RICE UNIVERSITY

Assessing Biological Interactions and Potential Impacts of Emerging Carbonaceous Materials to Terrestrial Organisms

by

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ABSTRACT

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This research addresses the potential ecotoxicity of two emerging carbonaceous materials: C\textsubscript{60} and biochar. The use of these materials is rapidly increasing, as well as their potential for widespread applications. Thus, information about unintended consequences associated the widespread use, incidental or accidental release, and disposal of these emerging materials is needed. The environmental impacts of C\textsubscript{60}, its stable water suspension (nC\textsubscript{60}), and biochar are assessed here using bacteria and earthworms as model receptors.

The antibacterial activity of nC\textsubscript{60} can be mitigated by the presence of natural organic matter as a soil constituent or dissolved in the water column. Sorption to soil might decrease the bioavailability of nC\textsubscript{60} and thus its toxicity to bacteria. Aqueous organic matter also may mitigate nC\textsubscript{60} toxicity. Pristine C\textsubscript{60} showed toxicity to the earthworm’s reproduction and was rapidly bioaccumulated by earthworms, although to a lower extent than smaller phenanthrene molecules that are more hydrophobic; thus, the large molecular size of C\textsubscript{60} hinders its bioaccumulation. Less bioaccumulation occurred at higher C\textsubscript{60} concentration in soil, which is counterintuitive and reflects that higher C\textsubscript{60} concentrations that exceed the soil sorption capacity exist as larger precipitates that are less bioavailable. Earthworms avoided soils amended with high concentrations of dry
biochar, and experienced significant weight loss after 28-day exposure. The avoidance response was likely to avert desiccation rather than to avoid potential toxicants (i.e., PAHs formed during biochar production by pyrolysis) or nutrient scarcity. By wetting the biochar to field capacity before exposing the worms, this adverse effect can be completely mitigated. Overall, this research provides a foundation for ecotoxicity assessment associated with exposure to C₆₀ or biochar, and establishes a method by which other emerging materials can be evaluated for their potential environmental impacts.
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CHAPTER 1
INTRODUCTION

1.1 Introduction

"Technology ... is a queer thing. It brings you great gifts with one hand, and it stabs you in the back with the other."


The discovery, development, and application of novel materials have brought great benefits to human beings; however, in the development of these applications, there is often uncertainty associated with unintended adverse effects of these materials to the environment and human health. Past instances have proven that many new materials, which were initially considered to be completely benign, turned out to have negative impacts to ecosystem and public health. After the harsh experiences of asbestos (a fire retardant causing lung disease), thalidomide (an effective tranquilizer and painkiller causing birth defect), DDT (a powerful pesticide and also a carcinogen), chlorofluorocarbons (widely used in refrigeration and electronics that also contributes to ozone depletion), and many other examples, the importance of a better understanding of the uncertain impacts of emerging material is obvious. This thesis aims to assess the potential ecotoxicity of two kinds of carbonaceous materials (buckminsterfullerene (C$_{60}$) and biochar), which have been considered for a wide variety of novel and widespread applications and can thus be considered as emerging materials.

Fullerenes, discovered in 1985, represent the third allotrope of carbon [1]. There are many kinds of fullerenes depending on the numbers of carbon atoms (i.e., C$_{60}$, C$_{70}$,
C$_{78}$, C$_{84}$), and among these C$_{60}$ is the most commonly studied. C$_{60}$, which has 60 carbon atoms arranged in hexagonal and pentagonal arrangements in the shape of a soccer ball, has a particle size of 7 Å [2]. Its unique properties such as small size, large surface area, photosensitivity and surface reactivity have made C$_{60}$ and its derivatives promising candidates for many applications, including cancer therapeutics, medicine, drug delivery, and computer sensors [3-7].

Although pristine C$_{60}$ is nearly insoluble in water (estimated solubility $1.3 \times 10^{-11}$ g/L) and only soluble in a few organic solvents [8-10], researchers have successfully prepared stable water suspensions containing nano-sized C$_{60}$ aggregates of up to 100 mg/L via several methods [11-15]. The nC$_{60}$ aggregates differ from pristine C$_{60}$ in many aspects, such as color, hydrophobicity, toxicity and reactivity [13]. It has been proposed that water molecules stabilize the formation and existence of nC$_{60}$ [16]. Particle size, morphology and surface characteristics are affected by preparation methods and solution chemistry [14]. When there is a spill of C$_{60}$ or C$_{60}$ dissolved in solvents, nC$_{60}$ may be formed in the environment. While it is recognized that nC$_{60}$ may be the most environmental relevant form of C$_{60}$ [13, 14], it is important to consider the bioavailability and toxicity of both C$_{60}$ and nC$_{60}$ for a complete understanding of its environmental fate and potential impacts.

Biochar is defined as the carbon-rich product produced by heating biomass in a closed container with little or no available air [17]. Application of biochar to soil has been shown to increase soil water and nutrients retention (e.g., by trapping them in micropores [17]), improve soil fertility, and enhance soil structure (e.g., enhanced porosity and soil aeration) [18-20]. The relatively stable nature of biochar and its
subsequent long soil residence time make biochar soil amendment a promising approach to enhance plant growth and increase CO₂ capture (through photosynthesis) to mitigate CO₂ emissions [21]. Biochar is also effective in removing organic contaminants from water [22, 23] and has been demonstrated to be six times more effective in absorbing heavy metals compared to activated carbon [24]. Therefore, it is very likely that biochar will be broadly used in the near future, underscoring the need to proactively assess and mitigate any unintended consequences. In particular, the literature has not yet addressed the potential impact of biochar amendment on terrestrial organisms and the organisms’ associated response.

The interest and use of both C₆₀ and biochar are in upswing. Nanomaterials have already been incorporated into more than 1000 commercial products, and this number is rapidly increasing [25] (Figure 1.1). It has been predicted that the nano-related goods and services market could reach 2.6 trillion dollars by 2015 [26]. Frontier Carbon are producing fullerenes and carbon nanotubes in large quantities in factories with capacities as high as 1500 ton/year [27]. Although the biochar industry is still very small, it has already drawn worldwide attention. Using biochar to replenish the soil carbon pool, restore soil fertility and sequester CO₂ has been proposed to the United Nations Conventions to Combat Desertification and is under discussion [28]. It is very likely that biochar will be used widely in the near future. Because of the potential for widespread application of these two materials, it is essential to proactively assess and mitigate any unintended consequences associated with their applications.
Figure 1.1. Number of new products containing nanomaterials rapidly increases from 2005 to 2009. Number of total products listed, by date of inventory update, with regression analysis [25].

1.2 Statement of purpose

This research provides a foundation for ecotoxicity assessment associated with exposure to two emerging carbonaceous materials: C\textsubscript{60} and biochar. The use of these materials is rapidly increasing, as well as their potential for widespread applications. Thus, information about unintended consequences associated their widespread use, incidental or accidental release, and disposal is needed. Building a fundamental, quantitative understanding of material behavior in natural and engineered systems enables the development of accurate fate and transport models useful for material life cycle impact assessments necessary for risk mitigation.
We focus on bacteria and earthworms as model organisms in terrestrial ecosystems that are likely to be exposed to both C\(_{60}\) (incidentally released through land application of biosolids from wastewater treatment plants that received such engineered nanoparticles, or accidentally released through spills) and biochar (intentionally released as agricultural soil amendment). Bacteria are the basis of all known ecosystems and are important agents of biogeochemical cycles and food webs. Earthworms perform many essential and beneficial functions in soil ecosystems, including decomposition, nutrient mineralization, and soil structure improvement [20], and their ability to perform these functions can be inhibited upon exposure to harmful substances. Thus bacteria and earthworms can serve as a model of chemicals that would be harmful to human health. The methods discussed in this research can also offer a method for evaluating the environmental impact of other emerging materials.

This research first studied the antibacterial activity of nC\(_{60}\) and how this activity would be affected once geosorbents were introduced. We then shifted our focus from bacteria to higher level organism, earthworms, to further assess the potential ecotoxicity of C\(_{60}\) in terrestrial systems and the effect of organic matter on bioavailability of C\(_{60}\). Both ecotoxicity and bioaccumulation potential of C\(_{60}\) were investigated. Finally, we applied the same ecotoxicity assessment approach to another emerging carbonaceous material, biochar, to assess its potential environmental impacts.

1.3 Objectives and Hypotheses

1) Evaluate the potential terrestrial ecotoxicity of C\(_{60}\) including its antimicrobial activity, and discern the effect of natural organic matter on bioavailability and
impact. We hypothesize that soil-associated or dissolved NOM attenuates nC₆₀ toxicity by decreasing its bioavailability (e.g., coating of the nanoparticles by humic acids or sorption by soil organic matter) and/or modifying its surface chemistry. Toxicity of C₆₀ to soil invertebrates can be detected by avoidance behavior and other viability parameters (e.g., weigh loss and reproduction).

2) Assess the uptake and bioaccumulation of C₆₀ by soil invertebrates (i.e., earthworms) and the factors affecting its bioavailability. We hypothesize that bioaccumulation of C₆₀ depends not only on particle characteristics (e.g. size, Kₐw) but also the concentration of soil organic matter, with higher organic matter content decreasing bioavailability and bioaccumulation.

3) Investigate the potential toxicity of biochar to earthworms. We hypothesize that the toxicity of biochar is concentration-dependant and can be evaluated by earthworm avoidance behavior.

1.4 Thesis outline

Chapter 1 provides the background and rationale for the ensuing research. Chapter 2 provides a literature review of relevant topics discussed in the thesis. It begins with an introduction to fullerenes and fullerene water suspensions. A review of bioavailability and ecotoxicity of fullerene is presented next. The interactions of fullerene and natural organic matter are also discussed. Definition and applications of biochar are then reviewed. Methods and relevance of earthworm toxicity testing for toxicity assessment are discussed next, and the chapter finishes with a discussion of bioaccumulation potential of carbonaceous materials to earthworms. In Chapter 3, methods and materials
used in the research are described in details to help the readers with successful reproduction. The remaining chapters address the following goals of this research:

Chapter 4, entitled “Effect of soil sorption and aquatic natural organic matter on the antibacterial activity of a fullerene water suspension”, investigates the association of C60 water suspension (nC60) with natural organic matter, present as a soil constituent or dissolved in the water column, and its effect on the antibacterial activity of nC60. The following paper was published based on these results: “Li, D., Lyon, D.Y., Li, Q., Alvarez, P.J., (2008). Effect of soil sorption and aquatic natural organic matter on the antibacterial activity of a fullerene water suspension. Environ Toxicol Chem 27, 1888-1894”.

Chapter 5, entitled “Assessing the potential ecotoxicity of C60 using earthworm Eisenia fetida”, uses earthworms as a biological system model to continue exploring the toxicity of C60 to terrestrial systems. Acute avoidance and reproduction test results are presented.

Chapter 6, entitled “Bioaccumulation of 14C60 by the Earthworm Eisenia fetida”, studies the bioaccumulation potential of 14C-labeled C60 in earthworms. Bioaccumulation potential of 14C-labeled phenanthrene was also investigated as comparison. Effects of C60 concentration in soil and soil organic matter on bioaccumulation were studied. The following paper was published based on these results: “Li, D., Fortner, J.D., Johnson, D.R., Chen, C., Li, Q. and Alvarez, P.J.J. (2010) Bioaccumulation of 14C60 by the earthworm Eisenia fetida. Environ. Sci. Technol 44(23):9170-5”.
Chapter 7, entitled “Assessing the potential ecotoxicity of biochar using earthworm *Eisenia fetida*”, uses this model organism to study the potential impact of another emerging carbonaceous material, biochar. This chapter is taken from the following paper: “Li, D., Hockaday, W.C., Masiello, C.A., and Alvarez, P.J.J. (2010) Earthworm avoidance to biochar can be mitigated by wetting. Submitted”.

