amendment series was not fully documented. These deposits are thought to be castings, containing MWCNT consumed in the test soil.

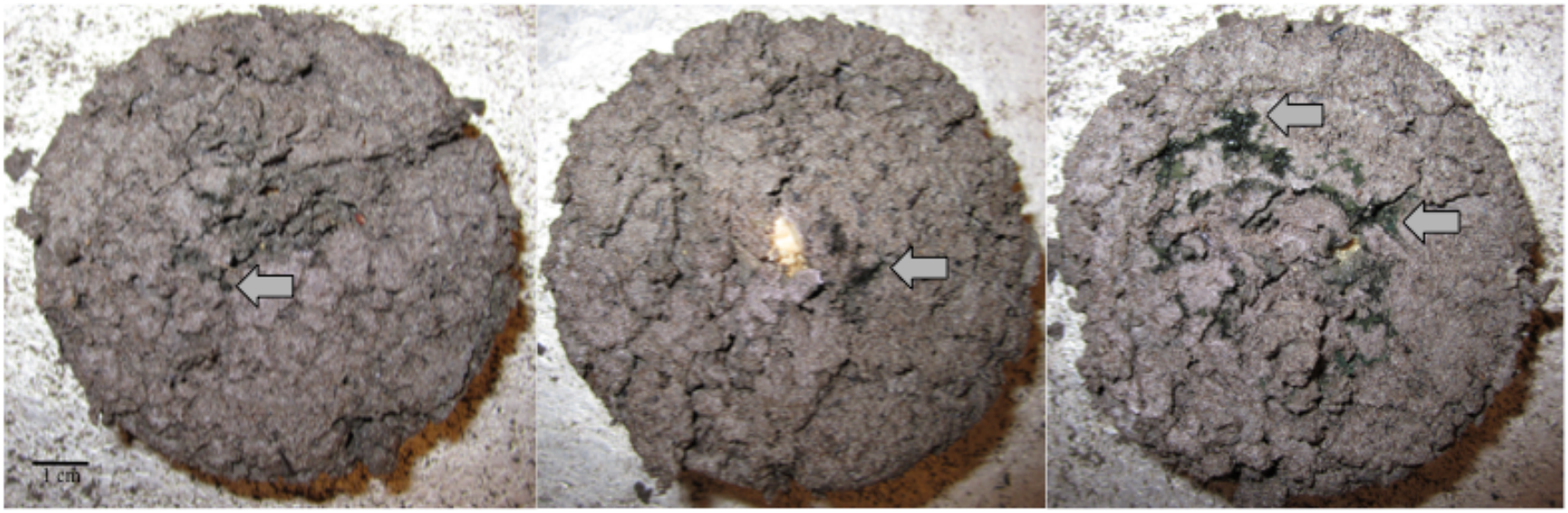

Fig. 3.1.2. Photographs of *Eisenia andrei* test soil from test units after the soil was removed and before adult removal. Image A is taken from test unit 560-4, B from 1000-1, and C from 1800-8. Note the black deposits (marked by arrows) seen in all three soil samples, with the most intense deposits found in C.

3.1.2 Effects of 63-day exposure on progeny production and survival

The soil colour and texture varied with the intensity of amendment; soils containing higher MWCNT concentrations were darker and wetter. Analysis found that there were no statistically significant differences in the number of progeny produced between or among treatments (Fig. 3.2.3).

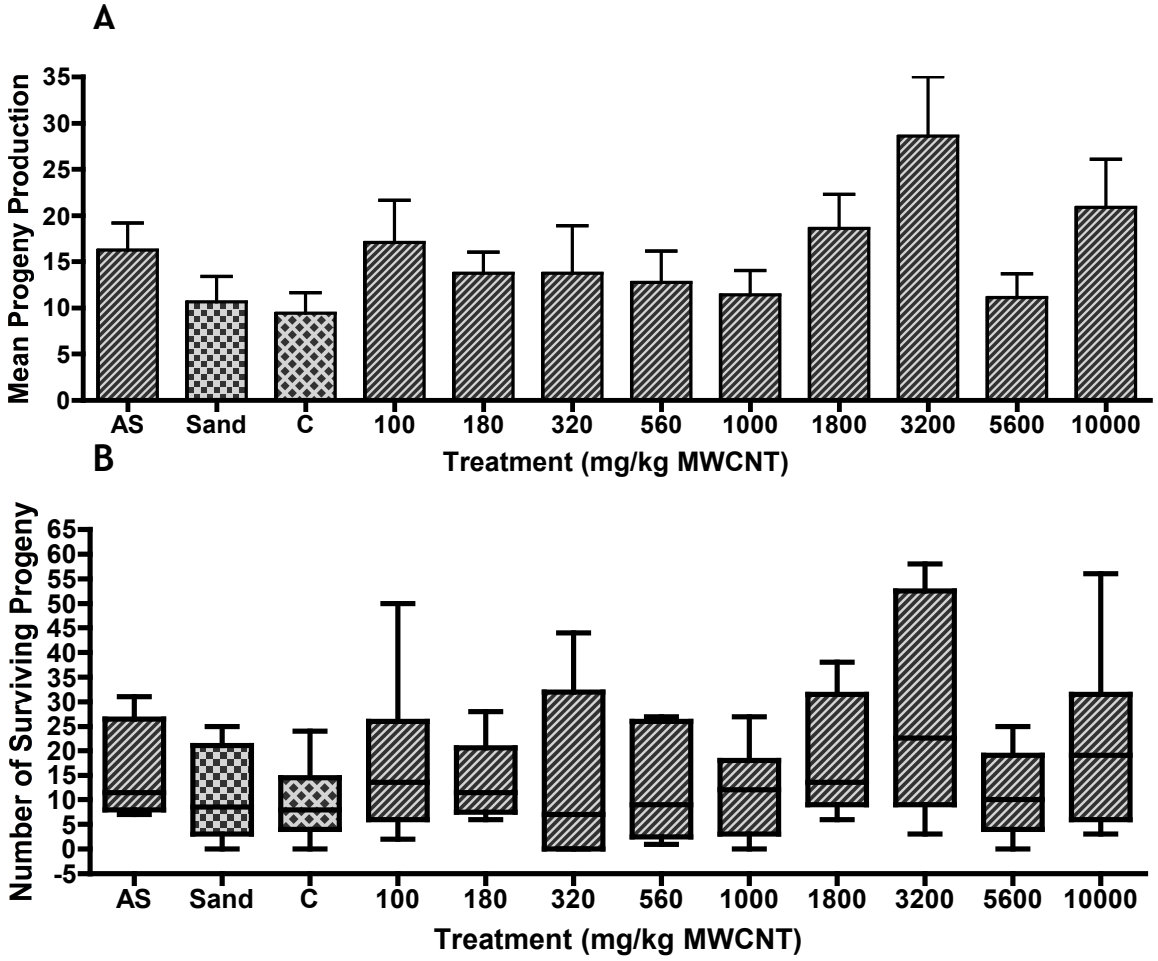

Fig. 3.2.3. A. Mean progeny production of *Eisenia andrei* in each treatment after 63-day exposure to various concentrations of MWCNT slurry. B. Box and Whisker plot showing data range for each treatment.

Analysis of progeny wet mass data showed a significant increase in mean progeny wet mass (Fig. 3.2.4) ($p = 0.0001$) for individuals exposed to treatment of 3200 mg/kg MWCNT dry soil compared to artificial soil control. Analysis also found significant difference increase in progeny wet mass in 1800, 3200, and 10 000 mg/kg MWCNT treatments compared to sand slurry control and activated carbon control, as well as an increase in progeny wet mass in 3200 mg/kg MWCNT treatment and 180 mg/kg MWCNT treatment.

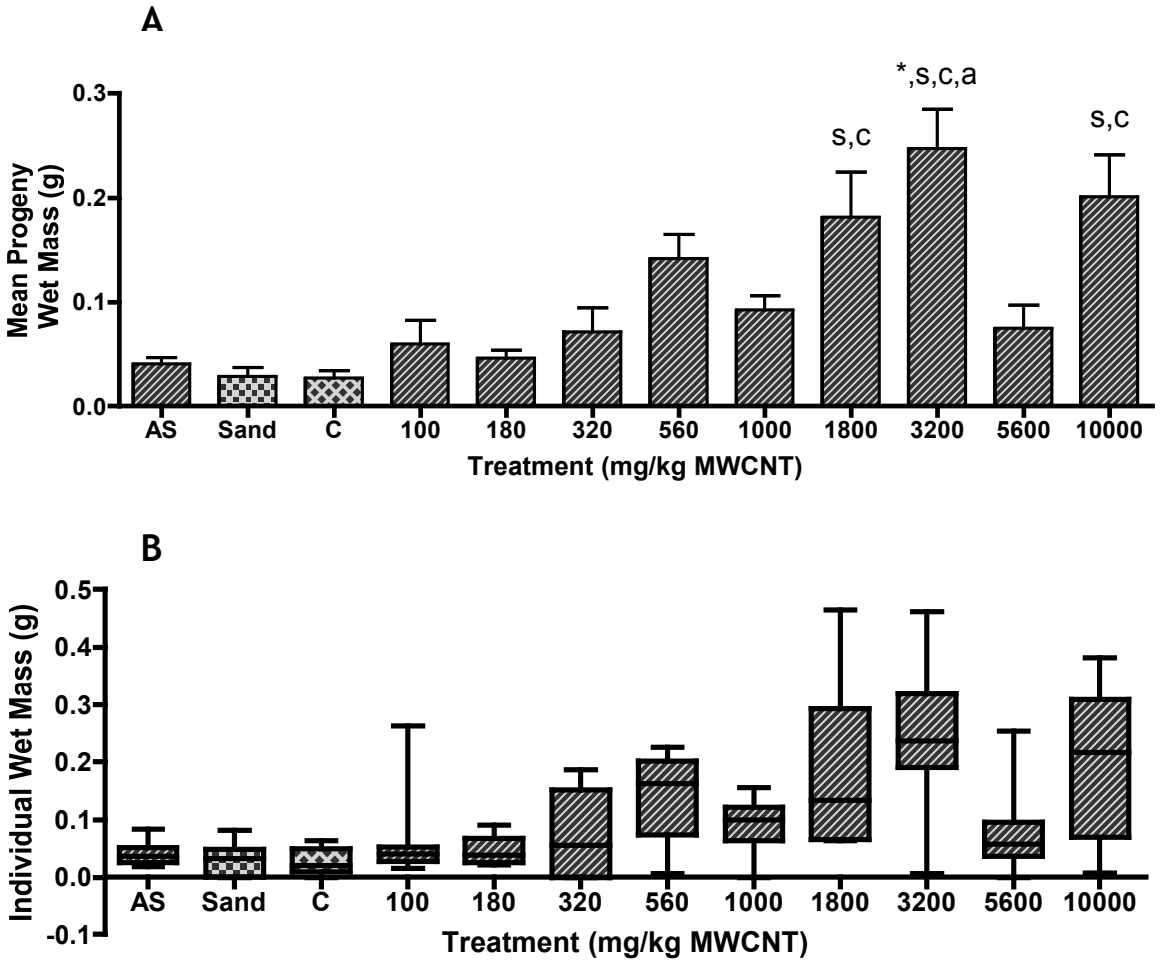

Fig. 3.2.4. A. Mean *Eisenia andrei* progeny wet mass for each treatment after 63-day exposure to various concentrations of MWCNT slurry. Asterisks denote significant difference from the AS control, "s" denotes significant difference from sand control, "c" denotes significant difference from activated carbon control, "a" denotes significant difference from 180 mg/kg MWCNT amendment. B. Box and Whisker plot showing data range for each treatment.

Mean dry mass of individual progeny did not differ significantly from that in the control treatments (Fig. 3.2.5). This would suggest that the mass difference found in wet organisms was due to water mass; however, there was no significant variation found between any treatments when mean progeny percent moisture content was analyzed (Fig. 3.2.6).

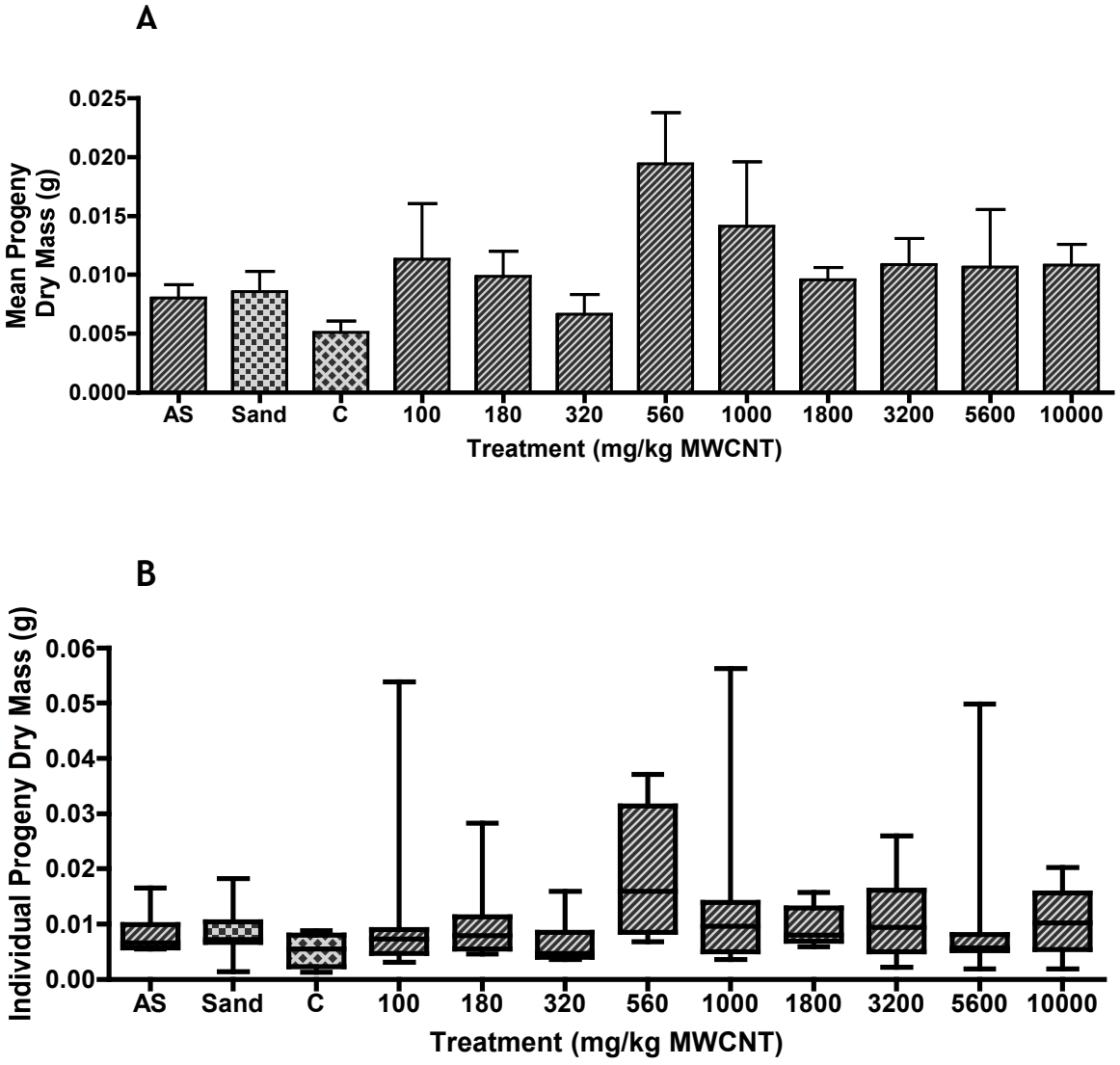

Fig. 3.2.5. A. Mean individual *Eisenia andrei* progeny dry mass for each treatment after 63-day exposure to various concentrations of MWCNT slurry. B. Box and Whisker plot showing data range for each treatment.