Finally, Chapter 8 wraps up the thesis with a discussion of the engineering significance of this work, conclusions, and recommendations.
CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

2.1 Introduction to fullerene and fullerene water suspension

2.1.1 Discovery and applications of fullerene

Fullerene was first discovered in 1985 by Kroto et al. at Rice University [1]. Kroto, Curl, and Smalley were awarded the 1996 Nobel Prize in Chemistry for the discovery of this class of compounds. There are many kinds of fullerenes depending on the number of carbon atoms (i.e., C\textsubscript{60}, C\textsubscript{70}, C\textsubscript{78}, C\textsubscript{84}) Among these, buckminsterfullerene (C\textsubscript{60}) is the most commonly studied. C\textsubscript{60}, which has 60 carbon atoms in the shape of a soccer ball, has a particle size of 7 Å [2]. The name of Buckminsterfullerene (C\textsubscript{60}) was chosen after Richard Buckminster Fuller, who was known for popularizing the geodesic dome. The structure of C\textsubscript{60} was first proposed in 1970 by R.W. Henson [29]. Although his work was not accepted even by his colleagues, Carbon magazine acknowledged his achievement in 1999. Naturally occurring fullerenes can be found in soils impacted by lightning and geological formations at very low concentrations [30, 31]. In 1990, Huffman et al. developed the simplified method to produce fullerenes in large amount [32], allowing more scientific research focused on exploring physico-chemical properties of C\textsubscript{60}.

Because of its molecule structure with 30 carbon double bonds, C\textsubscript{60} has been reported to react with up to 34 methyl radicals per molecule, so C\textsubscript{60} was considered as world’s most efficient free radical scavenger and described as a “radical sponge”[33]. On the other hand, when photosensitized, C\textsubscript{60} could be excited and produce reactive oxygen species (ROS) [34-36]. Thus C\textsubscript{60} and its derivatives have been considered as promising
candidates in many medical and cosmetic applications [37]. C\textsubscript{60} was able to localize within the cell to mitochondria and other cell compartment sites where free radicals were produced in diseased conditions [38]. Facial products that contain engineered C\textsubscript{60} nanoparticles have already been released into market: Radical Sponge\textsuperscript{®}, made by Tokyo-based Vitamin C\textsubscript{60} BioResearch; Dr. Brandt Lineless Cream, from New York City-based Dr. Brandt; and Zelens Fullerene C-60 Day Cream made by Zelens in London [39]. C\textsubscript{60} can also be used in catalysts, sensors [7], and carrier for gene and drug delivery [38].

2.1.2 Preparation and properties of fullerene water suspension

Although pristine C\textsubscript{60} is nearly insoluble in water (estimated solubility $1.3 \times 10^{-8}$ mg/L) and only soluble in a few organic solvents [8-10], researchers have successfully prepared stable water suspensions containing nano-sized C\textsubscript{60} aggregates of up to 100 mg/L via several methods [11-15]. C\textsubscript{60} is usually first dissolved in an organic solvent (e.g., tetrahydrofuran (THF) or toluene), which is subsequently evaporated after the addition of water. Other methods employ encapsulation (in polyvinylpyrrolidone or cyclodextrins), sonication, or stirring in water for an extended period of time [15]. In this study, the nano-sized C\textsubscript{60} aggregates prepared by toluene/THF will be referred to as nC\textsubscript{60}, with particle size ranging from 2 to 500 nm [13].

The nC\textsubscript{60} aggregates differ from pristine C\textsubscript{60} in many aspects, such as color, hydrophobicity, and reactivity [13]. It has been proposed that water molecules stabilize the formation and existence of nC\textsubscript{60} [16]. Particle size, morphology and surface characteristics are affected by preparation methods and solution chemistry [14]. nC\textsubscript{60}
nanoparticles have been shown to be hydrophilic and negatively-charged, indicating that nC\textsubscript{60} is stable in the suspension and likely to persist in the environment [40].

2.2 Ecotoxicity of C\textsubscript{60} and nC\textsubscript{60}

Despite the widespread research attention drawn by the C\textsubscript{60} nanoparticles, little is known about their potential environmental and human health impacts. To date, only a few studies have assessed the biological impact of pristine C\textsubscript{60} and C\textsubscript{60} did not show much toxicity in most of the studies. Fullerene dissolved in benzene did not show any acute toxic effect to mouse epidermis [41]. While 90-95\% of the injected \textsuperscript{14}C-labeled C\textsubscript{60} nanoparticles accumulated in rat liver, no acute toxicity was observed [42]. Oral administration of C\textsubscript{60} into Sprague-Dawley rats also showed no acute toxicity [43]. In the direct cell exposure studied, C\textsubscript{60} did not cause genetic damage to either bacterial or Chinese hamster lung cells [43]. The C\textsubscript{60} molecule was able to strongly bind to nucleotides and caused significant deformation of DNA, and therefore may interfere with the biological functions performed by DNA resulting in long-term negative effects to the organisms [44].

Several toxicological studies have focused on nC\textsubscript{60}, showing that it is toxic to bacteria, eukaryotic cell lines, water fleas, and several kinds of fish [3, 13, 45, 46]. Research also indicates that nC\textsubscript{60} delays zebrafish embryo and larva development and exerts teratogenic effects [47]. nC\textsubscript{60} prepared by simply stirring in water elicited genotoxicity in bacteria [40]. In some studies, nC\textsubscript{60} has shown anti-viral properties [48]. Antibacterial activity of nC\textsubscript{60} was affected by preparation methods, particle size, storage conditions, and the age of C\textsubscript{60} used to make nC\textsubscript{60} [49, 50]. The mechanism of
antibacterial activity of nC$_{60}$ was first attributed to its ability to generate ROS when photosensitized [34, 35]; however, recent studies have shown that the toxicity effect of nC$_{60}$ required direct contact of the nanoparticles with the cells. It appears that nC$_{60}$ exerts ROS-independent oxidative stress in the cells, causing protein oxidation, changes in cell membrane potential, and interruption of cellular respiration [51, 52].

Some of these toxicological studies used nC$_{60}$ prepared with THF as an intermediary solvent, and trace THF residual was found trapped between nC$_{60}$ particles even after filtration and evaporation [53], raising concerns that the toxicity of nC$_{60}$ is due to THF residual, which is classified by many regulatory bodies as a neurotoxin, rather than nC$_{60}$ itself [53-56]. Another suggestion is that the toxicity of THF-mediated nC$_{60}$ was a result of the formation of g-butyrolactone via the oxidation of THF during the nC$_{60}$ production [57]. In contrast, some studies showed toxic effects of both THF-mediated nC$_{60}$ and nC$_{60}$ prepared by long-term stirring [49, 56]. And THF was not antibacterial at the highest possible concentrations found in nC$_{60}$. Furthermore, nC$_{60}$ suspensions retain their toxicity for more than two years, conflicting with the statement that THF exerts toxicity via forming peroxides which are short-lived [27]. Therefore, THF residual is not a sole determining factor in the toxicity of nC$_{60}$.

2.3 Interactions of natural organic matter (NOM) with C$_{60}$

Most previous toxicological studies were conducted in simple systems with well-defined aqueous media. Little is known about how natural organic matter (NOM), ubiquitous in soil or suspended in the water column, affects bioavailability and toxicity of nC$_{60}$. Bioavailability is defined here as the extent to which humans and ecological
receptors are exposed to xenobiotics (e.g., manufactured nanomaterials) in soil and sediment. When toxicity requires direct contact with the toxicant, bioavailability controls both acute and chronic toxicity (bioaccumulation and biomagnification). Usually bioavailability depends on matrix properties (type and concentration of organic matter, pore size distribution, mixing) and material properties (hydrophobicity, volatility, phase, solubility, etc.). Bioavailability of chemicals is considered to be important in controlling their environmental impacts. When exposed to soil, it is suggested that C₆₀ partitions into soil organic matter decreasing the solution-level bioavailability [58]. Recent research by Tong et al. [58] demonstrated that soil may eliminate the high toxicity of nC₆₀ that has been observed in low-salt mineral media [13-15, 45]. Johansen et al. [59] studied the effect of C₆₀ on soil bacteria and protozoans, and found only fast-growing bacteria were inhibited just after incorporation of nC₆₀. nC₆₀ also caused small differences in the soil community structure by affecting a minor part of the soil microorganisms resulting in dissimilar Dice coefficients compared to the control soil [59]. This indicates the need to consider nC₆₀ interactions with common constituents in environmental matrices to obtain representative results of potential environmental impacts. In addition to toxicological tests, flow-through column studies using glass beads, clays and natural soil have demonstrated the relatively limited mobility of nC₆₀ [60-63]. Clay minerals have also been demonstrated to have a strong propensity to associate with nC₆₀ [63], corroborating the notion that nC₆₀ is unlikely to disperse widely in a natural soil setting.

Soil is a complex system consisting of minerals, weathered rock fragment, organic matter, gases, water, and living organisms. Soil organic matter (SOM) includes all the organic substances in or on the soil, such as living organisms, active fraction
organic matter, surface residue and stabilized organic matter (humus). Active fraction organic matters are organic compounds that can be used as food by microorganisms. Humus are complex organic compounds that remain after many organisms have used and transformed the original material; humus make up most of SOM. There are three general groups of humus: humic acid, fulvic acid, and humin. Among these humic substances, humin is not soluble in water at any pH nor in alkaline solutions. Humic acid is not soluble in water under acidic conditions (pH < 2) but is soluble at higher pH values. It can be extracted from soil by various reagents but is insoluble in dilute acid. Humic acid is the major extractable component of soil humic substances. It is dark brown to black in color. Fulvic acid is soluble in water under all pH conditions. It remains in solution after removal of humic acid by acidification. Fulvic acid is light yellow to yellow-brown in color (http://www.landfood.ubc.ca).

Previous studies have demonstrated that NOM greatly enhanced C_{60} dispersion in water [64], and increased its aqueous stability [65, 66]. Although the presence of divalent cations may work to bridge C_{60} with soil particles, it is suggested that C_{60} partitioning into SOM is most likely the major factor controlling the solution-level bioavailability [58]. Results of physico-chemical characterization suggested that C_{60} may have been chemically transformed when dispersed in an NOM solution in the presence of sunlight [64]. NOM caused disaggregation of nC_{60} crystals and aggregates, thus significantly changing their particle size and morphology, indicating that NOM may play a critical role in the transport and toxicity of C_{60} [67].

2.4 Introduction to biochar
2.4.1 Definition and history of biochar

Biochar is defined as carbon-rich product generated by heating the biomass in a closed container with little or no available air [17]. This process is called pyrolysis. The feedstock to produce biochar can be any kind of biomass, such as wood chips, leaves, manure, and crop residues. Once the pyrolysis process starts, it is self-sustaining and requires no outside energy input (Figure 2.1).

Figure 2.1. The overall process of pyrolysis. (Courtesy Johannes Lehmann, Cornell University)

Using biochar for soil amendment has a history of more than 2500 years. Native Indians in the Amazon basin, Brazil, originated the technique of using charcoal to increase soil fertility. This anthropogenic soil (locally known as Terra Preta de Indio in Brazil) (Figure 2.2) covers more than 50,000 ha in Central Amazonia. It remains highly fertile today, even with little or no application of fertilizers [68]. In 1804, Young
discussed use of ‘pairing and burning’ where soil is heaped onto peat after setting it on fire with reportedly significant increases in crop yields [69]. In China and Japan, humans have traditionally produced biochar in agriculture by covering waste biomass with soil and burning it for days.

Figure 2.2. Typical profiles of “Terra Preta” (right) and oxisol (left) [68].

2.4.2 Applications of biochar

Application of biochar to soil has been shown to increase soil water and nutrient retention (e.g., by trapping them in micropores [17]), improve soil fertility, and alter soil structure (e.g., enhanced porosity and soil aeration) [18, 19]. Adding biochar to soil has been shown to significantly increase seed germination, plant growth, and crop yield in poor soils [18]. Figure 2.3 presents the result from a field experiment comparing the biochar amended soil with plain soil, showing that biochar amended soil increased plant growth of corn significantly. Biochar is able to retain nutrients in the soil and reduce leaching losses. Furthermore, biochar by itself contains high carbon content and a range
of plant macro- and micro-nutrients and can be considered as a fertilizer. Most of the cations present in ashes contained in the biochar are dissolvable salts and readily available for plant uptake [70]. Studies also showed that soil amendment with biochar increased soil cation exchange capacity and pH, thus increased efficiency of applied fertilizers [18].

![Biochar amended soil compared with plain soil](http://www.biochar.info)

Figure 2.3. Biochar amended soil compared with plain soil ([http://www.biochar.info](http://www.biochar.info)).

Due to the relatively stable nature of biochar, adding it to soils is considered a promising way to reduce CO₂ emissions [21]. Instead of letting dead plants and other biomass waste decompose in soil, they can be pyrolyzed into biochar which can be used
to promote plant growth and (through photosynthesis) sequester some of the carbon into stable C pool for centuries [68, 71]. Biochar mixed with soil thus mitigates CO₂ emission to the atmosphere and stores it in a temporary carbon sink, making it a carbon-negative process. Transforming biomass waste into biochar can also mitigate other potent greenhouse gas (GHG) emissions (e.g., CH₄ and N₂O produced under anaerobic conditions) from conventional waste management. Research suggested that CH₄ and N₂O emissions from soil may be lessened by soil amendment with biochar [72]. A number of mechanisms have been proposed to explain decreased N₂O emissions following biochar application, including increased soil pH and aeration, reaction between N₂O and aromatic carbon in biochar, biochar serving as habitat for nitrifiers, and metallic and metal oxide catalytic N₂O reduction on biochar surface. The mechanisms responsible for decreased CH₄ production when biochar is applied likely involve increased soil aeration and redox potential [17]. The application of biochar may also displace use of fertilizers resulting in reduction of GHG emissions associated with the manufacture of fertilizers [17].

Another purpose of biochar application is to prevent the leaching of nutrients and pesticides from soils to reduce pollution and eutrophication risk. The carboxylic groups produced on the edges of the aromatic backbones of the charcoal during pyrolysis are responsible for the high nutrient-holding capacity [68]. Experiments have proven biochar is a good sorbent for eutrophication-causing nutrients such as phosphate and ammonium [19]. Biochar is also effective in removing organic contaminants from water [22, 23] and has been demonstrated to be six times more effective in absorbing heavy metals compared to activated carbon [24].
At present, it is not clear whether there is a maximum amount of biochar that can be safely added to soils without compromising other soil functions [73]. The appropriate application rate of biochar varies with soil type, crop type, weather condition, and so on. At loadings up to 140 t C ha\(^{-1}\), positive yield effects have been reported [21]. However, opposite effects of biochar addition to soil were also observed. Negative yield effects were reported on beans at application rates of 150 t C ha\(^{-1}\), while at application rates up to 50 t C ha\(^{-1}\), positive yield effects were observed on the same crop [74]. This may be due to reduced N uptake from soil by plants (increased C/N ratio) and/or allelopathic effects associated with hydrocarbons or heavy metals leached from the biochar [74]. Not only the relation between biochar loading rate and crop outcome is undetermined, but also the effect of biochar to terrestrial biota is unclear. For example, conflicting results have been reported for impact of biochar application to earthworms. Chan et al. (2008) found that earthworms preferred soil amended with biochar produced from poultry litter pyrolyzed at 450°C. However, this effect was not reproducible with biochar from the same feedstock pyrolyzed at a 550°C [75]. In another study with biochar derived from papermill waste, earthworms exhibited a slight but statistically indiscernible attraction to biochar [76]. The underlying mechanisms still require further work.

2.5 Toxicological studies with earthworms

2.5.1 Rational of using earthworms for toxicological studies

The earthworm is one of the important soil organisms. It performs many essential and beneficial functions in decomposition, nutrient mineralization, and soil structure improvement. Upon exposure to harmful contaminants, the earthworms’ ability to
perform these vital functions may be inhibited. Many ecotoxicological studies have used earthworms as model systems to assess potentially toxic soils [77-87]. Earthworms are very mobile in soil, and may avoid soil sites contaminated with hazardous wastes. This may affect soil properties, biomass, the community structure of flora, and even the entire food chain [88]. Therefore, not only the mortality of the earthworms, but also the behavior of the earthworm has significant ecological implications [88].

Earthworms are suitable test organisms for bioavailability assessment because of their close contact with the soil, thin and permeable cuticle, and consumption of large amounts of soil. Earthworms are also known for their importance in the terrestrial food chain, and high ability of pollutant accumulation. Furthermore, the earthworms are inexpensive, widely available, and suitable for handling in laboratories.

2.5.2 Common toxicological tests with earthworms

Traditional earthworm bioassays include acute and chronic toxicity tests operated under different ranges of sensitivity (lethal, sublethal). In acute toxicity tests, earthworms are usually exposed to soils containing different concentrations of test chemicals for 7-14 days using mortality as endpoint. Only relatively high concentration of certain pollutants will affect the survival percentage of earthworms. Chronic toxicity tests employ relatively low (sublethal) concentrations of the hazardous materials for prolonged exposure to earthworms using endpoints such as body mass loss, reproduction, and subsequent growth of progeny. Chronic toxicity test takes at least 8 weeks [89].

The acute avoidance test was first developed in 1996 [88]. In this test, earthworms are simultaneously exposed to a soil sample spiked with a test chemical, and to a control
soil. The location of live animals is observed after 2 days. The International Standards Organization (ISO) has established earthworm avoidance test guidelines for rapid screening and evaluation of soil function and influence of contaminants and chemicals on earthworm behavior [90]. Environment Canada also published a standard method to test toxicity of contaminated soil using the earthworm avoidance test [89]. Compared to the toxicity tests described above, the avoidance tests have been proven to have higher sensitivity to some contaminants and require less experimental time (Table 1) [77, 88]. In a study by Schaefer et al., earthworms avoided soil containing 29 mg TNT/kg soil significantly, while soils with concentrations less than 1,142 mg TNT/kg soil showed no significant acute or reproduction toxicity effect [77]. This study also showed that earthworms showed a total avoidance effect to naphthalene at concentration of 50 mg/kg, while the LC$_{50}$ of naphthalene to earthworms was 173.8 mg/kg. Hence, the avoidance test is a faster, reliable candidate for assessment of contaminated soils.

### Table 2.1. Comparing three different earthworm test methods

<table>
<thead>
<tr>
<th>Test name</th>
<th>Biological endpoint</th>
<th>Test duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute lethality test</td>
<td>Mortality</td>
<td>7-14 days</td>
</tr>
<tr>
<td>Earthworm reproduction test</td>
<td>Total dry wt, number of cocoon and live juvenile worms</td>
<td>&gt;=8 weeks</td>
</tr>
<tr>
<td>Earthworm avoidance test</td>
<td>Number of live worms per treatment</td>
<td>48 hours</td>
</tr>
</tbody>
</table>

### 2.6 Bioaccumulation of C$_{60}$ by earthworms

#### 2.6.1 Bioaccumulation potential of C$_{60}$ by earthworms
The bioavailable fraction of a chemical in soil is considered as the portion in the matrix that can be taken up by biota under environmental conditions [91]. Therefore, the bioavailability of fullerene can be envisaged by measuring how much of the nanoparticle is accumulated in the body tissue of environmental organisms.

The earthworm, as the prey of many vertebrates and invertebrates [20], is a potential entry point to terrestrial food webs for contaminants [86] as uptake can happen through skin contact as well as oral consumption [92]. Many studies have been performed to investigate the bioavailability and bioaccumulation of polyaromatic hydrocarbons (PAH) using earthworms, showing PAHs are readily accumulated in the fatty tissue of the organisms [93-95]. Thus C\(_{60}\), which is also a large, hydrophobic, polycyclic molecule [96], might be able to accumulate in earthworm body tissue. If earthworms successfully take up and accumulate C\(_{60}\), these nanoparticles could potentially be transferred to higher trophic levels, such as insects, birds, rodents, and other carnivores, potentially even human beings.

Currently, our knowledge of earthworm uptake and bioaccumulation of nanomaterials is limited. It is claimed that nanoparticles could easily be absorbed by earthworms, allowing possible transfer to other animals higher up the food chain [97, 98]. Compared to bulk particles, nanomaterials seemed to be easier for earthworms to take up. Results showed that earthworms accumulated more nano-sized Al\(_2\)O\(_3\) than micro-sized Al\(_2\)O\(_3\) at same treatment concentrations [99]. However, Petersen et al. found that only small amounts of carbon nanotubes could be absorbed into earthworm tissues, possibly due to the strong interaction between carbon nanotubes and soil organic matter and the relatively large size of the nanotube [85]. Compared to carbon nanotubes, C\(_{60}\)
nanoparticles, which have smaller particle size in all dimensions, might be absorbed at a higher rate. For C_{60}, food uptake may be the major pathway to exposure, since accumulation via the digestive route increases with the hydrophobicity of the compound [92].

Bioaccumulation of chemicals by organisms can be described by the bioconcentration factor (BCF) for water, biomagnification factor (BMF) for food, or bioaccumulation factor (BSAF) for soil/sediment. BSAF is defined as the ratio of chemical concentration in the organism to that in the immediate environment (soil, water or sediment). In this study BSAF is calculated according to the following equation [81]:

$$\text{BSAF} = \frac{C_{\text{earthworm dry weight}}}{C_{\text{soil dry weight}}}$$ (2.1)

where $C_{\text{earthworm dry weight}}$ is the pollutant concentration in earthworm at a certain time and $C_{\text{soil dry weight}}$ is the pollutant concentration in soil at the same time. BSAF values are usually lower than 0.1 for compounds with a log $K_{OW}$ less than 3, and may increase to approximately 10, for compounds with a higher log $K_{OW}$ [100, 101]. However for substances with $K_{OW}$ higher than 6, the large molecule size and strong absorption to soil may prevent bioaccumulation resulting in a lower BSAF [95].

2.6.2 Factors affecting bioaccumulation

2.6.2.a Concentration of the pollutant in soil/food

The relationship between chemical concentration and BSAF is not always consistent. Zhang et al. demonstrated BSAF increased with increasing RDX concentration in soil [101], while Kelsey and his coworker suggested an inverse
relationship between \( p,p' \)-DDE concentration and BSAF by three different earthworm species [102]. Decreasing BSAF with increasing pollutant concentration was also observed in the bioaccumulation study of avermectin B1a by earthworms [103]. It seems that the effect of contaminant concentration to BSAF depends on physico-chemical properties of the contaminant, soil concentration, and test organisms.

### 2.6.2.b Incubation time

It has been noted that timescale can significantly affect the final concentration of pollutant in organism body tissue because absorption, accumulation, and elimination processes might occur at different rates. It was observed in many studies that tissue concentration increased with incubation time and steady state levels were reached between 9-42 days [85, 86, 103-106]. Although the OECD guidelines recommend a 14-day incubation time when earthworm toxicity is of interest [107], 8-28 day exposure duration is commonly reported in the literature. However, cumulative tissue concentration might keep increasing up to 56 days for some compounds [104].

### 2.6.2.c Soil organic matter

Soil is a complex matrix in which the bioavailability of pollutant is governed by a three-way interaction between the compound, soil, and the organisms [91, 108]. Thus the organic matter in soil strongly affects the sorption/desorption mechanism of the organic compound and will play an important role in determining the BSAF. The most widely accepted theory describing the relation between the chemical concentration in soil and that in an organism is the equilibrium partitioning theory (EPT) [109]. EPT was first proposed in 1988 based on the premise that the bioavailability of organic chemicals
sorbed to soil is controlled by equilibrium partitioning between pore water, soil and organisms. Some deviations from EPT are observed mostly due to sequestration of pollutants caused by biological and physical-chemical factors [91, 100]. In a study by Petersen et al., BSAFs of pyrene decreased dramatically with increasing organic carbon content of the soils, consistent with equilibrium partitioning expectations, while BSAFs of carbon nanotubes stayed constant with varying organic carbon content. This may be explained by the stronger irreversible sorption of nanotubes to organic matter associated with the soil particles [85]. In our previous study, nC$_{60}$ also showed strong adsorption onto soil indicating soil organic matter may be an important factor influencing bioavailability of C$_{60}$ [110].

2.6.2.d Particle size

Size of the molecules or particles is an important factor affecting uptake of lipophilic compounds by organisms [85, 95, 111]. Apparently, it is difficult for large compounds to pass through the gut membrane; therefore, uptake rate is limited [95]. On the other hand, compounds with higher molecular weight usually exhibit high K$_{ow}$, which suggests higher sorption and lower desorption. To respond to questions concerning whether the compound was really taken up to the earthworm body or only adsorbed to the worm gut wall, Belfroid et al. fed earthworms food contaminated with different concentrations resulting in different concentrations in the earthworm and thus confirmed the real uptake [95].