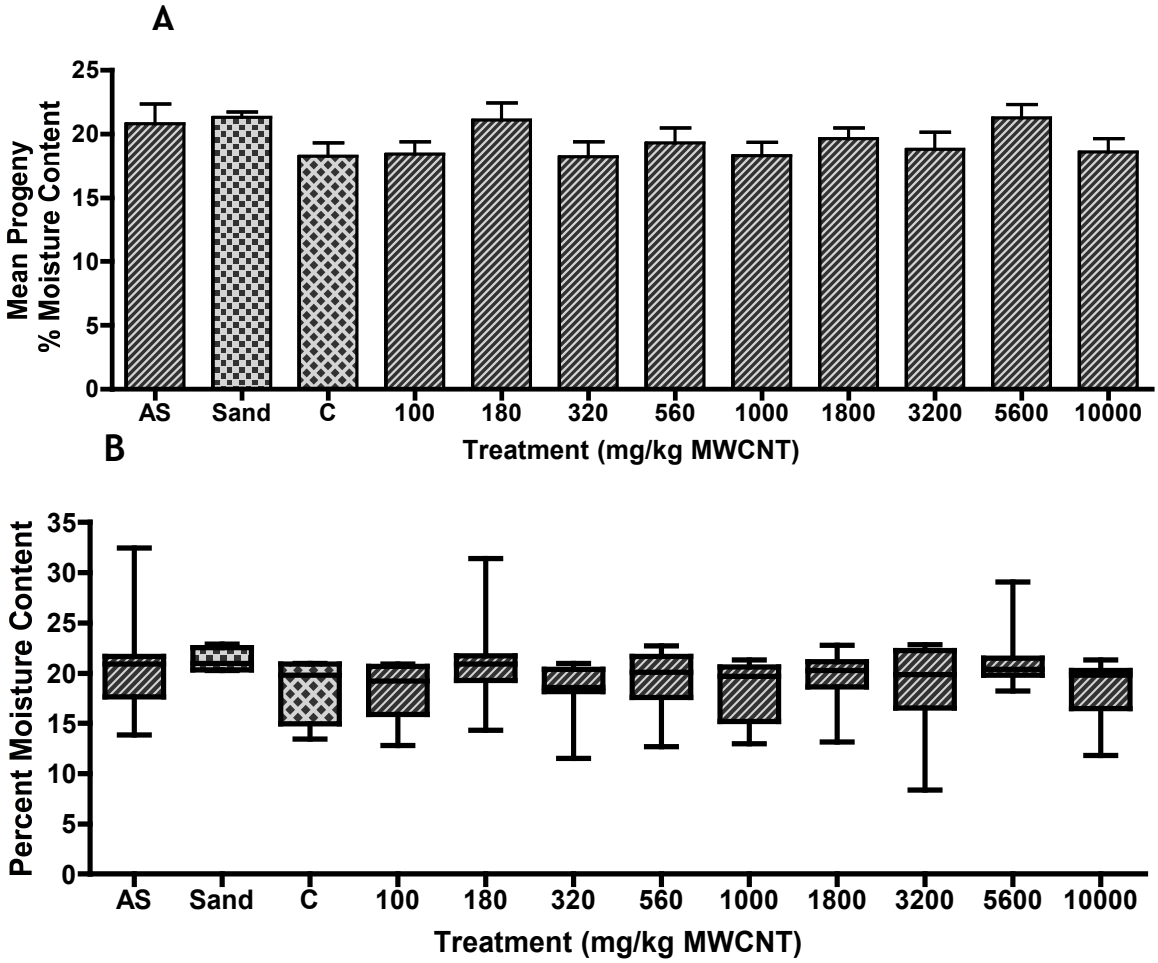

Fig. 3.2.6. A. Mean *Eisenia andrei* progeny moisture content for each treatment after 63-day exposure to various concentrations of MWCNT slurry. B. Box and Whisker plot showing data range for each treatment.

3.2 *Folsomia candida*

3.2.1 Effects of 28-day exposure on adult survival

Following 28-day exposure to amended soils, the appearance of surviving adult *Folsomia candida* was uniform throughout treatments, with largely vigorous, healthy-looking adults common. Adults with dark abdomens were observed with consistent (low) frequency throughout treatments. Eggs were found in all treatments, with no noticeable differences in colour or morphology.

No significant adult mortality was found in any amendment (see Fig. 3.2.1). Comparisons of mean adult survival among control treatments of artificial soil, sand slurry, activated carbon slurry, and equivalent multi-walled carbon nanotube treatment revealed no significant variation.

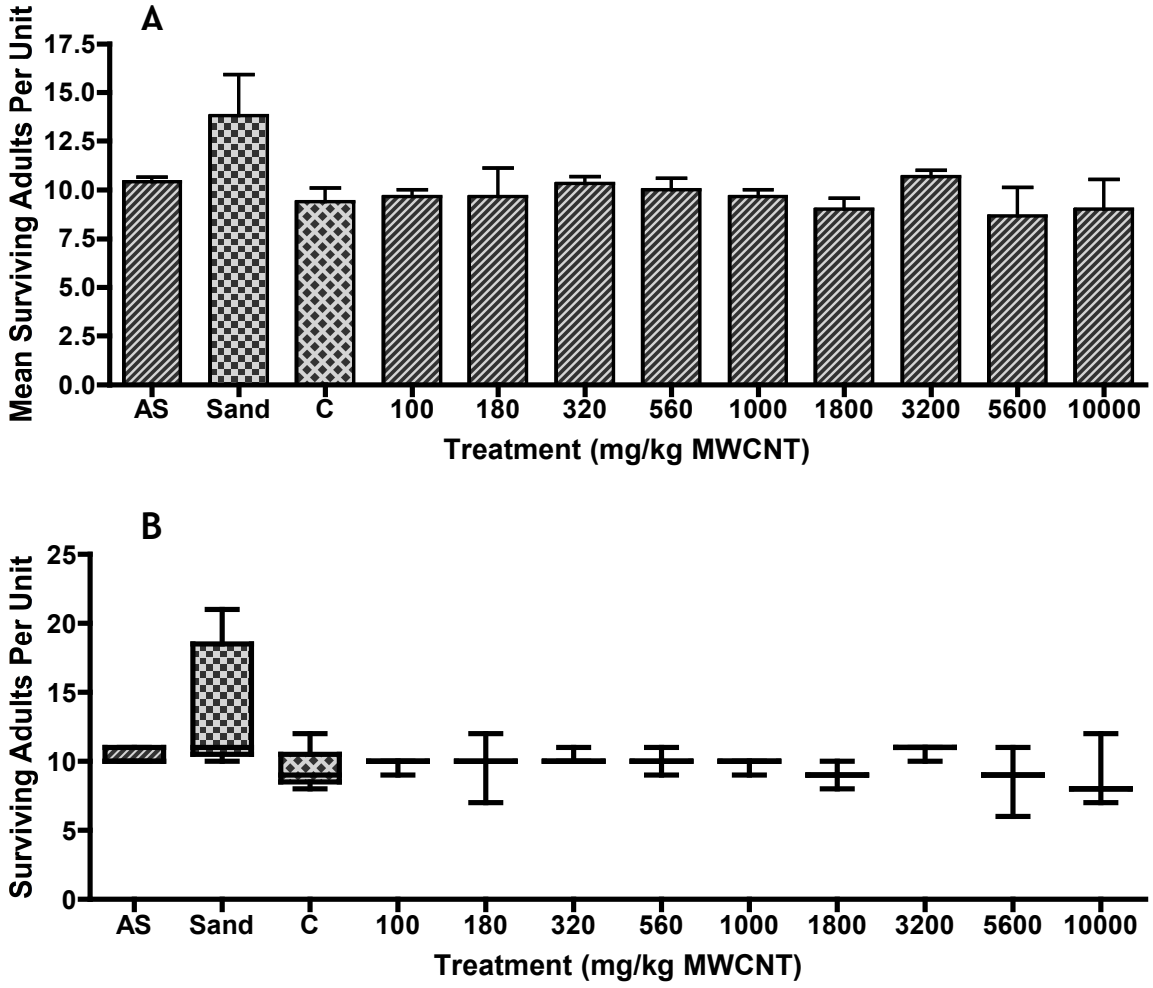

Fig. 3.2.1. A. Survival of adult *Folsomia candida* exposed to multi-walled carbon nanotube-amended artificial soil with controls of artificial soil (AS), sand slurry (Sand), and activated carbon slurry (C). B. Box and Whisker plot showing data range for each treatment.

3.2.2 Effects of 28-day exposure on reproduction

Following the 28-day exposure period, the number of surviving *Folsomia candida* progeny in each unit was determined using the floatation method. Progeny were observed to be generally healthy and active, with no trends in differing morphology, colouring, or behavior noted. There was no significant difference in progeny production amongst treatments at $p \leq 0.05$. Comparison of surviving progeny among control treatments of artificial soil, sand slurry, activated carbon slurry, and 1000 mg/kg multi-walled carbon nanotube amendment showed no significant difference (See Fig. 3.2.2).

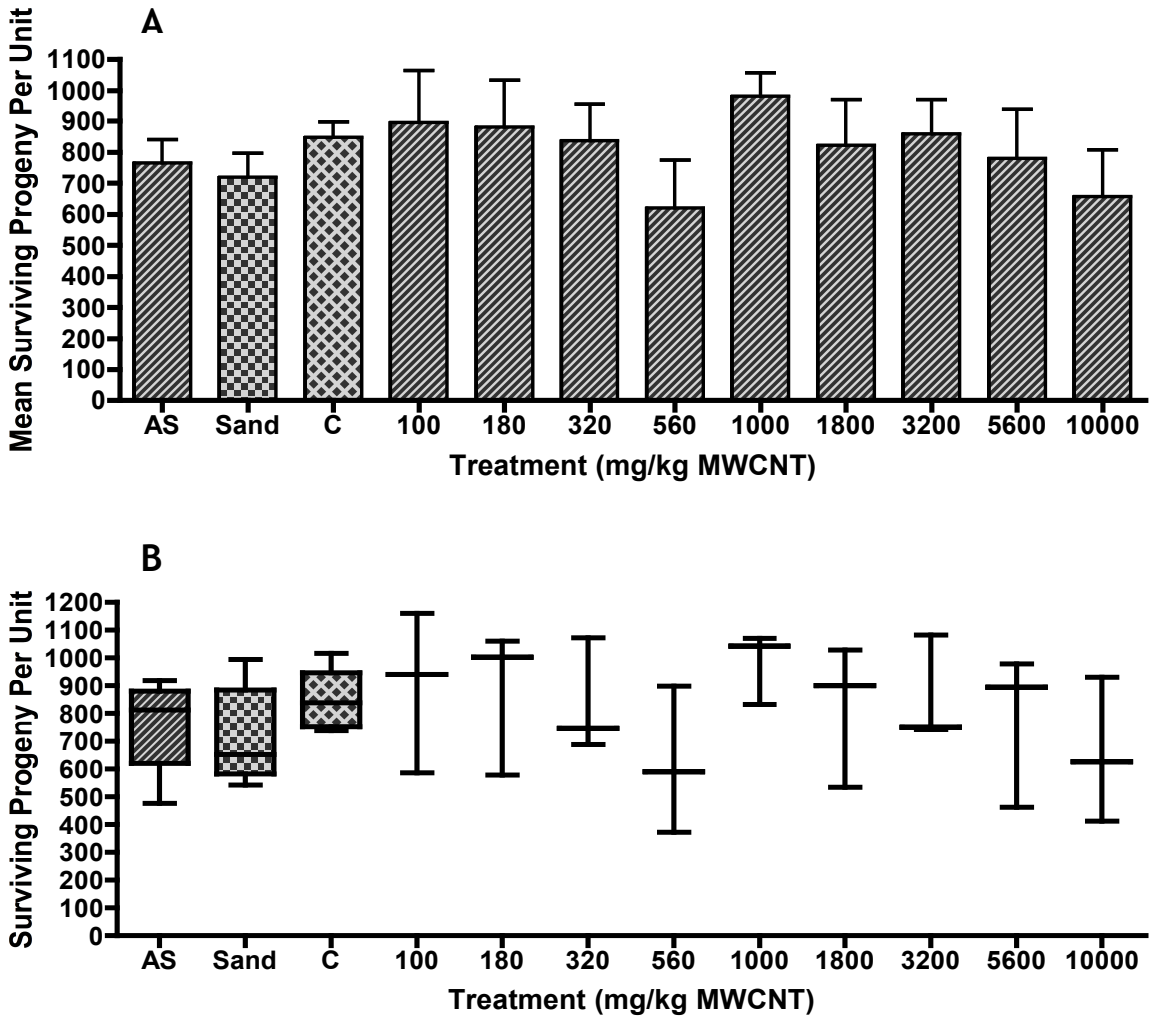

Fig. 3.2.2. A. Mean surviving *Folsomia candida* progeny exposed to multi-walled carbon nanotube-amended artificial soil with controls of artificial soil (AS), sand slurry (Sand), and activated carbon slurry (C). B. Box and Whisker plot showing data range for each treatment.

3.3 *Hordeum vulgare*

3.3.1 Effects of 14-day exposure on seedling emergence

After the 14-day exposure period, test units containing *Hordeum vulgare* (barley) seedlings were removed from growth chambers at the University of Guelph and returned to the Stantec soil laboratory for processing (see Fig. 3.3.1). No *Hordeum vulgare* seedlings had emerged in the 10 000 mg/kg MWCNT treatment during the test period, although It is believed that this is a result of the test soil being over-watered on day 0, resulting in a sediment-like consistency in the test units. These units were therefore left out of final statistical analysis; however, it should be noted that statistical significance was found in all measured parameters when the 10 000 mg/kg units were included.