2.6.2.e Organisms
Earthworms consume organic matter from soil and their feeding strategies differ from different species. Epigeic species live close to the surface and consume coarse particulate organic matter and surface litter; endogeic species live under soil and live on soil humus; and anecic species are burrowed deep in the soil and consume principally surface litter [102]. The difference between feeding strategies may significantly impact the bioaccumulation of organic compound by worm species. In a study of bioaccumulation of \( p,p' \)-DDE and \( p \)-chlorophenyl by three earthworm species, the BSAFs for *Eisenia fetida* (epigeic species) were 10-fold higher than anecic and endogeic species. Aging of the compounds could be an explanation for the different BSAFs of the earthworms because some species preferred litter to soil organic matter and were not able to uptake the weathered compounds efficiently [102].

### 2.7 Relation of this work to current state of knowledge

To date, little work has been done to explore the impacts of \( C_{60} \) and biochar to the terrestrial biological system. For \( C_{60} \), most of the toxicological studies have been conducted in the well-defined aqueous conditions, and effect of natural organic matter, which is ubiquitously present in soil or suspended in the water column, on the antibacterial activity of \( nC_{60} \) is unclear. Current understanding of the potential impacts of \( C_{60} \) to terrestrial invertebrates (e.g., earthworms) is limited, and the potential for \( C_{60} \) bioaccumulation in the terrestrial system has not been addressed, primarily due to the unavailability of quantification methods in the complex matrix. For biochar, the knowledge of impacts of biochar soil amendment to terrestrial organisms is limited, and the mechanisms of potential toxicity of biochar are unexplored.
This thesis investigated the potential toxicological effects of C₆₀ in terrestrial ecosystems, and the effect of NOM on the antibacterial activities of nC₆₀. The application of ¹⁴C-labeled C₆₀ in this study enabled accurate quantification of bioaccumulation of C₆₀ in biomass. The rate and extent of ¹⁴C₆₀ bioaccumulation were quantified using the earthworm *E. foetida* as a model organism. The effects of soil organic carbon content and C₆₀ concentration on bioaccumulation were also investigated. This thesis also studied the potential impacts of biochar to earthworms and explored the mechanisms of observed toxicity. It is important to raise these questions to prevent these materials from becoming a new class of pollutant. Thus this thesis provides a foundation for risk assessment of these materials and appropriate practices in their manufacture, use, and disposal.
CHAPTER 3.

MATERIALS AND METHODS

3.1 Materials preparation

3.1.1 Preparation of nC₆₀

nC₆₀ was prepared following a protocol described by Lyon et al. (2006) with some modifications [14]. C₆₀ (100 mg of 99.5% pure, SES Research, Houston, TX, or MER Corp., Tucson, AZ) was dissolved in 4 L of tetrahydrofuran (THF) (certified spectra-analyzed, Fisher Scientific, Houston, TX). The THF was sparged with nitrogen for 10 minutes to prevent oxidation before and after C₆₀ was added. The mixture was stirred overnight at room temperature in the dark. The solution was filtered through a 0.22-μm-pore-size Osmonics nylon membrane (Fisher Scientific) to remove undissolved C₆₀. A 250 mL aliquot of the C₆₀ tetrahydrofuran solution was stirred vigorously while adding an equal volume of Milli-Q water (Millipore, Billerica, MA) at a rate of 1 L/min. THF was evaporated using a Büchi Rotavapor (Büchi Labortechnik AG, Flawil, Switzerland) with a hot water bath, a refrigerated condenser, and a vacuum pump. One liter of the mixture was heated to 65°C to evaporate the tetrahydrofuran until a final volume of 300 mL was reached. Prior to concentration, the nC₆₀ suspension was filtered through a 0.45-μm-pore-size Osmonics nylon membrane filter to remove large particles. nC₆₀ was concentrated with a Büchi Rotavapor at 70°C to a final concentration of 10 to 15 mg/L nC₆₀. The concentrated suspension was filtered-sterilized through a 0.22 μm-pore-size cellulose syringe filter or a 0.22 μm-pore-size MCE membrane vacuum filter (Fisher Scientific). The resulting suspension was stored in the dark at room temperature.
3.1.2 Preparation of biochar

We prepared biochar in a custom-built demonstration-scale batch reactor at Rice University from apple wood sawdust purchased from Allied Kenco, Houston, Texas. The feedstock was composed of wood fragments which were 5-10mm x 0.5-1mm x 0.5-1mm when added to the biochar reactor; the particle size decreased by 25-50% following pyrolysis. The Rice reactor (Figure 3.1) used in this experiment produces approximately 2 kg biochar per 4 hour run. Biomass is sealed within a 20 liter reactor vessel constructed from 306 stainless steel and heated in a propane-fired furnace. Exhaust gases were passively vented to a series of heat exchangers at ambient temperature to remove condensable liquids and bio-oils and prevent their condensation into the biochar. Non-condensable gases were combusted in a secondary (venturi-style) burner to heat the reactor. The initial heating rate was approximately 5°C/minute to a temperature of ~400°C. Thereafter, the heating rate slowed to 1°C per minute, to a maximum temperature of approximately 525°C. Total heating time was approximately 250 minutes. The reactor was allowed to cool to ambient temperature (overnight) before removing the biochar. When removed from the reactor, the biochar was odorless and visually homogenous.
3.1.3 Preparation of artificial soil

Artificial soil (AS) was prepared as described by Environment Canada [89]. It consists of 10% *Sphagnum* peat moss (previously sieved through 2mm mesh), 20% kaolin clay and 70% quartz sand. The pH of dry soil is adjusted with CaCO₃ to the optimal range (6.5-7.5).

3.2 Characterization methods

3.2.1 Transmission Electron Microscopy (TEM)

TEM images (resolution of 0.2 nm) were obtained with a JEOL 2100 high resolution microscope (Figure 3.2) operated at 120 kV. The TEM samples were prepared by placing drops of sample on 300 mesh copper grids (Ted Pella, Inc.), which were placed on filter paper to remove excess water, and then dried overnight.
3.2.2 Particle size and zeta potential measurement

Particle size and zeta potential were determined using a non-invasive back-scatter (NIBS) device (Zetasizer Nano, Malvern Instruments, United Kingdom) (Figure 3.3). NIBS detects light scattering at 173°, which extends the range of sizes and concentrations of samples that can be measured. The mean diameters were weighted according to the number of particles in each size fraction.
3.2.3 Determination of nC₆₀ concentration

The concentration of nC₆₀ was determined by high-performance liquid chromatography (HPLC) (Waters 4695, Milford, MA) (Figure 3.4) analysis at 336 nm as described by Lyon *et al.* [15]. One ml of 100 mM magnesium perchlorate and 2 ml of toluene were added to 2 ml of the nC₆₀ suspension to extract nC₆₀ from the aqueous phase. The vial was sealed and the mixture was stirred for 2 hours. The vial was then placed in a -20°C freezer to aid in the removal of the toluene phase for analysis. A previous publication showed that this approach extracted more than 94% of the C₆₀ from the aqueous phase with toluene [13]. The HPLC analytical column was a Delta Pak C18 column (150 mm × 3.9 mm I.D., 300 Å, Waters, Milford, MA, USA). The chromatographic separation was performed at a constant flow rate of 1 ml/min with a
mobile phase of 100% toluene. \( \text{C}_{60} \) has a retention time of 1.58 min at these settings. A standard curve was prepared by dissolving varying amounts of \( n\text{C}_{60} \) in toluene, and the absorbance of each test sample at 336 nm was compared to the standard curve.

Figure 3.4. High-performance liquid chromatography (HPLC) (Waters 4695) coupled with radiocromatographic detection.

3.2.4 BET surface analyzer

The surface area of materials was determined based on the Brunauer-Emmett-Teller method using a Quantachrome Autosorb-3B Surface Analyzer (Quantachrome Instruments, Boynton Beach, FL, USA) (Figure 3.5).
3.3 Microbial toxicity tests

3.3.1 Toxicological test with respirometer

A respirometer (Oxymax-ER, Columbus Instrument, OH) (Figure 3.6) was used to monitor the heterotrophic activity of bacteria dosed with nC₆₀ (positive control) or a mixture of sorbents and nC₆₀.
3.3.2 Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial material that will inhibit the visible growth of a microorganism after overnight incubation [112]. MIC tests were performed using Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) methodology. Samples were prepared in 5-ml glass tubes with a series of concentrations of the test material. Test bacteria were added into the samples to a final OD$_{600}$ of 0.002. Then the samples were incubated overnight at 37 °C. MIC was determined as the minimum concentration of the test chemical resulting in an absence of growth of the bacteria in the tubes described before.
3.4 Earthworm toxicity tests

3.4.1 KCl reference toxicant assay

This test is aimed to evaluate the health condition of earthworm before all other experiments. Earthworms were exposed to soil (10 worms/200 g soil) containing a series of concentrations of KCl (5000 ppm to 8000 ppm) for 7 days. After 7 days, worms were hand-sorted from the soil and the numbers of live worms were recorded.

3.4.2 Avoidance test

Soil avoidance bioassays were conducted following a modified method developed by Environment Canada [89]. This test was conducted in stainless-steel avoidance wheels with six pie-shaped compartments connected to a circular, center chamber that served as the test arena (Figure 3.7). Negative control soil (unamended artificial soil) was placed into alternating compartments (3 compartments/unit, 250 g of soil/compartment), while test soil was transferred to the remaining compartments. Test soil and control soil were separated by removable aluminum partitions. Soil in each compartment was hydrated with Milli-Q water to 85% of water holding capacity. Partitions were removed afterwards. Ten worms were selected for each avoidance wheel. Worms were introduced to the center of the avoidance wheel individually. After the addition of ten worms to an avoidance wheel, a lid was placed to prevent escape and time was recorded (t=0). At least five replicates were conducted for each concentration of biochar. The wheels were placed in a dark area at 22°C for 48 h. At the end of 48 h time period, the lid was removed and the partitions were inserted back to the chambers to prevent further worm movement between the compartments. The numbers of worms in each compartment were recorded.
If a worm was cut by the partition and separated in two compartments, 0.5 was recorded for both of the compartments. The percentage of worms (10 in total) in test soils was then calculated.

Figure 3.7 Avoidance test wheel.

3.4.3 Growth and reproduction test

To measure the effects of prolonged exposure of biochar to earthworms on their growth and reproduction, we conducted a 28-d toxicity test according to Environment Canada protocols [89]. After depuration on filter paper hydrated with DI water for 24 h, earthworms were transferred to 500-ml glass jars filled with soil (10 worms/200 g soil) containing different concentrations of test materials for 28 d. Worm weight was measured and recorded at day 0 and day 28 after depuration. Cocoon numbers in each treatment
were counted and recorded. All treatments were prepared in triplicate. Weigh loss was calculated using the following equation:

\[ \text{Weigh loss} = \frac{(W_0 - W_{28})}{W_0} \times 100\% \quad (3.1) \]

Where \( W_0 \) represents the total weight of earthworms on day 0 and \( W_{28} \) represents the total weight of earthworms on day 28.

### 3.4.4 Determination of radioactivity in biomass

Test samples were first freeze-dried in a freeze dryer (Freeze dryer 4.5, Labconco, Kansas City, MO) (Figure 3.8) for 24h and then combusted in a biological oxidizer (OX-600, R.J. Harvey instrument, Tappan, NY) (Figure 3.9). \(^{14}\text{CO}_2\) captured in the scintillation cocktail was measured in a liquid scintillation counter (LS 6500, Beckman, Brea, CA) (Figure 3.10).

![Figure 3.8. Freeze dryer.](image)
3.5 Statistical analysis
Statistically significant differences in treatments were determined using Student t-tests and ANOVA at the 95% confidence level. Goodness of fit to the model was assessed using the Chi-Square test ($\alpha = 0.05$) in Excel.
CHAPTER 4
EFFECT OF SOIL SORPTION AND AQUATIC NOM ON THE
ANTIBACTERIAL ACTIVITY OF nC_{60}

4.1 Introduction

As the nanotechnology industry develops, C_{60} is expected to be produced and consumed in great amounts [113], so there is little doubt that this nanomaterial will increasingly be found in the environment. Thus, it is imperative to understand how C_{60} may interact with abiotic and biological components of the ecosystem and assess the potential environmental impacts resulting from its widespread use and disposal.