During visual assessment of foliage, leaves were found to be generally healthy. Occasional chlorotic (yellowing/loss of colour) leaves, leaf wrinkling, and some necrosis (brown/black portions due to cell death) were observed among seedlings in all treatments; however this was a rare occurrence and is not considered to be a phytotoxic effect from amended soil.

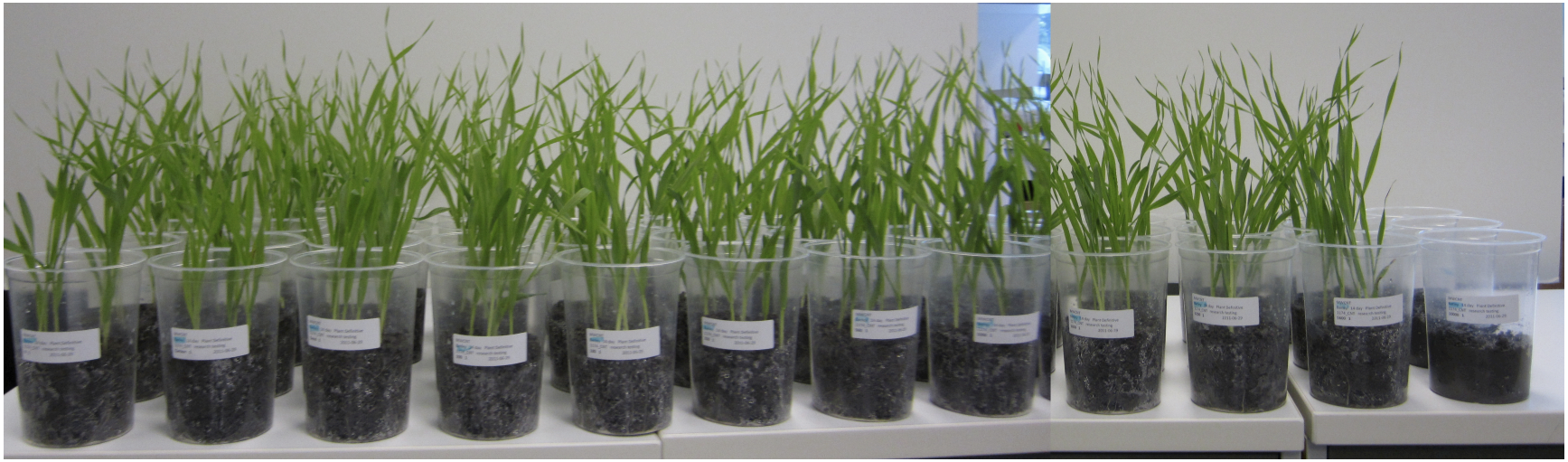

Fig. 3.3.1. *Hordeum vulgare* test units with seedlings after 14-day exposure, before processing. Units are arranged from left to right in ascending order according to nanotube amendment: Artificial Soil, Carbon, Sand, 100 mg/kg MWCNT, 180 mg/kg, 320 mg/kg, 560 mg/kg, 1000 mg/kg, 1800 mg/kg, 3200 mg/kg, 5600 mg/kg, 10 000 mg/kg. Note that the 10 000 mg/kg amendment had no seedling growth due to over-saturation of the test units.

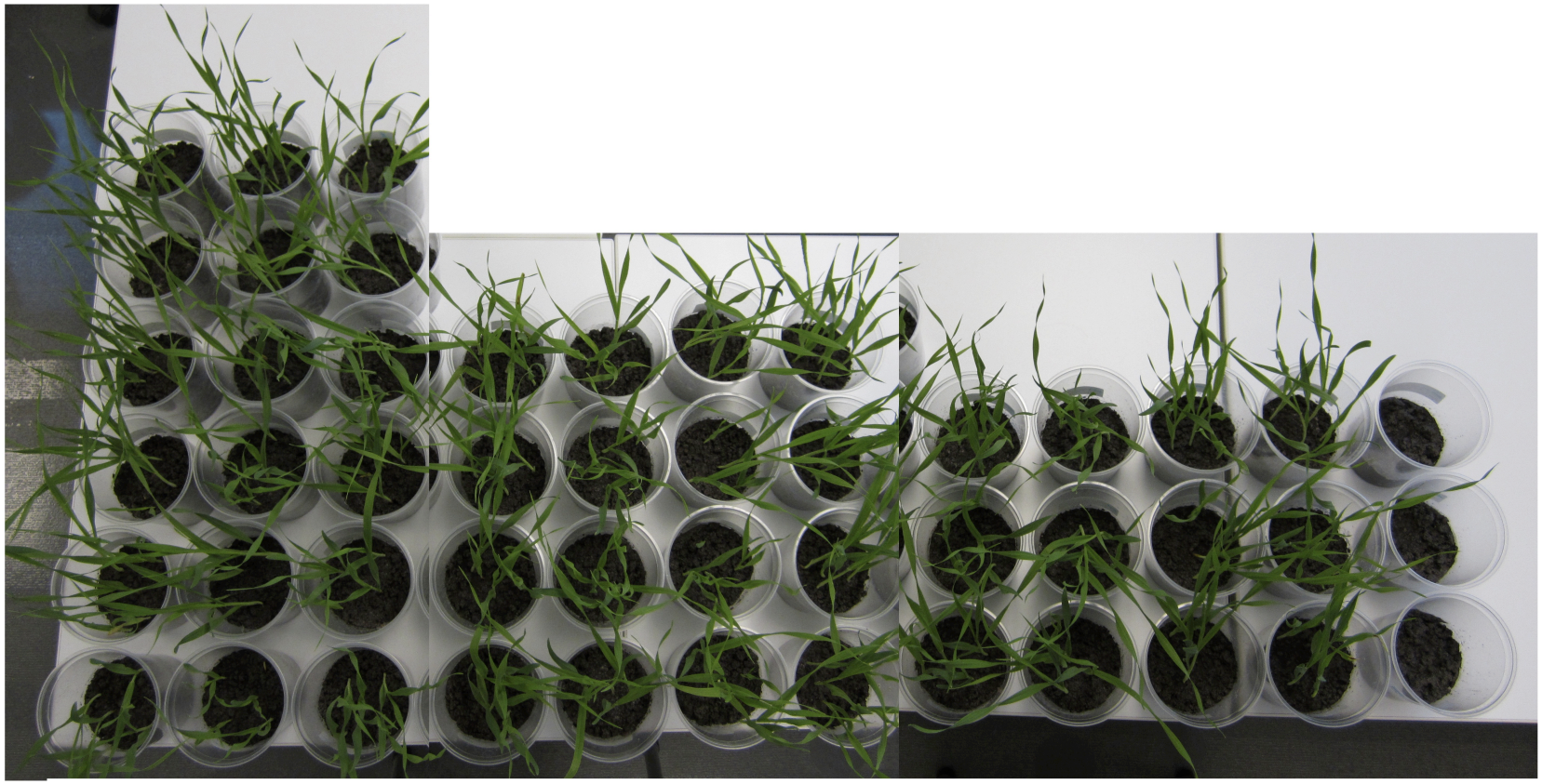

Fig. 3.3.2. *Hordeum vulgare* test units with seedlings after 14-day exposure, before processing as seen from above. Units are arranged from left to right in ascending order according to nanotube amendment: Artificial Soil, Carbon, Sand, 100 mg/kg MWCNT, 180 mg/kg, 320 mg/kg, 560 mg/kg, 1000 mg/kg, 1800 mg/kg, 3200 mg/kg, 5600 mg/kg, 10 000 mg/kg. Note that the 10 000 mg/kg amendment had no seedling growth due to over-saturation of the test units.

As shown in Figure 3.3.3, there was no significant variation in seedling emergence and emergence among treatments. The 10 000 mg/kg treatment was not included in statistical analyses due to oversaturation of the soil which likely resulted in the inhibition of seedling emergence in this treatment.

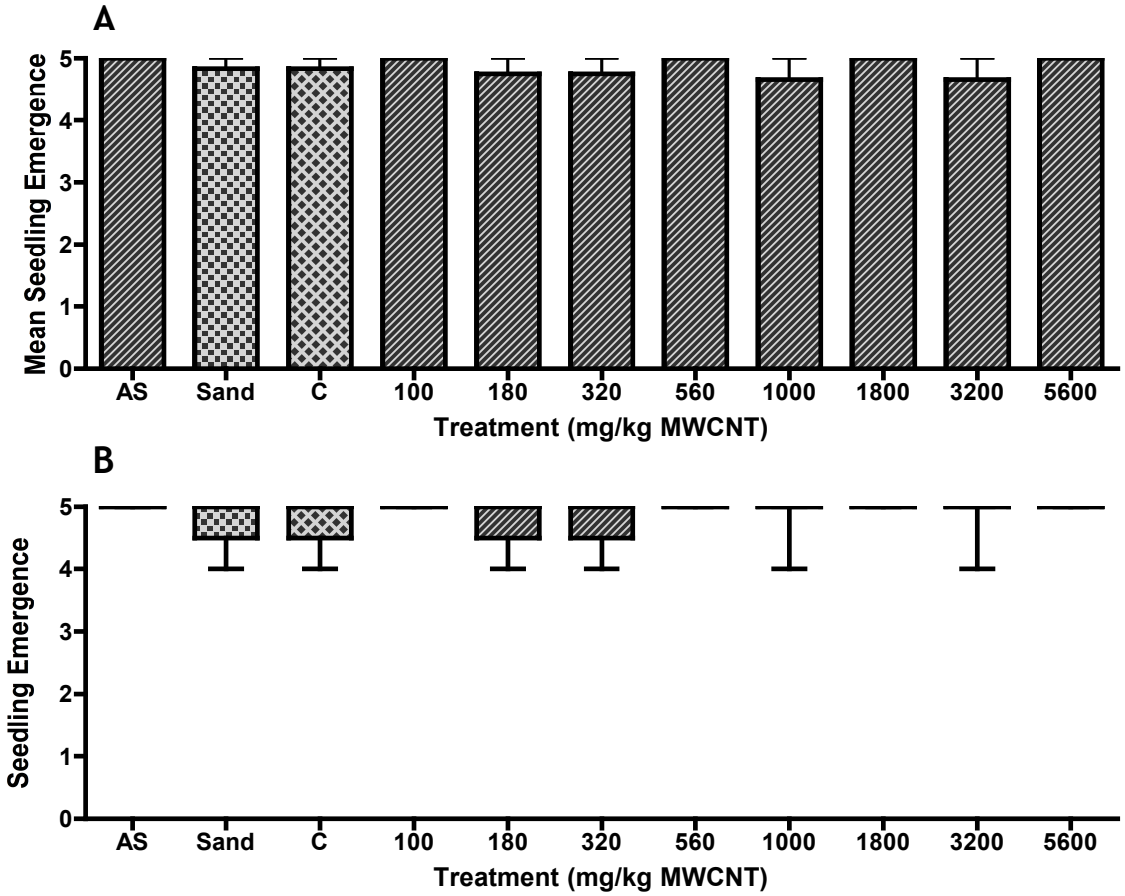

Fig. 3.3.3. A. Mean number of emerged *Hordeum vulgare* seedlings per test unit for each MWCNT treatment and controls of artificial soil (AS), sand slurry (Sand), and activated carbon slurry (C). B. Box and Whisker plot showing data range for each treatment.

3.3.2 Effects of 14-day exposure on root and shoot growth

Exposure to multi-walled carbon nanotube slurry-amended soil for 14 days resulted in no observable differences in barley shoots and foliage among treatments (See Fig. 3.3.4). Chlorosis was noted rarely, and without any correlation to treatment. Figure 3.3.8 shows *Hordeum vulgare* seedlings after being liberated from the test soil. As seen in Figures 3.3.1, 3.3.2, and 3.3.8, shoots and foliage were generally health throughout all treatments.

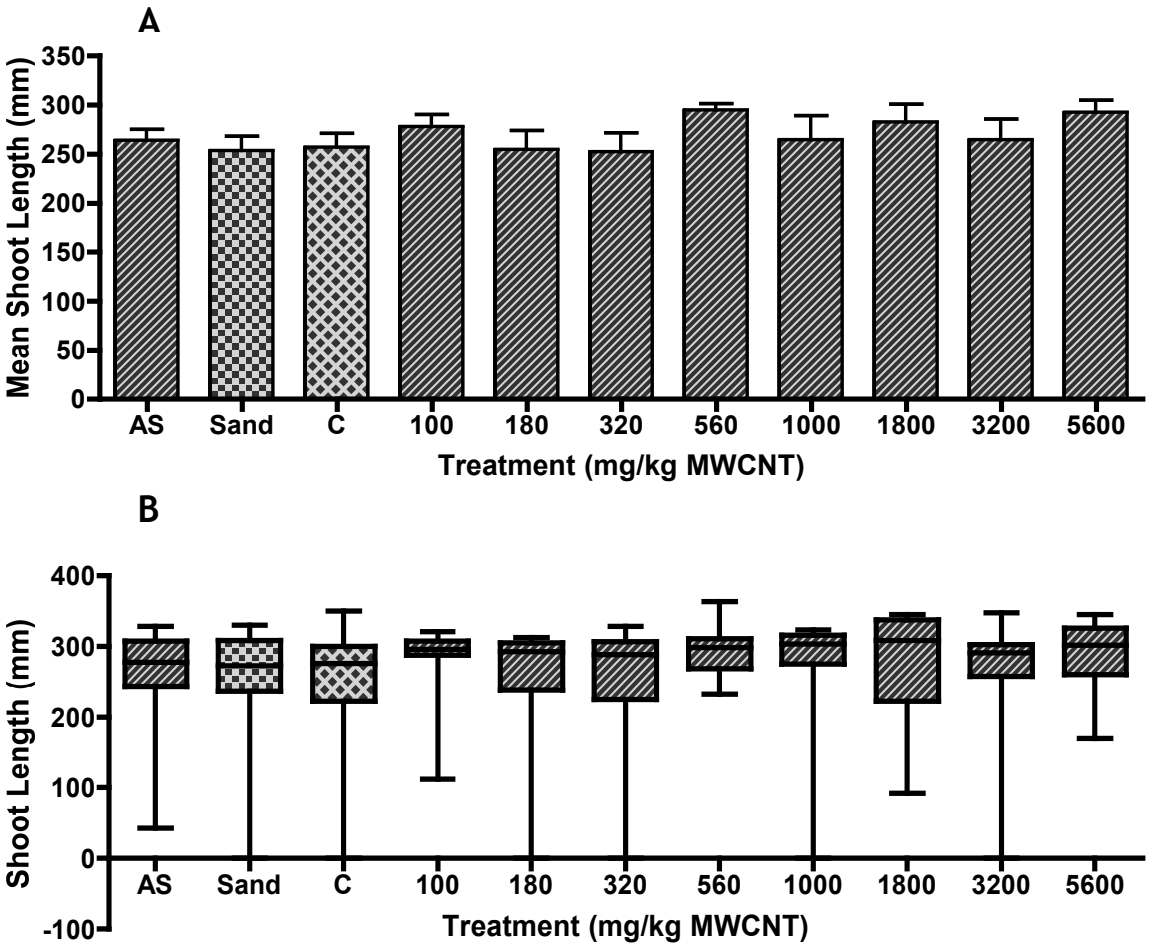

Fig. 3.3.4. A. Mean shoot length of *Hordeum vulgare* exposed to all MWCNT treatments as well as controls of artificial soil (AS), sand slurry (Sand), and activated carbon slurry (C). B. Box and Whisker plot showing data range for each treatment.

Shoot dry mass was similarly unaffected by exposure to the multiwalled carbon nanotube slurry (Fig. 3.3.5).

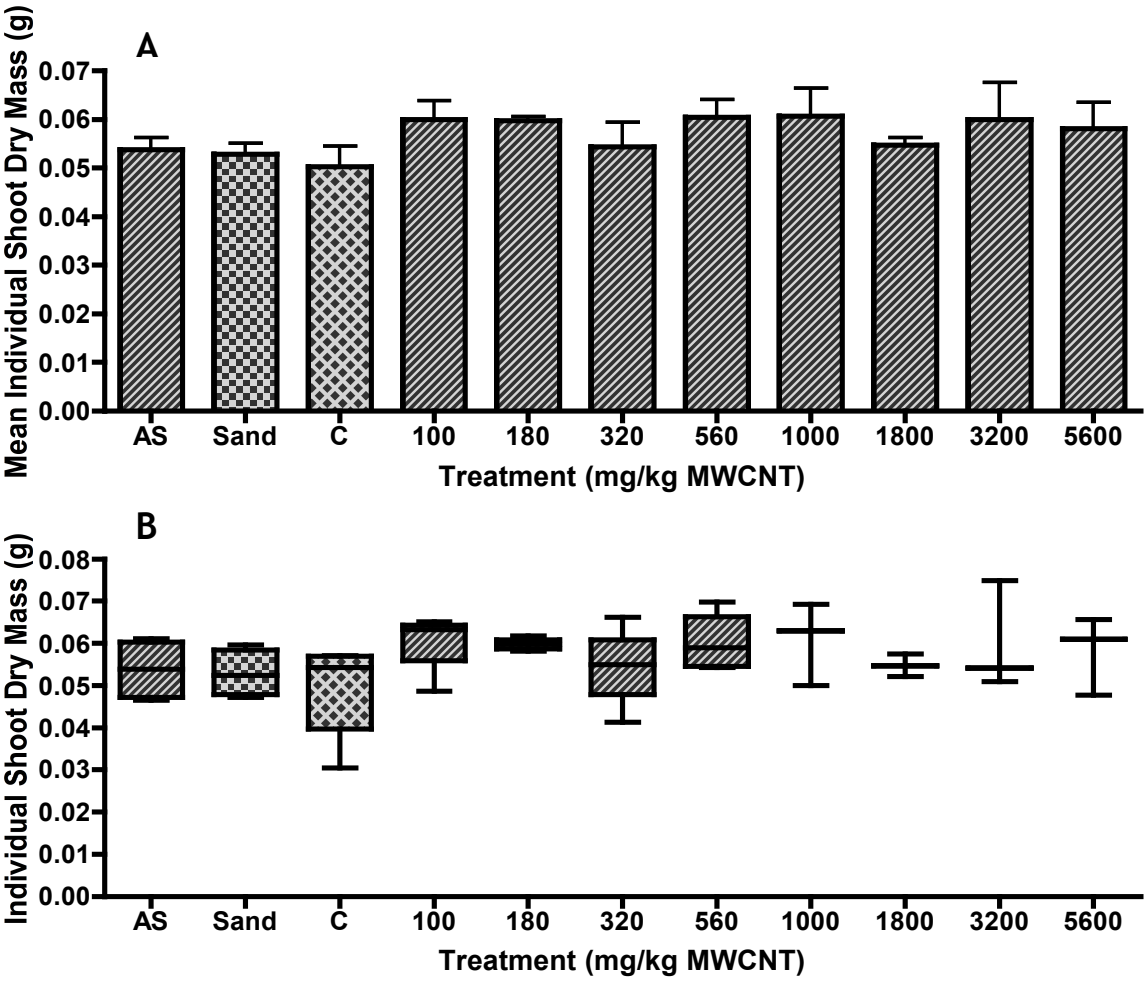

Fig. 3.3.5.A. Mean shoot dry mass of *Hordeum vulgare* exposed to all MWCNT treatments as well as controls of artificial soil (AS), sand slurry (Sand), and activated carbon slurry (C). B. Box and Whisker plot showing data range for each treatment.

Analysis of root elongation found no significant difference between treatments (Fig.3.3.6). Mean individual root dry mass was also unaffected by growth within the amended media (Fig. 3.3.7). Visual observation of root growth similarly found no physical variation (Fig. 3.3.8).

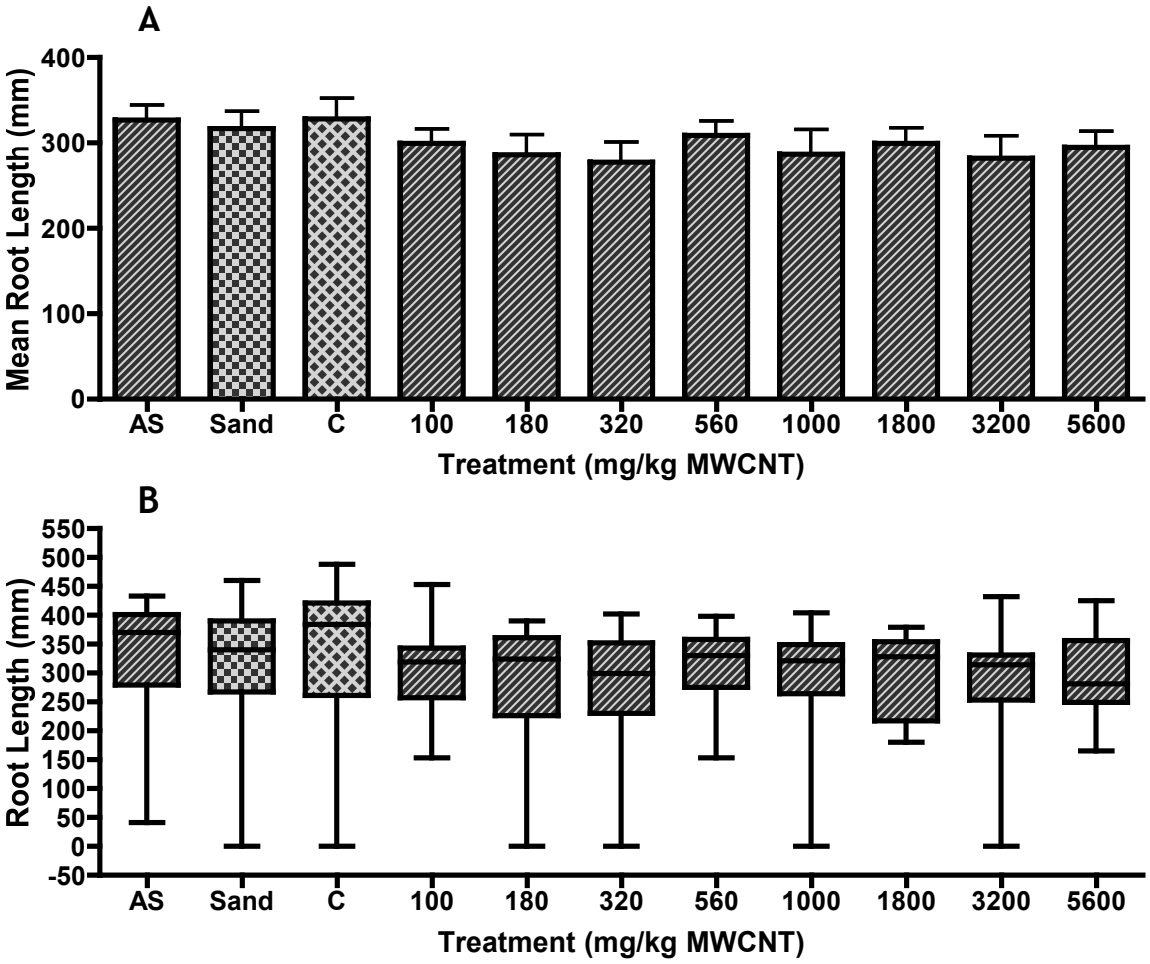

Fig. 3.3.6. A. Mean root length (mm) of *Hordeum vulgare* exposed to all MWCNT treatments as well as controls of artificial soil (AS), sand slurry (Sand), and activated carbon slurry (C). B. Box and Whisker plot showing data range for each treatment.

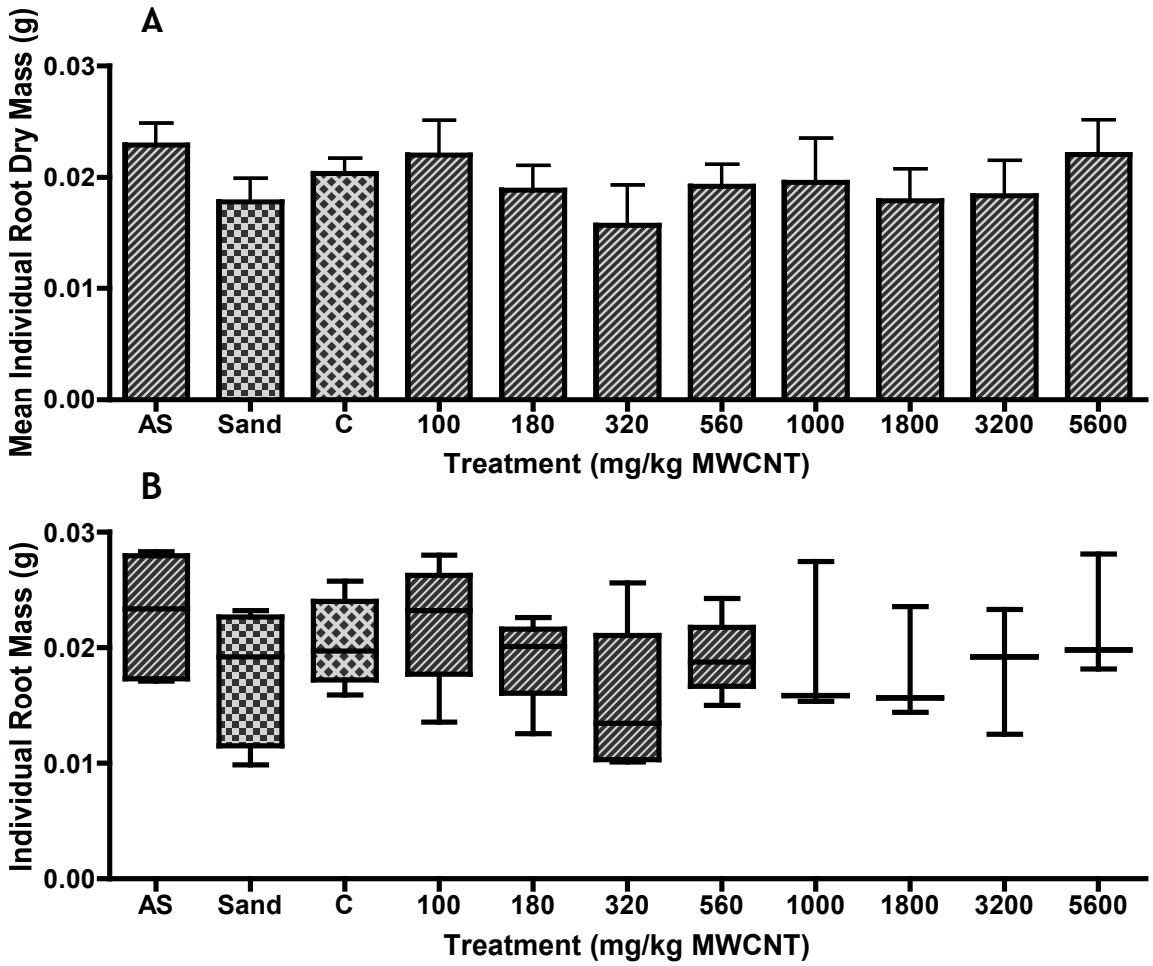

Fig. 3.3.7. A. Mean root dry mass of *Hordeum vulgare* exposed to all MWCNT treatments as well as controls of artificial soil (AS), sand slurry (Sand), and activated carbon slurry (C). B. Box and Whisker plot showing data range for each treatment.

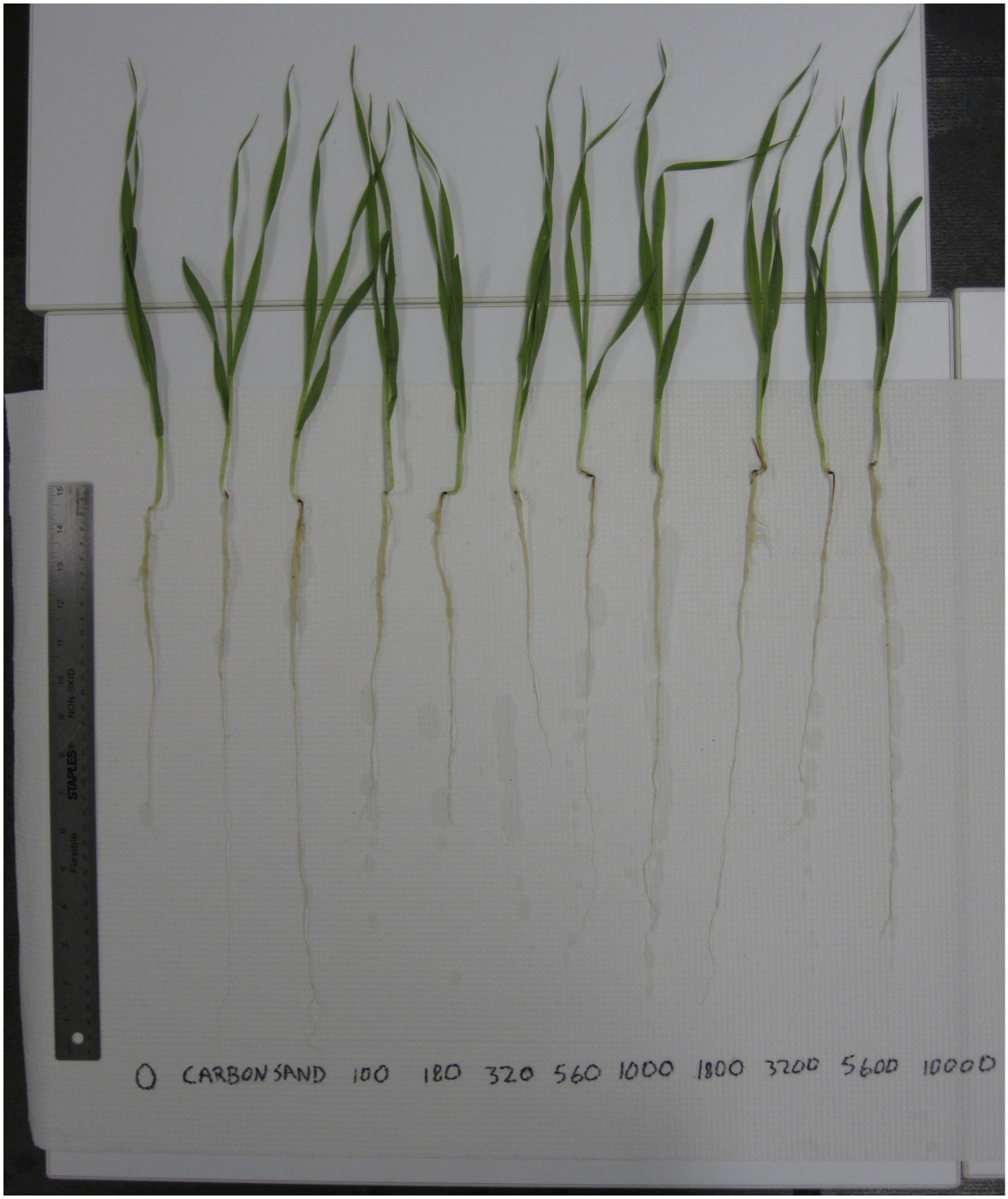

Fig. 3.3.8. *Hordeum vulgare* seedlings after liberation from amended test soil. No visible difference was noted between seedlings grown in any concentration of MWCNT amendment.