It has been suggested that nC_{60} is the most environmentally relevant form of C_{60} when there is a spill of C_{60} powder or C_{60} solution in a solvent [13]. Toxicological studies have shown that nC_{60} is toxic to bacteria, eukaryotic cell lines, water fleas and fish [13, 45, 46, 114]. However, most previous studies were conducted in simple systems with well-defined aqueous media. Little is known about how natural organic matter (NOM), ubiquitously present in soil or suspended in the water column, affects nC_{60} toxicity.

Recent research by Tong et al. [58] demonstrated that soil may eliminate the high toxicity of nC_{60} that has been observed in low-salt mineral media [13-15, 45]. This indicates the need to consider nC_{60} interactions with common constituents in environmental matrices to obtain representative results of potential environmental impacts. In addition to toxicological tests, flow-through column studies using glass beads, clays and natural soil have demonstrated the relatively limited mobility of nC_{60} [60-63]. Clay minerals have also been demonstrated to have a strong propensity to associate with
nC$_{60}$ [63], corroborating the notion that nC$_{60}$ is unlikely to disperse widely in a natural soil setting. However, only a limited amount of information is currently available regarding the bioavailability of C$_{60}$ in the natural environment, which is an important factor in controlling environmental impacts.

Previous work has demonstrated that NOM enhances the aqueous stability of carbon-based nanoparticles including nC$_{60}$ and multi-walled carbon nanotubes [65, 66]. Furthermore, it has been postulated that C$_{60}$ partitioning into soil organic matter controls the solution-level bioavailability and thus reduces the toxicity of nC$_{60}$ [58]. However, the extent to which nC$_{60}$ toxicity decreases as a function NOM type and concentration has not been addressed in the literature.

This chapter addresses the hypothesis that soil-associated or dissolved NOM attenuates nC$_{60}$ toxicity by decreasing its bioavailability and/or modifying its surface chemistry. Specifically, the solution-level bioavailability and antibacterial activity of nC$_{60}$ were examined in the presence of sorbents (powdered activated carbon [PAC] and soils) and low concentrations of dissolved humic substances to advance our understanding of the risks associated with environmental contamination by nC$_{60}$.

4.2 Materials and Methods

4.2.1 Preparation and characterization of nC$_{60}$

nC$_{60}$ was prepared as described in 3.1.1. Particle size and zeta potential were determined by Zetasizer Nano as described in 3.2.2. Zeta potential measurements were conducted in minimum Davis (MD) medium, which is described in the following section. C$_{60}$ particles were also analyzed by transmission electron microscopy (TEM) as described in 3.2.1. The concentration of nC$_{60}$ was determined as described in 3.2.3.
4.2.2 Bacterial Growth

The Gram-negative bacterium *Escherichia coli* K12 (ATTC #25404) was chosen as the test organism in order for the results to be comparable with previous publications that used *E. coli* [14, 15]. In addition, *E. coli* has been well-studied and is easy to grow on the minimum mineral medium that is necessary for precluding nC<sub>60</sub> coagulation and precipitation [15]. *E. coli* K12 was maintained on Luria-Bertani (LB) plates and in LB broth. Minimal Davis (MD) medium was made according to the recipe described by Lyon et al. [14] in which the potassium phosphate concentration was reduced by 90% compared with Davis medium. Bacterial growth was quantified by measuring optical density at 600 nm (OD<sub>600</sub>) using a Turner SP-830 spectrophotometer (Barnstead, Dubuque, IA, USA).

4.2.3 Sorbents

Powdered activated carbon (PAC), one of the most commonly used and well-studied sorbents, was used first to study how the sorption of nC<sub>60</sub> influences antibacterial activity in a well-defined system. PAC was purchased from Fisher Scientific (Pittsburgh, PA). The average diameter of PAC was 80.6 µm according to the manufacturer. The surface area of PAC was determined by the Brunauer-Emmett-Teller (BET) method to be 754.4 m<sup>2</sup>/g using a Quantachrome Autosorb-3B Surface Analyzer (Quantachrome Instruments, FL). Pore size distribution, calculated by the Barrett-Joyner-Halenda method based on N<sub>2</sub> adsorption/desorption data [115], is presented in Figure 4.1. The average pore size of PAC is 16.8 Å. Dry PAC was autoclaved at 120°C for 15 min before mixing with the nC<sub>60</sub> suspension.
Two kinds of soil were used in the experiments. One is Lula sandy soil (R.S. Kerr Environmental Research Laboratory, Ada, OK), which consists of 92% sand, 6% clay, about 1.5% silt, and 0.27% organic carbon [62]. The BET surface area of Lula soil has been reported to be 1.24 m$^2$/g [62]. The other soil was from Amana Colonies, Iowa, which is a silty loam to silty clay loam alluvium and contains 3.5% organic matter with a BET surface area of 34.1 m$^2$/g. Sand, one of the components of Lula soil, was also obtained from R.S. Kerr Environmental Research Laboratory.

4.2.4 Humic substances

Commercial humic acid (Sigma-Aldrich, MO) was used in initial experiments. Number average and weight average molecular weights of Sigma-Aldrich HA (AHA) determined by vapor pressure osmometry were reported to be 1630 and 4100 Da, respectively [116]. However, the impurity of AHA confounded the interpretation of some
results. Therefore, Suwannee River standard humic substances were used for subsequent experiments.

Suwannee River humic acid (SRHA) (Standard II, International Humic Substances Society (IHSS)) and Suwannee River fulvic acid (SRFA) (Standard II, IHSS) were used as model aquatic NOM. The molecular weight of SRHA was reported by Elimelech et al. (1997) [117] as 1,000-5,000 Da. The number average molecular weight of SRFA was determined by vapor pressure osmometry and reported to be 1360 Da [116]. SRHA and SRFA solutions are prepared by introducing 100 mg dry humic substance powder into 50 ml Milli-Q water and then stirring overnight. The solution was filtered through a 0.22 μm-pore size cellulose membrane filter and stored in the dark at 4°C.

4.2.5 Sorption of nC_{60} aggregates from aqueous solution to PAC

A sorption experiment was conducted to characterize the equilibrium partitioning of nC_{60} between water and PAC. The experiment was performed in triplicate, using different initial nC_{60} concentrations and a fixed PAC dose for each sample vial. The PAC (100 ± 0.1 mg) was mixed with 10 mL Milli-Q water to make a stock suspension of 10 mg/mL. For each 2 mL sample, 0.1 mL of the PAC stock suspension was added into a 5 ml vial. Then different amounts of nC_{60} (14.5 mg/L) were injected into the vials and corresponding volumes of Milli-Q water were added to complete the samples. The initial nC_{60} concentrations were 14.5, 7.25, 3.63, 1.81, 0.91, 0.45 mg/L. The mixture was stirred for 48 h, filtered with 0.45-μm-pore-size cellulose syringe filters, and analyzed for UV absorbance at 336 nm to determine the equilibrium aqueous phase nC_{60} concentration. The analysis of each sample was repeated three times. The average sorption losses of nC_{60} to membrane filters were determined in a preliminary study to be negligible, around
4.2.6 Assessing the effect of sorption on antibacterial activity of nC$_{60}$

To assess the effect of sorption on antibacterial activity of nC$_{60}$, a respirometer (Oxymax-ER, Columbus Instrument, OH) was used to monitor the heterotrophic activity of bacteria dosed with nC$_{60}$ (positive control) or a mixture of sorbents and nC$_{60}$. *E. coli* was grown over night and then diluted into 50 mL of MD medium to a final OD$_{600}$ of 0.002. Approximately 5-6 hours later (OD$_{600}$ about 0.08), while in exponential phase, bacteria were exposed simultaneously to nC$_{60}$ (0.5 mg/L) and/or PAC. Another set of similar experiments was conducted with PAC that had been equilibrated with nC$_{60}$ for two days prior to exposing to the bacteria. This modification was adopted to discern any sorption kinetics effect that may influence nC$_{60}$ bioavailability and toxicity to bacteria. Soils of different organic content (geosorbents) were tested subsequently. They were equilibrated with nC$_{60}$ for two days before the experiments.

4.2.7 Assessing the effect of aquatic NOM on nC$_{60}$ antibacterial activity

The effect of aquatic NOM on heterotrophic activity was investigated using respirometry, following a procedure similar to that described above. Both humic acids, SRHA (5.4 mg/L) and AHA (10 mg/L), were mixed with nC$_{60}$ for two days before exposing to the bacteria.

In addition, a cell growth inhibition assay was used to evaluate the toxicity of nC$_{60}$ in the presence of NOM. Varying levels of SRHA or SRFA and 1 mg/L nC$_{60}$ were mixed in MD medium in a 24-well plate and equilibrated for 2 days before being exposed to bacteria. *E. coli* was grown in LB medium at 37°C over night and was diluted in wells of the plate to a final OD$_{600}$ of 0.002. The plate was incubated for 48 hours, and growth of
cells in each well was recorded. All samples were tested in duplicate.

4.3 Results and Discussion

4.3.1 Sorption of nC₆₀ by PAC and soils

The equilibrium partitioning data between nC₆₀ and PAC were fitted with a linear isotherm (Figure 4.2). At equilibrium, the solution-phase concentrations decreased by 58-77% from the initial values of 14.5, 7.25, 3.63, 1.81, 0.91, and 0.45 mg/L, indicating that PAC is an effective adsorbent for nC₆₀. A linear isotherm in the form of \( q = K_d C_w \) was observed, where \( K_d \) denotes the partition coefficient, \( q \) (mg·g⁻¹) is the mass of nC₆₀ sorbed per unit mass of PAC at equilibrium, and \( C_w \) (mg·L⁻¹) is the nC₆₀ concentration in the solution phase at equilibrium. A \( K_d \) value of \( 10^{3.75±0.05} \) mL·g⁻¹ was obtained, indicating PAC is an effective sorbent for nC₆₀, although it has less affinity than for polynuclear aromatic hydrocarbons such as naphthalene, with a reported partition coefficient of \( 10^{5.17} \) [118]. We postulate that adsorption of nC₆₀ to PAC mainly occurred on the outer surface of PAC because most PAC pores were smaller than 25 Å (see Figure 4.1), which is much smaller than the average diameter of nC₆₀ (108 nm from NIBS measurement). This was also visualized by TEM. As shown in the insert in Figure 4.2, nC₆₀ aggregates were found attached on the outer surface of the PAC particles.
4.3.2 Effect of Sorbents on nC\textsubscript{60} Antimicrobial Activity

Experiments were conducted to test the hypothesis that sorption of nC\textsubscript{60} to potential geosorbents (e.g., soil constituents) or activated carbon would reduce its bioavailability (e.g., hinder direct contact with bacteria) and attenuate its antibacterial activity. A respirometer was used to monitor the heterotrophic activity (measured as CO\textsubscript{2} produced) of \textit{E. coli} exposed to nC\textsubscript{60} alone or in the presence of various sorbents (PAC, soil, and sand). Considering that nC\textsubscript{60} particles agglomerate and even precipitate in the presence of high salt and protein concentrations [15, 119], MD medium were chosen for bacteria culture throughout the antibacterial test.