3.4 *Medicago sativa*

3.4.1 Effects of 21-day exposure on seedling emergence

Following a 21-day exposure period, test units containing *Medicago sativa* (alfalfa) seedlings in amended soil were removed from growth chambers at the University of Guelph and returned to the soil laboratory at Stantec for processing (Fig. 3.4.1). Due to day-0 oversaturation of the test soil amended to 10 000 mg/kg multi-walled carbon nanotubes, these units were excluded from the final statistical analysis.

Visual analysis of foliage health noted general good health among alfalfa seedlings in all treatments. Instances of chlorosis (loss of pigmentation) and necrosis (dead tissue) were rare, but noted in some seedlings from all treatments and without significant trend (Fig. 3.4.2).

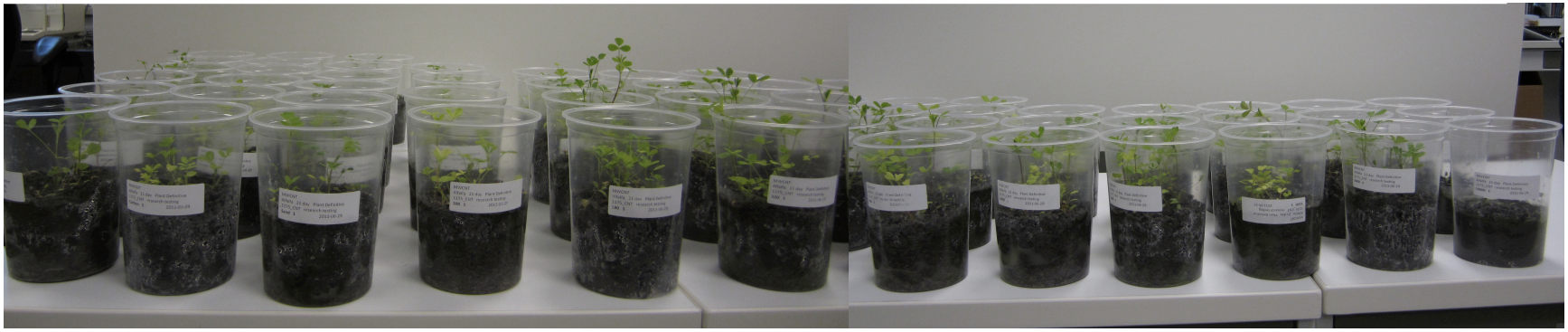

Fig. 3.4.1. *Medicago sativa* test units with seedlings after 21-day exposure, before processing. Units are arranged from left to right in ascending order according to nanotube amendment: Artificial Soil, Carbon, Sand, 100 mg/kg MWCNT, 180 mg/kg, 320 mg/kg, 560 mg/kg, 1000 mg/kg, 1800 mg/kg, 3200 mg/kg, 5600 mg/kg, 10 000 mg/kg. Note that the 10 000 mg/kg amendment had very little seedling growth likely due to over-saturation of the test units.

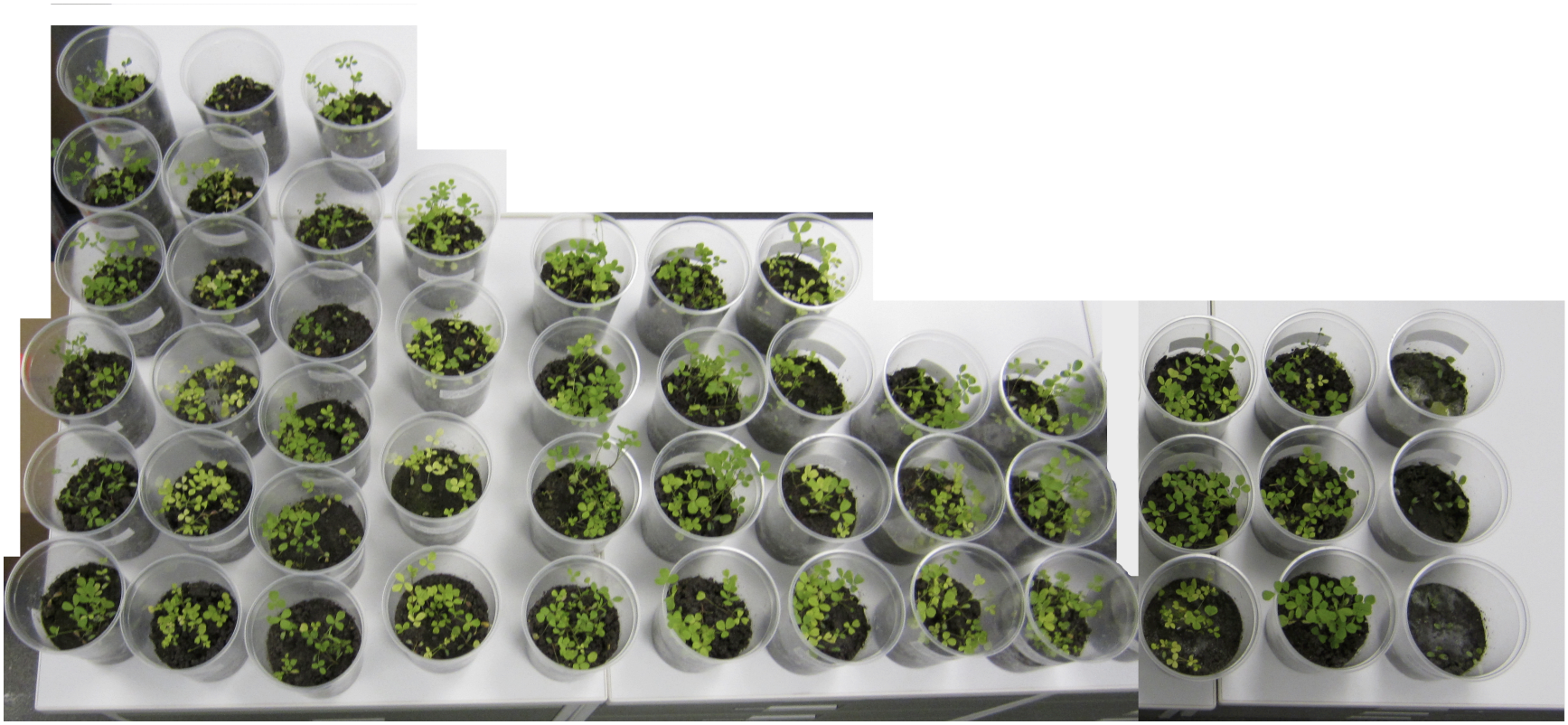

Fig. 3.4.2. *Medicago sativa* test units with seedlings after 21-day exposure, before processing as seen from above. Units are arranged from left to right in ascending order according to nanotube amendment: Artificial Soil, Carbon, Sand, 100 mg/kg MWCNT, 180 mg/kg, 320 mg/kg, 560 mg/kg, 1000 mg/kg, 1800 mg/kg, 3200 mg/kg, 5600 mg/kg, 10 000 mg/kg. Note that the 10 000 mg/kg amendment had litle seedling growth, likely due to over-saturation of the test units.

Statistical analysis of seedling emergence showed some significant variation in seedling emergence among treatments at $p \leq 0.05$ returned a p-value of 0.03. Using Bonferroni's multiple comparisons post-hoc test, the significant difference was found to be between the means of the 560 mg/kg MWCNT treatment and the artificial soil control (Fig. 3.4.3). This decrease in germination in the 560 mg/kg MWCNT treatment, while statistically significant, was not found to be part of a trend, and could be interpreted as experimental error.

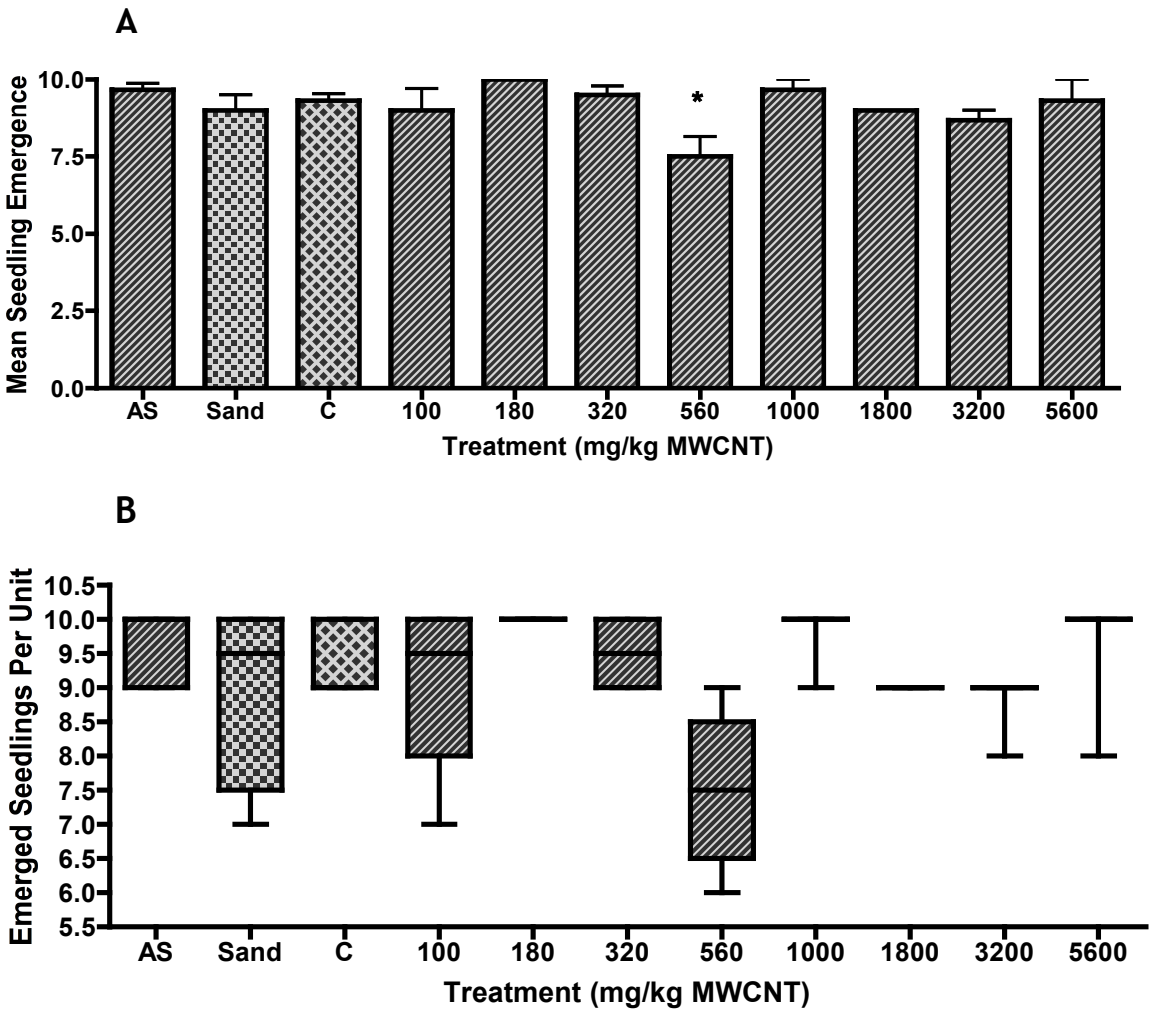

Fig. 3.4.3. A. Mean number of emerged *Medicago sativa* seedlings per test unit for each MWCNT treatment and controls of artificial soil (AS), sand slurry (Sand), and activated carbon slurry (C). Astericks placed above columns denote a significant variation from the control (AS). B. Box and Whisker plot showing data range for each treatment.

Effects of 21-day exposure on root and shoot growth

Shoot growth and foliage health in *M. sativa* seedlings exposed to MWCNT amended artificial soil did not differ significantly at any amendment concentration level when compared to seedlings grown in the negative control, artificial soil ($p > 0.05$). Seedlings grown in activated carbon amended soil, however, were found to have significantly less shoot growth than those plants grown in MWCNT treatments of 180, 320, 1000, and 1800 mg/kg soil dry mass ($p = 0.0001$). Additionally, enhancement of shoot and foliage growth was also seen in the 180 mg/kg MWCNT amendment in comparison to those grown in sand amended soil.

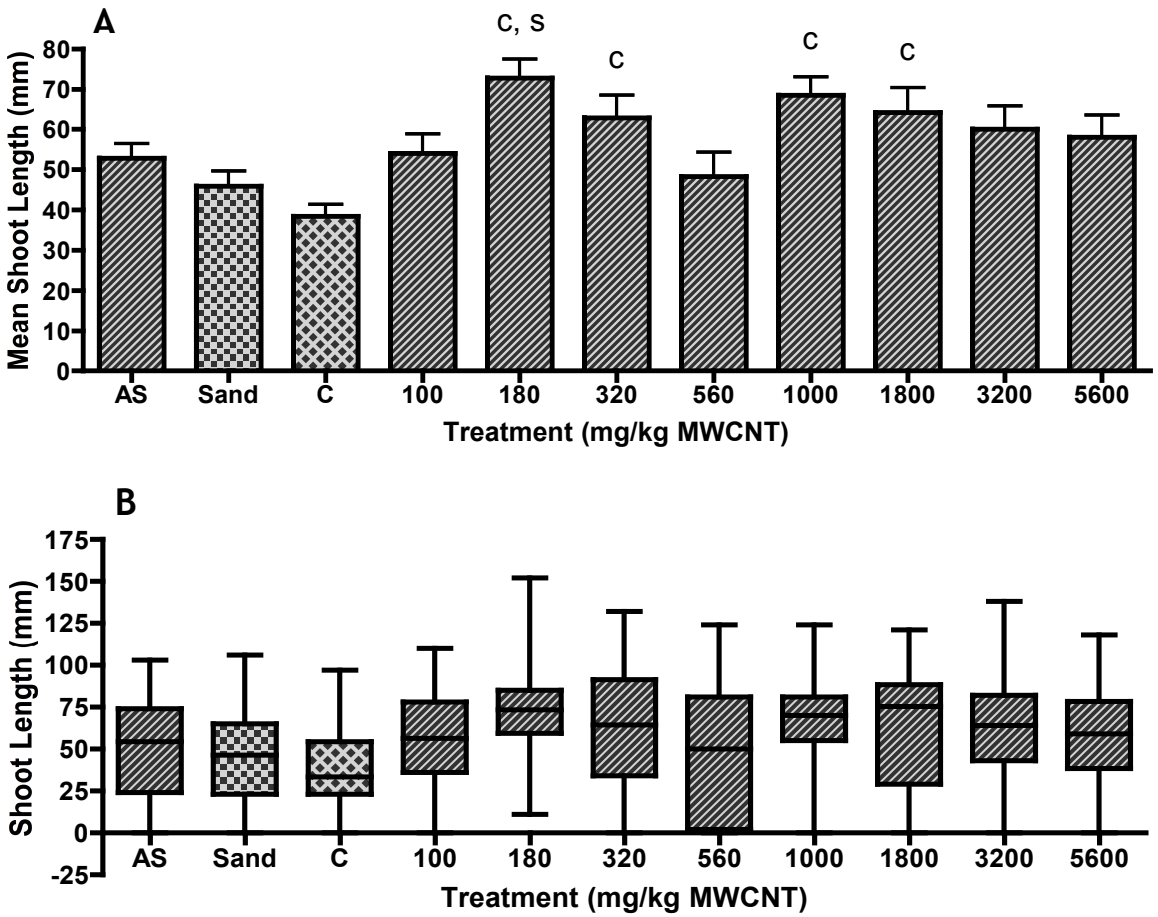

Fig. 3.4.4. A. Mean shoot length of *Medicago sativa* exposed to all MWCNT treatments as well as controls of artificial soil (AS), sand slurry (Sand), and activated carbon slurry (C). Columns marked with a "c" were found to be significantly different from activated carbon column, while columns marked with "s" were found to be significantly different from the sand treatment column. B. Box and Whisker plot showing data range for each treatment.

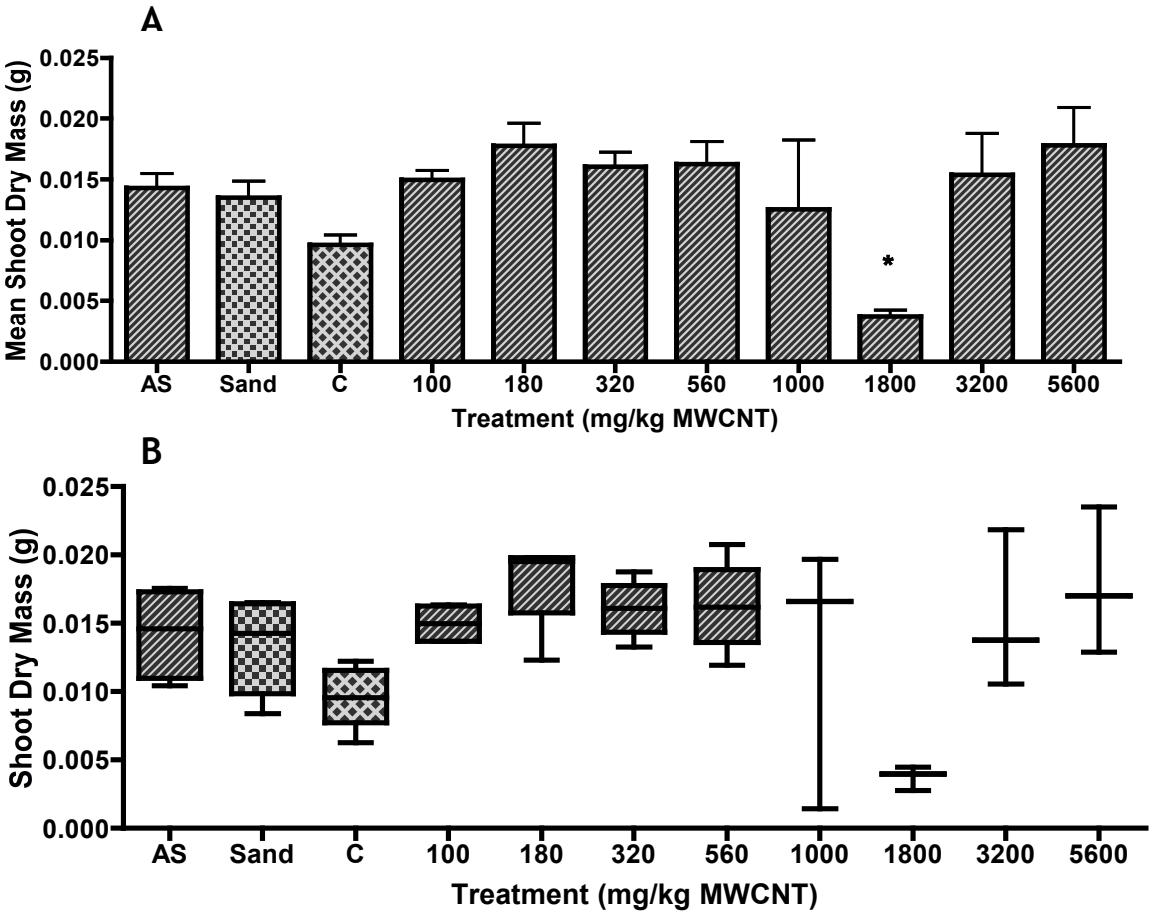

Fig. 3.4.5. A. Mean shoot dry mass of *Medicago sativa* exposed to all MWCNT treatments as well as controls of artificial soil (AS), sand slurry (Sand), and activated carbon slurry (C). Asterisks above columns denote a significant difference from AS. B. Box and Whisker plot showing data range for each treatment.

Analysis of root elongation found significant variation among median root length of *Medicago sativa* after 21 days of growth in treated artificial soils ($p \leq 0.05$). After returning a p value of less than 0.0001, it was determined using Dunn' multiple comparisons test that seedlings grown in activated carbon amended soils had significantly shorter roots than those grown in 180 mg/kg and 320 mg/kg MWCNT amended soils. The same analysis found that seedlings grown in sand amended soil had significantly longer roots than those grown in 560 mg/kg MWCNT amended soils, and significantly shorter than those grown in 180 mg/kg amended soils. No treatments varied significantly from the artificial soil control (Fig. 3.4.6).

Root dry mass analysis resulted in a p-value of 0.0074, suggesting significant variation among means. Dunn's multiple comparisons test revealed that there were significant differences between the artificial soil control (AS) and the activated carbon (C) control (Fig. 3.4.7). Root mass was significantly lower in seedlings grown in activated carbon amended soils compared to those grown in artificial soil.

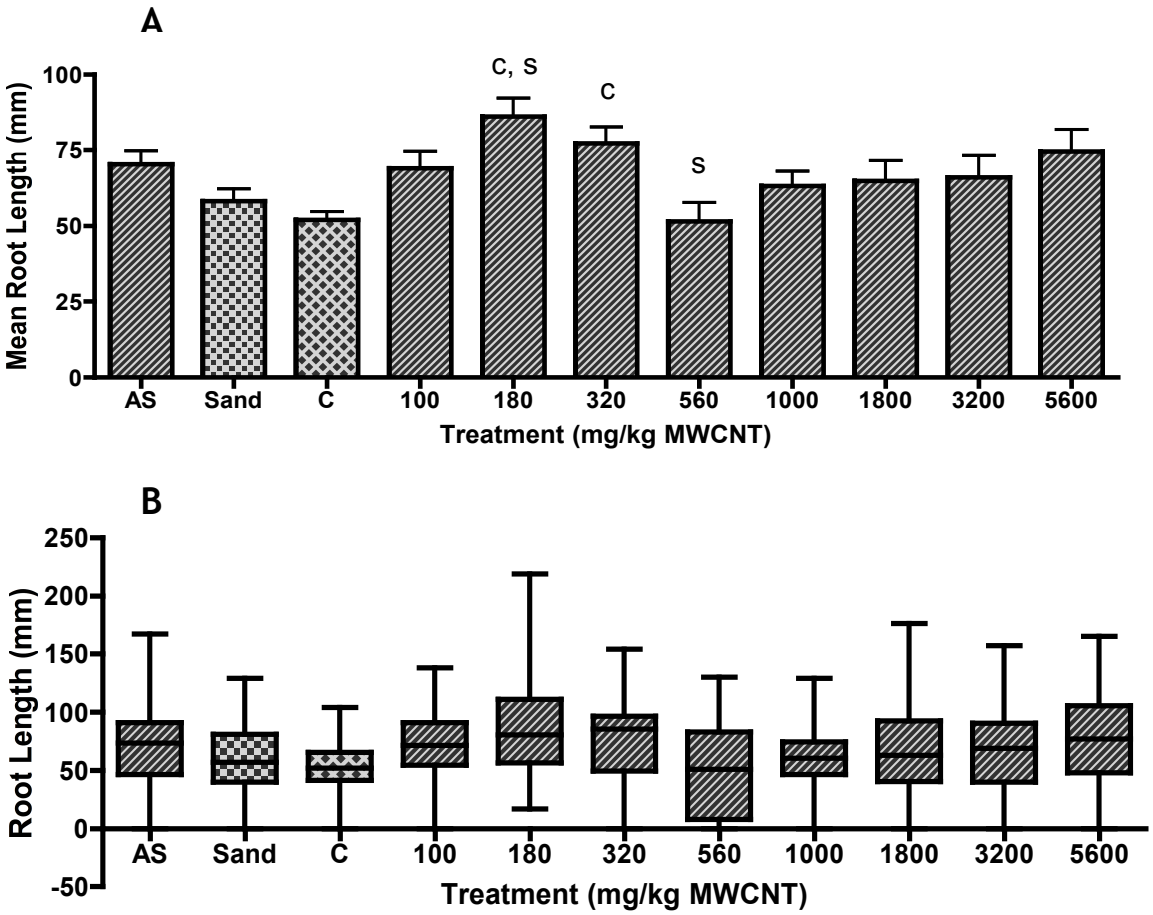

Fig. 3.4.6. A. Mean root length (mm) of *Medicago sativa* exposed to all MWCNT treatments as well as controls of artificial soil (AS), sand slurry (Sand), and activated carbon slurry (C). Columns marked with a "c" were found to be significantly different from activated carbon column, while columns marked with "s" were found to be significantly different from the sand treatment column. B. Box and Whisker plot showing data range for each treatment.

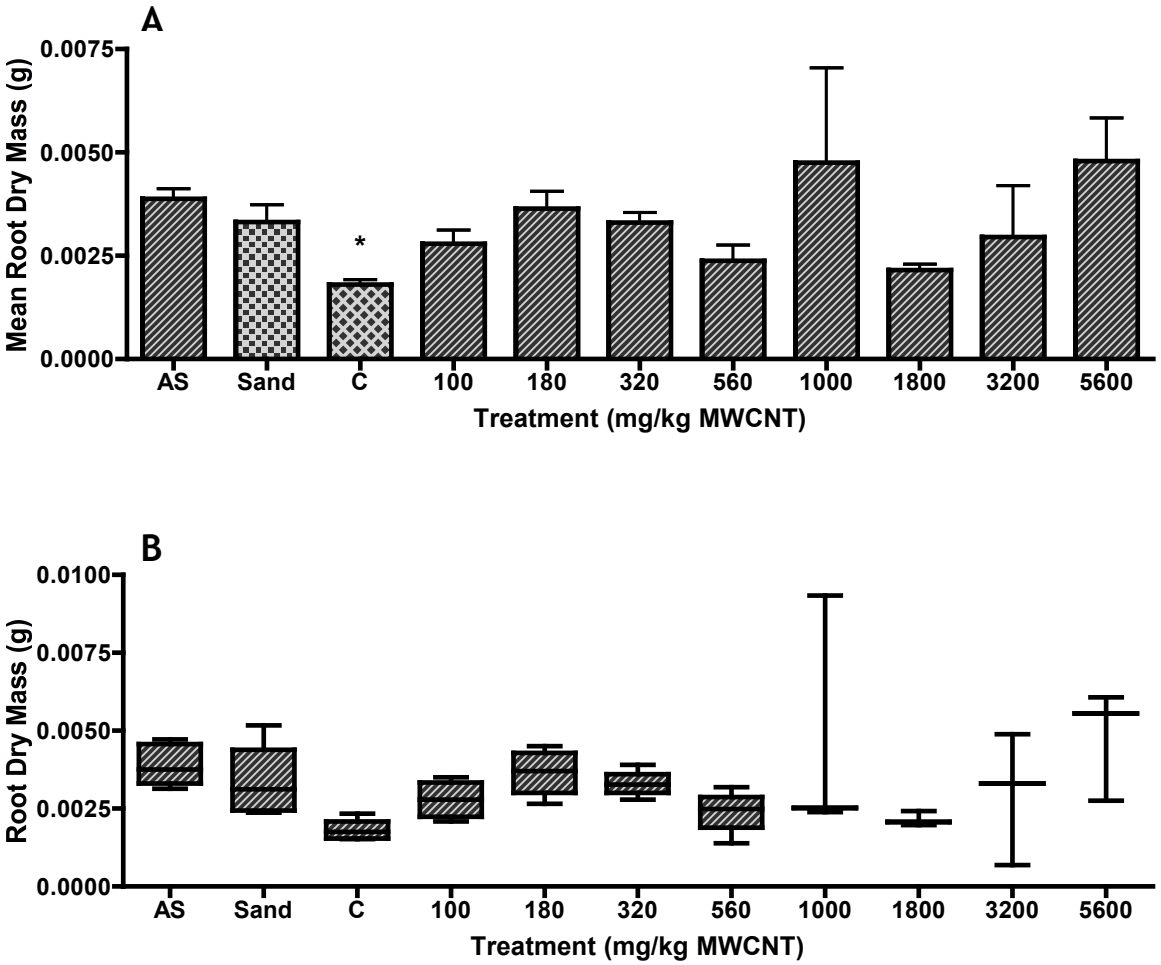

Fig. 3.4.7. A. Mean root dry mass of *Medicago sativa* exposed to all MWCNT treatments as well as controls of artificial soil (AS), sand slurry (Sand), and activated carbon slurry (C). Asterisks above columns denote a significant difference from AS. B. Box and Whisker plot showing data range for each treatment.