Figure 4.3 shows that the addition of nC\textsubscript{60} (0.5 mg/L, indicated by arrow)
significantly decreased the respiration rate of *E. coli* relative to an nC$_{60}$-free control. This bactericidal effect was mitigated by PAC. More than 90% of nC$_{60}$ was removed from the solution in samples containing various amounts of PAC when sorption reached equilibrium (data not shown). The residual concentrations, 0.001-0.05 mg/L, were much lower than the MIC of nC$_{60}$ for *E. coli*, which was previously determined to be between 0.1 and 0.5 mg/L, and even lower than the concentration needed to inhibit bacterial growth as the OD$_{600}$ of the bacteria was higher than 0.002 when nC$_{60}$ was added. When PAC was mixed with nC$_{60}$ at the time of exposure, higher dosage of PAC resulted in more pronounced attenuation in the antibacterial activity of nC$_{60}$ (Figure 4.3A). However, the beneficial effect of adding more PAC was not observed when PAC and nC$_{60}$ were equilibrated for 2 days prior to exposure (Figure 4.3B). These observations indicate that all PAC doses tested were able to remove nC$_{60}$ to below the concentration needed for growth inhibition, but longer nC$_{60}$-PAC contact time was required for lower PAC doses.
Figure 4.3. Attenuation of nC60 toxicity to E. coli by sorption onto PAC. The
addition of nC₆₀ (0.5 mg/L, indicated by the arrow) significantly decreased the respiration rate of E. coli relative to nC₆₀-free control. This effect was mitigated by PAC. Higher PAC amounts had a more pronounced attenuation effect when PAC was mixed with nC₆₀ at the time of exposure (Panel A). This sorption kinetic effect was not observed when PAC and nC₆₀ were equilibrated for 2 d prior to exposure (Panel B).

Two types of soils, a high organic content soil from Amana, Iowa and a sandy soil from Lula, Oklahoma, and the sand component of the Lula soil were also tested in this study. Both soils were found to reduce the toxicity of nC₆₀ to E. coli, assessed by respirometry (Figure 4.4). In contrast, sand with very low organic carbon content (<0.01%) was not effective in attenuating nC₆₀ toxicity. In a separate sorption experiment, the residual concentration of nC₆₀ (initial concentration 0.5 mg/L in MD medium) was 0.16 to 0.26 mg/L after sorption by the Lula soil, and 0.03 to 0.04 mg/L after sorption by Amana soil, using the same soil concentration as in the CO₂ production measurement, 100 mg/50 mL of MD medium. It is noted that although the residual nC₆₀ concentration after sorption by the Lula soil falls within the range of previously reported MIC values, the bacterial cell concentration used in the respirometry experiment was much higher than that used to evaluate the MIC (OD₆₀₀ = 0.002). The higher sorption capacity of the Amana soil is probably the result of larger surface area (34.1 m²/g versus 1.24 m²/g for the Lula soil), and higher concentration of organic matter (3.5% versus 0.27% in Lula soil). The unchanged toxicity of nC₆₀ in the presence of sand is probably due to the low organic carbon content of the sand and consequently low sorption capacity for nC₆₀. This suggests that the microbial community in soils with low concentration of organic matter...
and small surface area would be more susceptible to the presence of nC\textsubscript{60} than those in organic soils with larger surface area.

Figure 4.4. Lula soil (0.27% organic matter) and soil from Amana (3.5% organic matter) both reduced the antibacterial effect of nC\textsubscript{60} significantly at the concentration of 100 mg/50 mL MD media. Sand with very low concentration of organic matter (less than 0.01%) was not effective in attenuating nC\textsubscript{60} toxicity at the same concentration.

4.3.3 Effect of Dissolved NOM on nC\textsubscript{60} Antimicrobial Activity

To investigate the impact of dissolved NOM on nC\textsubscript{60} toxicity, CO\textsubscript{2} production by \textit{E. coli} in the presence of nC\textsubscript{60} and SRHA (5.4 mg/L) or AHA (10 mg/L) were measured. As shown in Figure 4.5, both SRHA and AHA significantly mitigated the antibacterial effect of nC\textsubscript{60}. nC\textsubscript{60} toxicity in the presence of NOM was also evaluated by assessing \textit{E.}
coli growth in a 24-well plate (Table 4.1). *E. coli* growth was completely inhibited by 1 mg/L of nC_{60}. However, SRHA at concentrations as low as 0.05 mg/L enabled growth, indicating mitigation of nC_{60} toxicity. No mitigating effect was observed at lower SRHA concentrations. Similar results were obtained with SRFA, although the minimum concentration that mitigated antibacterial activity was slightly higher (0.1 mg/L), indicating that SRFA was less effective than SRHA in attenuating nC_{60} toxicity. Typical humic acid concentrations in natural waters are much higher than the threshold toxicity-mitigating concentrations observed in our experiments. This underscores the fact that dissolved NOM in natural waters is likely to significantly mitigate nC_{60} toxicity.

![Graph showing cumulative CO2 production by E. coli](image)

**Figure 4.5.** SRHA (5.4 mg/L) and AHA (10 mg/L) attenuated the toxicity of nC_{60} to bacteria.
Table 4.1. Growth of E. coli in MD media with varying NOM addition and 1 mg/L nC$_{60}$. “+” denotes bacterial growth and “-”, no growth.

<table>
<thead>
<tr>
<th>Media+nC$_{60}$</th>
<th>NOM</th>
<th>E. coli growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MD + 1 mg/L nC$_{60}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MD + 1 mg/L nC$_{60}$</td>
<td>0.02 mg/L SRHA</td>
<td>-</td>
</tr>
<tr>
<td>MD + 1 mg/L nC$_{60}$</td>
<td>0.05 mg/L SRHA</td>
<td>+</td>
</tr>
<tr>
<td>MD + 1 mg/L nC$_{60}$</td>
<td>0.1 mg/L SRHA</td>
<td>+</td>
</tr>
<tr>
<td>MD + 1 mg/L nC$_{60}$</td>
<td>0.5 mg/L SRHA</td>
<td>+</td>
</tr>
<tr>
<td>MD + 1 mg/L nC$_{60}$</td>
<td>1 mg/L SRHA</td>
<td>+</td>
</tr>
<tr>
<td>MD + 1 mg/L nC$_{60}$</td>
<td>0.02 mg/L SRFA</td>
<td>-</td>
</tr>
<tr>
<td>MD + 1 mg/L nC$_{60}$</td>
<td>0.05 mg/L SRFA</td>
<td>-</td>
</tr>
<tr>
<td>MD + 1 mg/L nC$_{60}$</td>
<td>0.1 mg/L SRFA</td>
<td>+</td>
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<tr>
<td>MD + 1 mg/L nC$_{60}$</td>
<td>0.5 mg/L SRFA</td>
<td>+</td>
</tr>
<tr>
<td>MD + 1 mg/L nC$_{60}$</td>
<td>1 mg/L SRFA</td>
<td>+</td>
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</tbody>
</table>

Two hypotheses are proposed to explain how NOM attenuate the antibacterial effects of nC$_{60}$: (1) Adsorption of NOM on nC$_{60}$ surface interferes with direct contact of nC$_{60}$ with bacterial cells; (2) NOM may react with nC$_{60}$ or promote its disaggregation, change its surface chemistry and consequently antibacterial activity. Note that disaggregation alone, without NOM coating or changes in surface properties, would likely increase toxicity because of the higher surface area offered by smaller nC$_{60}$ particles [14]. Adsorption of NOM on nC$_{60}$ was evident from the zeta potential.
measurements. The zeta potential of nC₆₀ particles changed from \(-27 \pm 0.68\) mV to \(-30 \pm 0.77\) mV as soon as the negatively-charged SRHA was added into the suspension, suggesting that the adsorption of SRHA onto nC₆₀ occurred immediately.

The minimum concentration of SRHA needed to completely coat the surface of a 1 mg/L nC₆₀ particle suspension was estimated assuming that: a) C₆₀ and nC₆₀ are both rigid spherical particles with diameter of 1 nm [2] and 108 nm (number-averaged particle diameter measured by NIDS), respectively; b) the hydrodynamic diameter of SRHA ranges from 1.5 to 3.5 nm [120], with a molecular weight of 5000 Da [117]; and c) The adsorbed NOM forms a monolayer.

The number concentration of nC₆₀ is calculated as follows.

\[
nC₆₀\text{ number concentration} = \frac{nC₆₀\text{ concentration} \times C₆₀\text{ molecular volume}}{nC₆₀\text{ aggregate volume} \times 64\% \times C₆₀\text{ molecular weight}}
\]

\[
= \frac{1\text{ mg} / \text{L} \times \frac{4}{3} \times \pi \times (0.5 \text{ nm})^3}{\frac{4}{3} \times \pi \times (54 \text{ nm})^3 \times 64\% \times 720 \text{ Da}} = 1.037 \times 10^{15} \text{ L}^{-1}
\]

In this calculation, random packing density for rigid spheres, 64% is assumed for C₆₀ packing in an nC₆₀ particle (http://mathworld.wolfram.com/SpherePacking.html).

For SRHA with a hydrodynamic diameter of 1.5 nm, the concentration needed to form a monolayer coating on all nC₆₀ particles in a 1 mg/L suspension is:

\[
= nC₆₀\text{ number concentration} \times \frac{nC₆₀\text{ particle surface area}}{SRHA\text{ particle cross section area}} \times SRHA\text{ molecular weight}
\]

\[
= 1.037 \times 10^{15} \text{ L}^{-1} \times \frac{4 \times \pi \times (54\text{ nm})^2}{\pi \times (0.25\text{ nm})^2} \times 5000\text{ Da}
\]

\[
= 0.18 \text{ mg} / \text{L}
\]

If the hydrodynamic diameter of the SRHA increases to 3 nm, the concentration of SRHA needed for a monolayer coating is only 0.03 mg/L. This range of estimated
SRHA concentrations needed for a monolayer coating of nC₆₀ (0.03 to 0.18 mg/L) is consistent with the low concentrations observed to mitigate nC₆₀ antibacterial activity in the 24-well plate experiment.

High resolution TEM images of nC₆₀ before and after NOM addition show evidence of changes in nC₆₀ particle surface. In the absence of NOM, the crystalline structure of nC₆₀ is visible in Figure 6 B. After adding 0.05 mg/L SRHA, nC₆₀ loses some crystalline structure (Figure 6D, large areas without clearly identifiable crystal lattice). This was observed in many nC₆₀ particles. Apparently, in addition to coating the nC₆₀ surface, which hinders direct contact with cells, NOM may have also altered the structure of nC₆₀ through a yet undetermined mechanism. It is unclear whether this interaction is a redox reaction or simple disaggregation of C₆₀. The mechanism of such structural changes is recommended for further investigation.
Figure 4.6. TEM micrographs of nC\textsubscript{60} before and after addition of humic acid (0.05 mg/L). (A) nC\textsubscript{60} particles without humic acid. (B) Magnified part of nC\textsubscript{60} particle in picture A, showing crystalline structure. (C) nC\textsubscript{60} particles after addition of humic acid. (D) Magnified part of nC\textsubscript{60} particle in picture C, showing loss of crystallinity.
4.4 Summary

The antibacterial activity of nC$_{60}$ can be mitigated by the presence of NOM as a soil constituent or dissolved in the water column. Sorption to soil might decrease the bioavailability of nC$_{60}$ and thus its toxicity to bacteria, and this mitigating effect is likely to increase with the organic content and surface area of the soil. Aqueous organic matter may also mitigate nC$_{60}$ toxicity by coating nC$_{60}$, hindering direct contact of with cells, and possibly altering nC$_{60}$ surface chemistry through an undetermined mechanism. This notion is supported by zeta potential measurements, high-resolution TEM observations and theoretical coating calculations. Overall, this work implies that the impacts of nC$_{60}$ to indigenous microbial communities that are important to ecosystem health can be significantly mitigated by NOM, and suggests the need for further research to elucidate the mechanisms by which NOM reduces the toxicity of nC$_{60}$ nanoparticles.
CHAPTER 5.

ASSESSING ECOTOXICITY OF C_{60} USING THE EARTHWORM EISENIA FETIDA

5.1 Introduction

Knowledge of the impact of C_{60} on terrestrial organisms is limited. Tong et al. [58] demonstrated that soil may eliminate the high toxicity of nC_{60} that has been observed in low-salt mineral media [13-15, 45]. Our previous results also showed that association of nC_{60} with soil particles decreased the bioavailability of nC_{60} to bacteria and thus diminished its antibacterial activity [116]. In a recent toxicological study of carbonaceous nanoparticles to earthworms, cocoon production of earthworm Eisenia veneta was reduced by C_{60} administered through food at concentration of 1000mg /kg [121]. Since earthworms can take up contaminants through skin contact as well as by soil ingestion, toxicity tests other than food administration are needed to get a more complete understanding of effects of C_{60} to earthworms. In this study, we chose the earthworm Eisenia foetida which is widely used as a model soil organism in research and government guidelines, thus facilitating comparisons with previous studies. We conducted acute soil avoidance tests and viability assays (e.g., reproduction and weight loss) to assess the potential toxicity of C_{60} to the earthworms.