Fig. 3.4.8. *Medicago sativa* seedlings after liberation from amended test soil. Seedlings are arranged from left to right in ascending order according to nanotube amendment: Artificial Soil, Carbon, Sand, 100 mg/kg MWCNT, 180 mg/kg, 320 mg/kg, 560 mg/kg, 1000 mg/kg, 1800 mg/kg, 3200 mg/kg, 5600 mg/kg. No visible difference was noted between seedlings grown in any concentration of MWCNT amendment.

4.0 Discussion

4.1 General discussion

The toxicity of multiwalled-carbon nanotubes (MWCNT) in soil has been evaluated with earthworms, plants and springtails. This is the first study to examine the soil toxicity of MWCNT with this range of organisms. The overarching conclusion is that MWCNT had little impact on any of these organisms. This is discussed below in separate sections for the earthworms, plants and springtails. One interesting observation is the apparent ability of earthworms to aggregate MWCNT in soil. This observation is also discussed in consideration of how MWCNT in the soil might be degraded, measured, and transferred into terrestrial food chains.

4.2 Earthworm toxicity tests

The earthworm, *Eisenia andrei*, was not affected by MWCNT at exposure concentrations in soil of up to 10 000 mg/kg. Four earthworm response parameters were unchanged by the exposure to MWCNT: adult survival, reproduction, growth and moisture content of progeny. In general, this lack of a significant effect agrees with the overall conclusion of several reports on CBNP/NM and earthworms (Petersen *et al.*, 2008; Scott-Fordsmand *et al.*, 2008).

Perhaps the most studied class of CBNP/NM has been the fullerenes. These have been studied with three species of earthworms, *E. fetida, E. veneta,* and *Lumbricus rubellus*. Van der Ploeg *et al.* (2011) determined that for *L. rubellus,* exposure to C_{60} reduced cocoon production and juvenile growth rate and increased juvenile mortality (van der Ploeg *et al.*, 2011). The authors concluded that in the juvenile stage earthworms

were sensitive to C_{60} fullerenes and this could lead to long-term adverse effects on earthworm populations. In contrast, *E. fetida*, reproduction was impaired only at very high C60 concentrations, and even then, growth was not hindered (Li & Alvarez, 2011). The authors concluded that C_{60} in soil was unlikely to cause acute toxicity to *E. fetida*. A similar conclusion was reached with C_{60} and *E. veneta* (Scott-Fordsmand *et al.*, 2008). No effect was seen on hatchability, survival, or mortality atconcentrations up to 1000 mg of C60/kg.

Less attention has been given to CNT. Double-walled carbon nanotubes (DWCNT) were found to have a slight impact on *E. veneta* (Scott-Fordsmand *et al.,* 2008). Cocoon production was reduced at concentrations above 37 mg DWNT/kg. Conversely, hatchability, growth, and mortality were not affected at up to 495 mg DWNT/kg. Exposure of *E. fetida* to SWCNT and MWCNT at concentrations up to 3 g/kg had no effect on earthworm lipid content and dry mass (Petersen *et al.,* 2009a).

4.3 Springtail toxicity tests

This study represents the first on the effect of CNT on springtails (*Folsomia candida*), and as with the only study of MBNP and springtails, no effect was seen. MWCNT at concentrations in soil of up to 10 000 mg/kg of dry soil were found to have no effect on the survival and reproduction of springtails. In the case of MBNP, zinc oxide nanoparticles (ZnO-NP) were found not to alter survival but inhibited springtail reproduction (Kool *et al.,* 2011). This effect was not as a result of the presence of NP, but instead attributed to the release of zinc ions from the NP. The authors noted that the cuticle and ventral tube (diameter approximate 5 μm) were possible entry routes for chemicals and NP. Indeed the exoskeleton of arthropods might limit exposure to NP/NM.

Among arthropods, the most work on CBNP/CBNM has been done with *Drosophila melanogaster*, which is an insect rather than a member of the Collembola but still belonging to the subphylum hexapoda. When *D. melanogaster* larvae were exposed in their diet to fullerene C_{60}, carbon black (CB), SWCNT, and MWCNT, egg to adult survivorship was unimpaired (Liu *et al.,* 2009). When adult flies were exposed to the same CBNMs in dry form, CB and SWCNT adhered extensively to fly epicuticle surfaces. This overwhelmed natural grooming mechanisms, and led to impaired ability to climb test tube walls and ultimately to mortality. In contrast, C_{60} and MWCNT adhered weakly and could be removed by grooming. Locomotor function and survival were unchanged. Thus overall, MWCNT might just not be very toxic to arthropods.

4.4 Plant toxicity tests

MWCNT did not significantly influence seedling emergence, length of root growth, or dry mass of the two test species, *Hordeum vulgare* (barley) and *Medicago sativa* (alfalfa). This is similar to the majority of reports on the effects of CBNP/NM to plants. Some researchers have found negative effects and increasingly other researchers are discovering positive responses. These three very different outcomes are briefly discussed below.

Like many reports exploring the interactions between CBNP/NM and plants, MWCNT at 5600 mg/kg of dry soil had no effect on barley and alfalfa. MWCNT at up to 2560 mg/L did not negatively affect either the germination percentage or the germination index (GI) for alfalfa (Miralles *et al.,* 2012). Similar results have been seen for other species. The germination of lettuce, corn, cucumber, rape, radish or ryegrass was not affected by up to 200 mg/L CNT (Lin & Xing, 2007). Seeds of mustard and mung bean

germinated normally in 40 mg/L CNT (Ghodake *et al.*, 2010). MWCNT had no effect on wheat root and shoot growth (Wild & Jones, 2009).

Some studies have found CBNP/NM to have negative effects on plants, although these appear to depend on plant species, to some extent. Several studies focused on rice. When rice cells in suspension were exposed to MWCNT, the MWCNT wrapped around the cell walls, elicited hypersensitive responses, and eventually killed cells (Tan *et al.,* 2007). At low concentrations, death appeared to be by apoptosis; but at high concentrations, death was by necrosis. Rice seeds exposed to SWCNT in complexes with natural organic matter (NOM) had reduced biomass and delayed flowering (Lin *et al.,* 2009). SWCNT also appeared to cause apoptosis in *Arabidopsis* sp. protoplasts (Shen *et al.,* 2010). Functionalized and non-functionalized SWCNT inhibited root elongation in tomatoes but not in carrot and cabbage (Cañas *et al.,* 2008).

Reports of CNT having positive effects on plant development have sparked considerable interest as the results might be exploited in agriculture in the future (Gogos *et al.,* 2012). Non-functionalized SWCNT enhanced root elongation in onion and cucumber (Cañas *et al.,* 2008). Root elongation was also increased by MWCNT in alfalfa and wheat, but in alfalfa this was partially attributed to catalyst impurities (Miralles *et al.,* 2012). The most excitement has been caused by the discovery that MWCNT at 10 to 40 μg/mL greatly stimulated germination rates in tomato seeds (Khodakovskaya *et al.,* 2009). The mechanism appeared to be the mechanical penetration of the seed coat so that water uptake was enhanced, leading to increased germination. This led to a patent and to exaggerated claims in the popular press (Gogos *et al.,* 2012). An example of an over-the-top title in open source articles is “CNT are super-fertilizers”.

Shockingly, the original research paper by Khodakovskaya *et al.* (2009) was withdrawn on August 20, 2012 "due to violations of the Ethical Guidelines to Publication of Chemical Research of the American Chemical Society for reasons of unacceptable redundant inclusion of text and graphics from two works previously published in other journals". However, the core discovery seems to remain unchallenged.

Clearly much more research is required in order to discover the full range of effects to plants arising from exposure to CBNP/NM. From the broader perspective of studies on all NP/NM, many factors have been found to influence their toxicity to plants (Rico *et al.,* 2011), and possibly some of these might be important variables to consider in future studies with MWCNT. Some of these variables include: seed size, plant species, life cycle stage, and plant age. In addition, the exposure medium might be one of the critical factors influencing the outcome. In most of the studies to date, the growth media have varied. The strength of the current study is that the general toxicity testing was done as recommended by Environmental Canada guidelines, although there are no guidelines yet specifically for NP in soil.

4.5 MWCNT aggregates in soil

The structure of the test soil with high concentrations of MWCNT was noticeably changed in the presence of earthworms. Dark, irregular, cylindrical structures were evident in the soil after 35 days with MWNT but were not apparent when the MWNT were first mixed with the test soil, or when earthworms were absent. This was attributed to be the MWCNT being redistributed within the test soil through the activity of the earthworms. Soil structure has been defined as the arrangement of particles and associated pores (Oades, 1993). The soil particles are arranged into secondary units called

aggregates or peds. A hierarchy of soil aggregates has been proposed (Tisdall and Oades, 1982). Free particles and silt-sized aggregates (< 20 μm) clump together into micro aggregates (20 -250 μm), and in turn these stable micro aggregates cluster together into macro aggregates (>250 μm) (Six *et al.,* 2004). Abiotic and biotic factors contribute to aggregation, aggregate stability, and soil structure (Oades, 1993). The MWCNT might be considered as primary and secondary particles that during the course of the experiment selectively aggregate, and at high starting concentrations, become visible as dark streaks.

Why MWNT would selectively aggregate is a matter of speculation. Earthworms are however one biotic factor contributing to soil particle aggregation: one way that they do this is through the production of casts (Six *et al.,* 2004). Casts arise from earthworms ingesting soil, molding the material in their digestive tract, and egesting it. Many earthworm species (including *Eisenia andrei*) are selective in what they ingest. It is possible that earthworms selectively take up and egest MWNT-containing casts, which would be expected to be the black color of MWNT slurries. These casts could be more stable than conventional casts and be sites of additional aggregation, thus being responsible for the structures seen in the test soil. Little is known about the stability of MWCNT in soil.

4.6 Transformation of MWCNT in soil

To date, information on how CNT might change in the natural environment is limited (Petersen & Henry, 2011). Covalent reactions and biodegradation are thought to be the two types of transformations that are broadly possible.

Despite being generally inert, CNT can still undergo covalent additions (Petersen & Henry, 2011). The fullerene-like end-caps and defects on the sidewalls are potential

oxidation sites, although strong oxidative forces are required for chemical oxidation of CNT. Photooxidation is the most likely way that this could occur in the natural environment. When exposed to sunlight, carboxylated SWCNT produced reactive oxygen species (ROS) and these oxidize CNT. Oxidative treatments introduced oxygen-containing surface functional groups, such as hydroxyl, carboxyl, carbonyl, and ester groups, and open the end-cap (Petersen *et al*, 2011b). Inasmuch as the MWCNT were mixed into the test soil, light would be unlikely to penetrate sufficiently to cause any changes in the MWCNT.

Potentially, MWCNT could undergo biodegradation during the course of the experiments but this appears unlikely based on the length of the exposures. Generally, little information is available on the biodegradation of CNT in the environment, however one suggestion for deriving a potential timeframe is to look at what is known about the degradation of black carbon, which has properties similar to CNT. Black carbon degradation varies strongly with the soil type. In tropical soil the half-life has been estimated at approximately 50 years, whereas values of between 182 to 541 years were found for Russian steppe soil (Hammes *et al.,* 2008; Bird *et al.,* 1999).

Recently, possible biodegradation mechanisms have been investigated. Extracellular soil enzymes have been suggested to have a role in CNT degradation. This has been studied with horseradish peroxidase. This enzyme was found to modify carboxylated SWCNT but not SWCNT (Allen *et al.,* 2009). The changes included shortening of CNT and the addition of carboxyl groups. In the current study the test soil is unlikely to have abundant peroxidase content, which commonly arises from white rot and soft fungi (Sinasbaugh, 2010). Internalization of CNT into the phagocytic cells of

animals could also expose them to an environment that would support degradation. This has been studied with myeloperoxidases of neutrophils and phagolysosomal stimulant fluid (PSF). PSF is designed to mimic the low pH and chemical environment of phagolysosomes, which is where microbes and particulates accumulate inside phagocytes. Both the myeloperoxidase and PSF were found to degrade SWCNT. Over 90 days, PSF degraded carboxylated SWCNT to ultrafine solid carbonaceous debris (Liu *et al.,* 2010). It is known that earthworms contain peroxidases and phagocytes (Stein *et al.,*1977; Hassett *et al.,*1988) and thus might be able to degrade CNT. Springtails would also be expected to have peroxidases and phagocytes, although CNT might not be internalized as efficiently into springtails as in earthworms due to the comparatively impermeable Collembola epicuticle. Clearly the long-term fate of CNT in soil needs further work and whether CNT degradation will elicit different responses in the soil biota will be interesting to discover. One of the keys will be to develop effective methods to measure CNT in soil biota.

4.7 Measuring MWCNT

The analysis of the soil, earthworms, springtails, and plants for MWNT would be useful for understanding the movement of MWNTS in the environment but likely will be difficult. Imaging and/or analytical approaches could be tried. Localization of CNT in a carbonaceous background by transmission (TEM) or scanning (SEM) electron microscopy is difficult (Kammer *et al.,* 2012). This is due to the lack of contrast. Several quantitative analytical approaches (e.g. thermogravimetric analysis (TGA)) could be tried. TGA determines changes in weight in relation to a heating program in a controlled atmosphere. Thermal oxidation of raw SWNT and MWNT has been documented to occur

between 390 and 730 °C (Musumeci *et al.,* 2007). However, TGA might have trouble distinguishing CNT from background black carbon in soil (Sobek & Bucheli, 2009).

Another possible approach is a very new one, which could be described as microwave-induced heating (Irin *et al.,* 2012). This method is based on the fact that unlike most conventional materials, CNT evolve extreme amounts of heat when exposed to microwave field due to their strong microwave absorption. Irin *et al*. (2012) grew alfalfa seeds in the presence of CNT or microinjected alfalfa roots with known amounts of SWNT and MWNT. A thermocouple was used to measure the temperature increase in roots upon microwaving. The level of temperature increase depended on the CNT present inside the root, and calibration curves were generated. With this method, the threshold for detectable CNT concentration was < 0.1 μg, and was found to be more sensitive than Raman spectroscopy. The developers of this technique hope to analyze CNT in soil, earthworms, and tissue samples (Irin *et al.,* 2012). One question that could be addressed with development of effective measurement techniques is the possible transfer of MWCNT from soil into food chains.

4.8 Food chain transfer of MWCNT

As the earthworms appeared to have ingested and egested MWNT, as judged by the appearance of the casts, and as the earthworms still appeared healthy, the earthworms could act to transfer MWNT from the soil to food chains in the terrestrial ecosystems. In other words, the earthworms would be expected to have taken up MWNT into at least their digestive tract as a result of their geophagous behavior. Earthworms form the base of several food chains and thus could transfer internalized MWNT into a wide range of animals. Fish, amphibians, reptiles (e.g. snakes), mammals (e.g. moles), and birds (e.g.

robins) eat earthworms, as do invertebrates such as mites, beetles, snails and slugs (Edwards, 1994). Earthworms (*Eisenia fetida*) were placed in artificial soil media in which silver NP had been mixed and collected up to 15 days later and then fed to juvenile bullfrogs (*Rana catesbeina*) (Unrine *et al.,* 2012). Silver accumulated in the liver, kidney, spleen, muscle, stomach and intestine of the frogs. The results suggest that engineered NPs in soil may be taken up by earthworms and transferred to higher order consumers.

The springtail population appeared unaffected by exposure to MWNT, but whether springtails would pick up MWCT from the soil and potentially transfer MWNT up food chains is unclear. Springtails feed on fungal hyphae and possibly nematodes (Fountain & Hopkin, 2004). Therefore, if these organisms were to take up MWNT, springtails might internalize MWNT through ingestion. To date, little or no information is available on the uptake of NP by fungi and nematodes. Spiders, among other organisms, eat springtails, opening up the possibility of trophic level transfer of MWCNT (Vucic-Pestic *et al.,* 2010).

A concern has been expressed about edible plants transferring ENM into the human food chain (Rico *et al.,* 2011), but for this to occur plants would need to take up and store ENM. For carbon-based nanomaterial (CBNM), the literature available on these questions is limited. Research has been done on several crop species and on model research plants, such as *Arabidopsis thaliana*. The tested CBNMs have been the fullerene C_{70}, SWNT and MWNT. In some cases these materials formed complexes with natural organic matter (NOM).

Several experiments suggest the uptake of CBNM by plants. SWNT appeared to enter *A. thaliana* leaf cells and *Nicotiana tabacum* cells in suspension (Shen *et al.,* 2010;

Liu *et al.,* 2009) through an endocytotic process. In developing tomato seedlings, the seeds and root systems were reported to take up MWNT (Khodakovskaya *et al.,* 2009). In another study MWNT were observed initially on the root surface of wheat seedlings and then piercing the epidermal and root hair cell walls and root cap (Wild & Jones, 2009). NOM-suspended fullerene C_{70} appeared to be taken up into rice plants (Lin *et al.,* 2009). The presence of C_{70} in the form of black aggregates was seen in the seeds and roots, and was judged to be less abundant in the stems and leaves. The presence of NOM-C_{70} aggregates in leaves suggests that they had travelled through the xylem. In mature plants, the stem's vascular systems and leaves were the predominant sites of NOM-C_{70} aggregates, whereas the roots seemed to be devoid of C_{70}, supporting the claim that C_{70} was translocated from the roots to the aerial parts of the plant.

CBNM storage in plants and transfer up the food chain has yet to be demonstrated but at least two studies provide suggestive information. When a suspension of *N. tobaccum* cells was incubated with SWNT, this CBNM appeared to localize in vacuoles (Chan *et al.,* 2010). In the study of C_{70}-NOM in rice plants, a remarkable observation was made (Lin *et al.,* 2009). As mentioned previously, black aggregates were found in stems, leaves, roots and seeds. Additionally, black aggregates were spotted in the leaf tissues of second -generation plants. Thus it is possible that CBNM could be transferred to the next trophic level, for example, into ruminants and humans.

In the current study, visible signs of MWNT in plants were not apparent, and this is in agreement with other studies that show restricted movement of CBNM in plants. No uptake of SWCNT and functionalized SWCNT (F-SWCNT) was seen in the roots of cucumber seedlings (Cañas *et al.,* 2008). However, the SWCNT were found in the form

of nanotube sheets on the external surface of the main and secondary roots. Another study found that the entry of MWCNT into suspended rice cells was restricted by the cell walls (Tan & Fugetsu, 2007). MWCNT appeared as black clumps surrounding the cells (Tan *et al.,* 2009). It is clear that more research is needed on the potential of CBNM to enter the food chain through plants.

4.9 Summary

Multiwalled carbon nanotubes (MWCNT) in soil have been found to have no significant impact on ecological receptors, represented by the test species *Eisenia andrei*, *Folsomia candida*, *Hordeum vulgare,* and *Medicago sativa*. However, this should be considered just the beginning in evaluating the toxicity of introducing carbon nanotubes (CNT) into soil. This is because the number of functionalized CNT is large and continues to grow and because soil toxicity tests can have many additional endpoints and be done in a variety of ways. Currently, data and governance gaps exist for CNT (Philbrick, 2010). This thesis represents a start at filling the data gap.

Based on the analysis of data obtained in these tests, and the experience gained by their execution, a number of recommendations for future studies can be made:

1. The observation of black deposits in the earthworm test units after 35 days of exposure should be explored by conducting a test that is performed using the same procedures described for the definitive earthworm reproduction test used in this thesis. Instead of completing a 63-day reproductive test; however, the units should be processed after 35 days, with the deposits being harvested as a priority. Adults removed from the

units should be cleaned using deionized water, and placed into petri dishes lined with moistened filter paper (separate dishes for each treatment).

After 24 hours, the earthworms would be removed and the castings harvested. These could then be compared among treatments for colour, appearance under SEM, and analyzed using Raman spectroscopy or thermogravimetric analysis. Castings found in petri dishes would also be compared to those found in the test soil.

This test would continue to explore the hypothesis that *E. andrei* could be rearranging MWCNT in soil.

2. In order to ensure the exposure of *Folsomia candida* to MWCNT, the nanotubes could be applied more directly to the organisms. MWCNT could be used to amend *F. candida*'s food source (baker's yeast). Organisms would be placed into the standard test units containing artificial soil. A 28-day definitive test would be completed, with the organisms being fed the MWCNT amended food source. This would increase the likelihood of exposure and uptake of MWCNT in springtails through ingestion.

3. To further explore the effects of MWCNT amended soils on the emergence and growth of plants, different test media could be used. Comparison of the effects of germination and growth of seeds in amended artificial soil and an amended hydroponic growth medium could offer insight into the wide variation in effects seen among tests involving CNT and plants.

A potential continuation of these experiments would be to repeat the definitive tests used in this thesis (*E. andrei* definitive tests on survival, reproduction and growth; *F. candida* test on survival and reproduction; *H. vulgare* and *M. sativa* test on seedling emergence and growth) with the addition of equivalent treatments using MWCNT with various functional groups, MWCNT suspended using surfactants, and different types of CNT (single walled, double walled). This large-scale analysis would be invaluable in developing a base of CNT toxicological data in order to prevent human or ecological damage caused by the release of these materials.

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Appendix

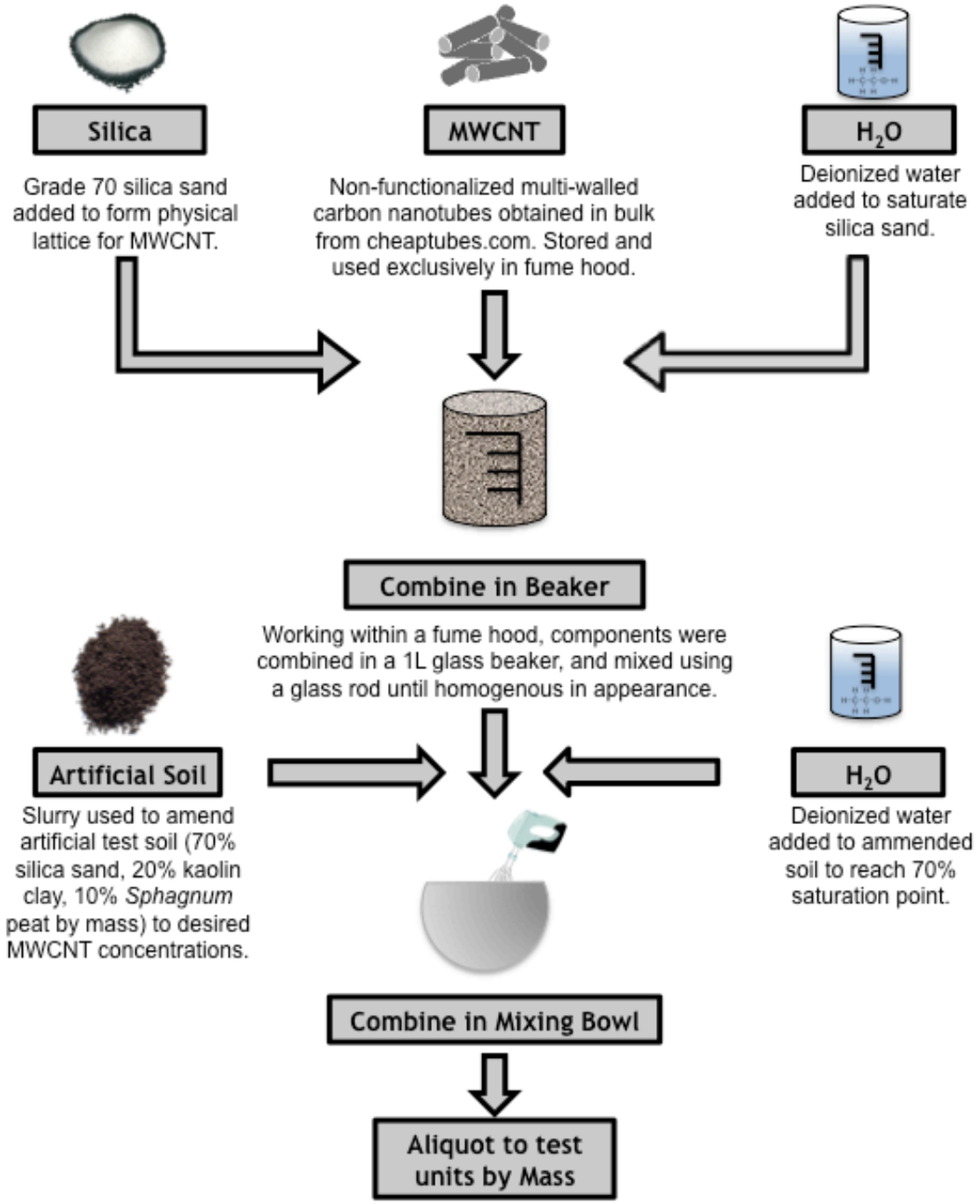

Fig. A.1 Flowchart describing the methods for the production of MWCNT slurry.

Prior to conducting the full definitive bioassays, range-finding tests using *Eisenia andrei* and *Folsomia candida* were employed to determine appropriate treatment ranges and experimental methods. These range-finding bioassays were completed using the same methods described for the definitive tests; however, a smaller treatment range was used with fewer units per treatment (in comparison to definitive tests). Two controls of artificial soil and slurry without MWCNT component were used, along with three concentrations of MWCNT amended soil (100, 1000, 10 000 mg/kg MWCNT). Additionally, a small amount of ethanol was included in the MWCNT slurry (approximately 1% of slurry final volume).

After 28 days, analysis of surviving *Folsomia candida* showed a significant decrease in both the production of progeny and in the survival of adults in MWCNT-treated soils. ANOVA with $p \pm 0.05$ showed significant difference between treatment of 10 000 mg/kg to the artificial soil control for mean number of surviving adults, as well as progeny production (Fig. A.2, A.3). Statistical power was very weak, but these tests were intended simply to provide an estimate for larger scale and longer termed tests.

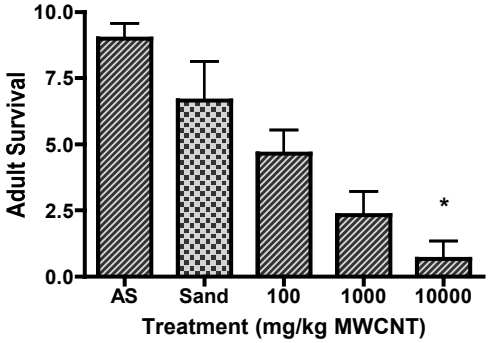

Fig. A.2. Mean number of surviving adult *F. candida* found in test units after 28-day exposure to MWCNT amended soil, controls of artificial soil (AS), and sand slurry (Sand). Treatments marked with "*" were shown to be significantly different from the AS control.

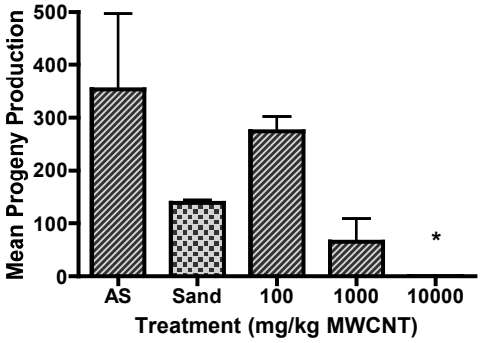

Fig. A.3. Mean number of *F. candida* progeny found in test units after 28-day exposure to MWCNT amended soil, controls of artificial soil (AS) and sand slurry (Sand). Treatments marked with "*" were shown to be significantly different from the AS control.

After 35 days of exposure, adult *E. andrei* were removed from each test unit, leaving only progeny. There were no adult mortalities in the 35-day exposure. After 63 days, the test was ended and the progeny were counted. Analysis of variance showed no significant difference between the treatments and the artificial soil control.

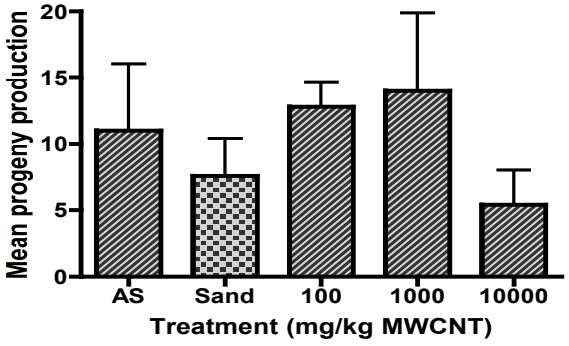

Fig. A.4. Mean *E. andrei* progeny production in 35-day range finding test exposure to soils amended with MWCNT, and controls of artificial soil (AS) and sand slurry (Sand).

After analysis of the data obtained from these tests, it was believed that while the "slurry" method of amending soils with MWCNT was successful, toxic effects found in the range-finding tests could possibly be attributed to the presence of ethanol in the slurry. This effect could be attributable to ethanol present in the amended soil, through direct toxicity to the organisms or by dehydration of the soil. It is also possible that there was an interaction between the ethanol component and the MWCNT. It was therefore decided that in future definitive testing, the ethanol would be left out of the slurry in order to avoid this complication.