5.2 Materials and Methods

C_{60} (99.5% pure) was purchased from SES Research (Houston, TX). Eisenia fetida (Figure 5.1) was purchased from The Worm Farm (Durham, CA). Worms were maintained in fiberglass bins with Premier Sphagnum peat moss hydrated to achieve a
moisture content of about 35% dry weight. The pH was neutralized with CaCO₃ (Fisher Scientific, Pittsburgh, PA). Earthworms were fed every other day with Magic Worm Food (Magic Products Inc., Amherst Junction, WI) containing 12% of crude protein, 1.0% of crude fat, and 6.0% of crude fiber. Prior to exposure to C₆₀, a potassium chloride (KCl) toxicity test was conducted to check the health of the worms, which was described in 3.4.1. Artificial soil was prepared as described in 3.1.3.

![Figure 5.1 Earthworm Eisenia fetida and its cocoon.](image)

Earthworm avoidance test of soil amended with C₆₀ was conducted according to the method described in 3.4.2. C₆₀ was amended into soil directly in the form of powder. Two concentrations of C₆₀ were tested in the avoidance test (2,000 and 10,000 ppm). Growth and reproduction tests were conducted with three C₆₀ concentrations (5,000, 10,000, and 50,000 ppm) according to the methods described in 3.4.3.

5.3 Results and Discussions
5.3.1 $C_{60}$ avoidance assessment

Earthworms did not significantly avoid soil amended with $C_{60}$ powder with concentration up to 10,000 ppm at 95% confidence interval (Figure 5.2). Due to the requirement of a large amount of materials in this test, no higher concentrations were tested. This result indicated that earthworms could not differentiate unamended soils and soils with $C_{60}$ at relatively high concentrations at least in a 48-h period.

Figure 5.2 Percentage of worms recovered in $C_{60}$-amended versus unamended soil compartments after 48-h avoidance tests. Worms did not significantly avoid soil amended $C_{60}$ for up to 10,000 ppm. Error bars represent ± one standard error ($n = 5$).
5.3.2 Effects of C$_{60}$ on earthworm growth and reproduction

Since no food was provided during the tests, worms in control soil lost 32.65% of total weight after 28-d incubation (Figure 5.3). Worms in soil amended with C$_{60}$ did not experience significant weight loss compared to the worms in control soils. Thus soil with C$_{60}$ up to 50,000 ppm did not affect the weight loss of earthworms.

![Figure 5.3 Worm weight loss after 28-day incubation in control soil or soils amended with C$_{60}$. C$_{60}$ did not have a significant effect on worm weight loss. Error bars represent ± one standard error (n = 3).](image)

At 50,000 ppm, the highest C$_{60}$ applied in this study, C$_{60}$ significantly decreased earthworm’s cocoon production after 28-d incubation (Figure 5.4), indicating that C$_{60}$ could have negative effect to earthworm reproduction if present at such high
concentrations. However, at lower concentrations (5,000 and 10,000 ppm), this effect was not observed. Similar reproduction toxicity of $C_{60}$ was observed in a previous study of feeding earthworms with food containing $C_{60}$ at 1,000 mg/kg [121]. Obviously, exposing worms to soil with $C_{60}$ is less toxic than direct food administration. In other studies, $C_{60}$ also displayed reproduction toxicity in mice [122]. Pregnant mice were injected with $C_{60}$ solubilized with polyvinylpyrrolidone (PVP). After the injection, all the embryos died at 137 mg/kg, and 50% of the embryos were abnormal at 50 mg/kg. Other research has also indicated that n$C_{60}$ delays zebrafish embryo and larva development and exerted teratogenic effects [47]. Although the toxicity mechanism is still unclear, it is suggested that $C_{60}$ was incorporated into the conceptus and interrupted the function of the yolk sac and embryonic morphogenesis [122].
Figure 5.4 Worm cocoon production after 28-day incubation in unamended soil or soils amended with C$_{60}$. At 50,000 ppm, C$_{60}$ significantly decreased the cocoon production of earthworms. * indicates significant difference from controls at the 95% confidence level. Error bars represent ± one standard error (n = 3).

Although C$_{60}$ did not elicit a noticeable avoidance effect or a negative effect on the growth of the earthworms, the presence of C$_{60}$ decreased cocoon production by earthworms significantly. Since earthworms did not avoid soil containing C$_{60}$ even at 50,000 ppm, it is very likely that earthworm could reside in soil containing C$_{60}$ for a sufficient time to hinder cocoon production. Considering that earthworms have a relatively low reproduction rate, a small change in reproduction could significantly affect the whole population, and thus impact the food web and the wider environment [123].
Therefore, C\textsubscript{60} may have a significant sublethal impact to the terrestrial ecosystem due to its potential impact on reproduction.

5.4 Summary

Earthworms did not avoid soil containing C\textsubscript{60} at a relatively high concentration (10,000 ppm). No negative effect was observed for C\textsubscript{60} to earthworm’s growth even at a concentration of 50,000 ppm. However, the presence of C\textsubscript{60} decreased earthworm’s cocoon production significantly at 50,000 ppm. Therefore, C\textsubscript{60} may have a significant sublethal impact to the terrestrial ecosystem due to its potential impact on reproduction.
6.1 Introduction

Carbon fullerenes, particularly buckminsterfullerene (C\textsubscript{60}), represent a class of engineered carbon nanomaterials with unique photochemical and electronic properties. Many fullerenes, including C\textsubscript{60}, are being considered for applications in numerous products and processes such as cancer therapeutics, drug delivery, and computer sensors [4-7, 124]. Furthermore, fullerene nanomaterials are becoming increasingly available and affordable. For example, Frontier Carbon is on track to produce up to 1,500 ton/year of fullerenes and carbon nanotubes [27]. The growing use of fullerene materials increases the likelihood of accidental or inadvertent release to natural systems, which calls for a better understanding of their behavior, fate and impact in natural systems. Such understanding is critical to accurately inform the ecologically responsible use and disposal of fullerenes and support related risk assessment efforts.

Currently, little is known about the bioavailability of fullerenes (including C\textsubscript{60}) in the environment, and C\textsubscript{60} bioaccumulation potential has not been quantified. Bioavailability is defined here as the extent to which ecological receptors are exposed to hydrophobic organic contaminants (HOCs) in soil and sediment. Bioavailability is critical to the apparent toxicity of HOCs and controls their bioaccumulation and biomagnification potential [125]. Normally, bioavailability depends on matrix properties (e.g., soil type and organic carbon content [OC], pore size distribution, mixing) and HOC properties (e.g., hydrophobicity, volatility, solubility). As C\textsubscript{60} is nearly insoluble in water
(estimated solubility $1.3 \times 10^{-11}$ to $7.96 \times 10^{-9}$ g/L) and only soluble in certain organic solvents [8, 126, 127], most biological studies have focused on surface-charged, nano scale C$_{60}$ aggregates (nC$_{60}$) [13, 15].

Previous studies have shown that nC$_{60}$ associates with soil OC [58, 110], and that dissolved natural organic matter (NOM) decreases the deposition rate of nC$_{60}$ to solid surfaces due to steric repulsion [128], thus enhancing its potential mobility in aqueous systems. The hetero-aggregation of nC$_{60}$ with organic colloidal particles may also influence its bioavailability in the environment [129]. Although the interactions between pristine molecular C$_{60}$ and soil OC have received limited attention, if any, soil OC is known to affect the bioavailability of other polycyclic compounds that partition into the soil organic phase. For example, pyrene bioavailability decreases dramatically with increasing soil OC, consistent with equilibrium partitioning expectations [85].

Earthworms, including *Eisenia fetida*, are the prey of both vertebrates and invertebrates [20], and can act as an entry point for HOCs in soil into terrestrial food webs [88, 105]. *E. fetida* can assimilate dissolved contaminants through skin contact as well as by soil ingestion [92, 130]. Many studies have shown bioaccumulation of HOCs such as polynuclear aromatic hydrocarbons (PAHs) in the fatty tissue of earthworms [93-95]. Thus C$_{60}$, which is also a large, hydrophobic, polycyclic molecule, might similarly accumulate in earthworm fatty tissues. This raises the possibility of subsequent transfer to into upper trophic levels occupied by insects, birds, and rodents. Furthermore, bioaccumulation pathways in earthworms may also have relevant implications for potential bioaccumulation in higher order species including humans.
Current knowledge of earthworm uptake and bioaccumulation of nanomaterials is limited. Carbon nanotubes were reported to bioaccumulate with relatively low propensity in *E. fetida* (Biota-sediment accumulation factors, BSAF = 0.006 to 0.02) [85], possibly due to strong sorption to soil OC (which can decrease partitioning into fatty tissue) and the relatively large size of the nanotubes (which may physically hinder uptake processes). The potential for $C_{60}$ bioaccumulation in terrestrial systems has not been addressed in the literature, primarily due to the unavailability of accurate quantification methods for complex, carbon-rich matrices such as soil and biological tissues [85]. Scintillation counting of $^{14}$C-labeled $C_{60}$ enables such measurements, and $^{14}$C$_{60}$ has been previously synthesized to assess the uptake of $^{14}$C-labeled nC$_{60}$ by human keratinocytes and rat tissue [42, 131]. However, $^{14}$C$_{60}$ is not commercially available and its synthesis is challenging, which represents an obstacle to quantifying its bioaccumulation propensity.

To our knowledge, this is the first study to assess the bioaccumulation of “pristine” (i.e., untransformed) $C_{60}$ from soil into a terrestrial organism. The rate and extent of $^{14}$C$_{60}$ bioaccumulation were quantified using the earthworm *E. fetida* as a model organism. Bioaccumulation of $^{14}$C-phenanthrene was used as a positive control and baseline for comparison. The effects of soil OC content and $C_{60}$ concentration in soil were also investigated.

### 6.2 Materials and Methods

#### 6.2.1 Materials

$C_{60}$ (99.5% pure) was purchased from SES Research (Houston, TX). Radiolabeled ($^{14}$C) $C_{60}$ ($18.75 \pm 2.02$ mCi mmol$^{-1}$ or 500 mg L$^{-1}$ $C_{60}$ in toluene at > 99% purity) was custom synthesized by the Research Triangle Institute (Research Triangle Park, NC).
Toluene (ACS grade) and phenanthrene (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO). $^{14}$C-phenanthrene (55 mCi mmol$^{-1}$ or 324 mg L$^{-1}$) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

6.2.2 Organisms

*Eisenia fetida* was purchased from The Worm Farm (Durham, CA). Worms were maintained in fiberglass bins with Premier Sphagnum peat moss hydrated to achieve a moisture content of about 35% dry weight. The pH was neutralized with CaCO$_3$ (Fisher Scientific, Pittsburgh, PA). Earthworms were fed every other day with Magic Worm Food (Magic Products Inc., Amherst Junction, WI) containing 12% crude protein, 1.0% crude fat, and 6.0% crude fiber. Prior to exposure to C$_{60}$, a potassium chloride (KCl) toxicity test was conducted to check the health of the worms [89]. Only worm cultures that produced KCl LC$_{50}$ values $\geq$ 8,000 mg kg$^{-1}$ were chosen for the experiment. Sexually mature earthworms (i.e., clitellated), in a range of 0.3-0.6 g, were selected for C$_{60}$ experiments.

6.2.3 Soils

Three different soils were selected to investigate the effect of OC on C$_{60}$ bioaccumulation: (1) silty loam Grenada-Loring field soil (GL soil, 0.7% OC) collected from the Brown Loam Experimental Station (Learned, MS), (2) soil collected from Ft. Drum, NY (FD soil, 5.2% OC), and (3) Luia sandy soil (R.S. Kerr Environmental Research Laboratory, Ada, OK) (Luia soil, 0.3% OC). All the soils were air-dried, processed through a hammer mill, and sieved (1.4 mm mesh size) before use. The chemical and physical properties of these soils are listed in Table 6.1.
Table 6.1. Physicochemical Characteristics of the Tested Soils.

<table>
<thead>
<tr>
<th>Soil Properties</th>
<th>Grenada-Loring&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ft. Drum&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Lula&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.72</td>
<td>6.80</td>
<td>7.4</td>
</tr>
<tr>
<td>% Fines (silt and clay)</td>
<td>97.0</td>
<td>91.5</td>
<td>55</td>
</tr>
<tr>
<td>% Sand</td>
<td>3.0</td>
<td>8.5</td>
<td>45</td>
</tr>
<tr>
<td>Cation exchange capacity (mEq/100g)</td>
<td>10.8</td>
<td>27.0</td>
<td>6.2</td>
</tr>
<tr>
<td>Organic carbon content, %</td>
<td>0.7</td>
<td>5.16</td>
<td>0.27</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data from reference [132].

<sup>b</sup> Data from reference [133].

<sup>c</sup> Analyses performed by A&L Plains Agricultural Laboratory, Inc. Lubbock, TX

6.2.4 Soil amendment with C<sub>60</sub> and aging

Soils were amended with C<sub>60</sub> at concentrations of 0.25, 60, 100 and 300 mg kg<sup>-1</sup> dry soil (8,747, 5,382, 8,613, and 29,860 dpm g<sup>-1</sup> dry soil, respectively, measured with a biological oxidizer) by adding toluene solutions containing labeled and unlabeled C<sub>60</sub> (0.43, 30, 50, and 150 ml of toluene solution per kg soil, respectively). Toluene was then allowed to evaporate for 7 d during which the amended soil was thoroughly mixed three or four times daily. The residual toluene concentration at the time of exposure was determined by a commercial analytical laboratory (Pace Analytical, Lenexa, KS) to be below the detection limit (< 5 µg/kg), and thus inconsequential for the bioaccumulation experiments. Avoidance tests (unpublished data) showed that the worms did not differentiate between unamended control soils and soils exposed to toluene alone.
The lowest \( C_{60} \) concentration, 0.25 mg·kg\(^{-1}\), was estimated to be below the soil sorption capacity (explained in 6.3.2), allowing bioaccumulation measurements for primarily molecularly available, sorbed \( C_{60} \) in the soil. The higher \( C_{60} \) concentrations exceeded the soil sorption capacity, which simulates contamination after accidental releases of \( C_{60} \) powder such as during transportation, likely resulting in the presence of both aggregated/precipitated \( C_{60} \) and sorbed molecular \( C_{60} \) in the soil.

To investigate the possibility of \( C_{60} \) transformation, \( C_{60} \) was applied to GL and FD soils at 100 mg·kg\(^{-1}\) dry soil (8,441 DPM g\(^{-1}\)), and aged for 30 d. A mixture of \( C_{60} \) and \( ^{14}C_{60} \) dissolved in toluene was sprayed evenly onto 50 g of each soil. After toluene evaporated (7 d evaporation time), the soils were tumbled, mixed, and then stored open to the air at 22 °C. Triplicate soil samples (1 g each) were taken after 1 month of incubation. The samples were shaken and sequentially extracted for 24 h (each) with 10 ml of water, methanol, and toluene. Extracts were then centrifuged at 10,000 rpm for 30 min and supernatants were filtered through 0.22 \( \mu \)m cellulose syringe filters to remove suspended soil particles. Triplicate aliquots of 1 ml water and methanol samples were analyzed using a liquid scintillation counter (LSC) (LS 6500, Beckman Coulter, Brea, CA) to confirm the absence of \( ^{14}C \) in these hydrophilic fractions, while the supernatant of the toluene extract (containing \( ^{14}C \)) was analyzed by high-performance liquid chromatography (Waters 4695, Milford, MA) coupled with radiochromatographic detection (IN/US Systems, Inc., Tampa, FL) (HPLC-RC). The HPLC is also equipped with a photodiode array detector (Waters, 996, Milford, MA) which was used for non-radiolabeled \( C_{60} \) analysis. Separation was accomplished with a Delta Pak C18 column (150 mm × 3.9 mm I.D., 300 Å, Waters, Milford, MA, USA) at a constant flow rate of 1
ml/min with a mobile phase of 100% toluene. $^{14}$C$_{60}$ had a retention time of 1.51 min at these settings (Figure 6.1). Serial extractions of moist soils and analysis of the extracts were also conducted after bioaccumulation tests. The water-soil weight ratios were determined by weighing the moist soils and drying the soils at 60 °C to constant weight. The total radioactivity of the soils was determined by combustion in a biological oxidizer (OX-600, R.J. Harvey instrument, Tappan, NY) followed by measurement of trapped $^{14}$CO$_2$ by LSC.

6.2.5 Uptake experiments

$C_{60}$ uptake by earthworms from three different soils was assessed according to standard procedures described by Environment Canada [89] with modifications. Uptake patterns of $^{14}$C-phenanthrene, a representative PAH, were also investigated for comparison. Non-radiolabeled phenanthrene was dissolved in ethanol to make a 1 g L$^{-1}$ solution. $^{14}$C-phenanthrene was added to the phenanthrene solution and mixed thoroughly. The mixed solution was applied drop-wise to GL soil to a final concentration of 0.05 mg g$^{-1}$, with radioactivity of 5,818 dpm g$^{-1}$. The ethanol was then allowed to evaporate overnight. All test soils were tumbled overnight after the solvents evaporated. At least three 0.5-g random samples of each soil were analyzed for total radioactivity to assess uniform mixing.

Prior to the experiment, earthworms were depurated for 24 h on filter paper hydrated with deionized (DI) water. The depurated worms were then exposed to $C_{60}$-laden soils (hydrated overnight with DI water to 70%-80% water holding capacity) for 1, 3, 7, 14, and 28 d, respectively, with 10 earthworms per 200 g of soil (dry weight). Triplicate treatments were run for each data point. After exposure, the earthworms were
washed, depurated on hydrated filter paper for 24 h, freeze-dried, weighed, and analyzed for bioaccumulated radioactivity by combustion in the biological oxidizer with LSC. In order to investigate the potential biotransformation of C₆₀ assimilated by worms, one worm of each sample was sacrificed, freeze-dried, crushed into powder and sequentially extracted by water, methanol, and toluene prior to HPLC-RC analysis as described above.

6.2.6 Elimination experiments

C₆₀ elimination from worms was also investigated. After exposure for 14 d in C₆₀-laden FD soils (100 and 300 mg-C₆₀ kg⁻¹ dry soil), worms were depurated for 24 h, transferred to C₆₀-free (unamended) FD soil, and analyzed for radioactivity after 1, 2 or 7 d.

6.2.7 Data analysis

Bioaccumulation data were fit to a first-order accumulation rate model [134] using nonlinear regression with Prism (GraphPad Software, Inc., La Jolla, CA, USA):

\[
C_{\text{org}} = \frac{(k_s C_{s,0})(e^{-\lambda t} - e^{-k_e t})}{k_e - \lambda}
\]

(6.1)

where \(C_{\text{org}}\) is the ¹⁴C concentration in the organism (mg g⁻¹ of organism dry weight), \(C_{s,0}\) is the initial concentration in soil (mg g⁻¹ of soil dry weight, approximately equal to the total applied amount, SI), \(k_s\) is the uptake rate constant (g dry soil g⁻¹ dry organism d⁻¹), \(k_e\) is the elimination rate constant (day⁻¹), \(t\) is the duration (days), and \(\lambda\) is the rate constant for the decreasing bioavailability of the compound (day⁻¹) [134].

Because the uptake of C₆₀ or phenanthrene by E. fetida was expected to occur mostly through soil ingestion, the biota-sediment accumulation factor (BSAF) was used to quantify bioaccumulation. BSAF was defined as the ratio of the concentration of a compound in an organism to that in the sediment [135]. The BSAFs were calculated at
selected exposure times as the ratio of the $C_{60}$ or phenanthrene concentration in the earthworms ($C_{\text{org}}$, mg g$^{-1}$ dry weight biomass) to that in the soils ($C_s$, mg g$^{-1}$ dry soil):

$$BSAF_i = \frac{C_{\text{org}}}{C_s}$$ (6.2)

Whether differences in BSAF values between different treatments were statistically significant was determined using Student t-tests and ANOVA at the 95% confidence level. Goodness of fit to the model (Equation 6.1) was assessed using the Chi-Square test ($\alpha = 0.05$) in Excel.

6.3 Results and Discussion

6.3.1 Aging of C$_{60}$ in natural soil

Nanomaterials may undergo biological, chemical or physical transformations in soils; thus, characterizing the material form to which ecological receptors are exposed is necessary for risk assessment. Unfortunately, current analytical capabilities are insufficient to fully characterize the state of C$_{60}$ transformation, aggregation or agglomeration in complex matrices such as soil or organism tissue. Nevertheless, converging lines of evidence suggest that C$_{60}$ was not transformed nor formed aqueous aggregates (nC$_{60}$) after being aged in different soils. First, $^{14}$C was detected only in the toluene extract of the amended soils, not in the water or methanol extracts (0.01 mg L$^{-1}$ or 30 DPM ml$^{-1}$ detection limit) (Tables 6.2 and 6.3). Since surface charged nC$_{60}$ cannot be readily extracted by toluene without oxidizing agents [136] (which were not used in this study), and no $^{14}$C was found in water or methanol extracts, nC$_{60}$ formation and C$_{60}$ transformation to hydrophilic products (if any) are assumed to have been negligible. The single $^{14}$C peak detected by HPLC-RC analysis corroborates the initial purity of $^{14}$C$_{60}$ in
soil and the lack of transformation over the exposure period (Figure 6.1). A similar, single radiochromatographic peak in worm extracts in toluene and no detectable radioactivity in water or methanol extracts (data not shown) also indicates no transformation within worm tissue upon accumulation. Apparently, C$_{60}$ was not metabolized under the conditions studied, possibly due to its structural stability, insolubility, and/or relatively large molecular size (~0.7 nm in diameter [1]).

Table 6.2. Percentage of $^{14}$C$_{60}$ recovered in serial extractions of dry soils, based on the amount of $^{14}$C$_{60}$ added (100 mg kg$^{-1}$, 8,441 DPM g$^{-1}$). Data correspond to high-dose systems where C$_{60}$ was likely present in the soil in both aggregate/precipitate powder and sorbed molecular forms. Error bars represent ± one standard deviation.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Water extract</th>
<th>Methanol extract</th>
<th>Toluene extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL</td>
<td>0</td>
<td>0</td>
<td>98.5 ± 4.3</td>
</tr>
<tr>
<td>FD</td>
<td>0</td>
<td>0</td>
<td>109.2 ± 6.8</td>
</tr>
</tbody>
</table>

Table 6.3. Percentage of $^{14}$C$_{60}$ recovered in serial extractions of moist soils after bioaccumulation tests, based on the amount of $^{14}$C$_{60}$ added (100 mg kg$^{-1}$, 8441 DPM g$^{-1}$). Data correspond to high-dose systems where C$_{60}$ was likely present in the soil in both aggregate/precipitate powder and sorbed molecular forms. Error bars represent ± one standard deviation.

<table>
<thead>
<tr>
<th>Soils</th>
<th>Water extract</th>
<th>Methanol extract</th>
<th>Toluene extract</th>
<th>Combusted$^a$</th>
<th>Not extracted$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL</td>
<td>0</td>
<td>0</td>
<td>45.8 ± 11.8</td>
<td>98.7 ± 4.2</td>
<td>52.9 ± 12.6</td>
</tr>
<tr>
<td>FD</td>
<td>0</td>
<td>0</td>
<td>9.2 ± 1.9</td>
<td>102.6 ± 8.6</td>
<td>93.4 ± 8.8</td>
</tr>
<tr>
<td>Lula</td>
<td>0</td>
<td>0</td>
<td>64.5 ± 10.0</td>
<td>96.0 ± 8.7</td>
<td>31.5 ± 13.3</td>
</tr>
</tbody>
</table>

$^a$ Unextracted soils were combusted in a biological oxidizer to determine total $^{14}$C.

$^b$ Calculated as the difference of $^{14}$C recovered by combustion minus total $^{14}$C extracted.
Figure 6.1. HPLC UV chromatograms for total C$_{60}$ initially (A) and after aging in soil for 30 days (B). The corresponding radio-chromatograms for $^{14}$C$_{60}$ are shown as inserts. Red lines represent the radio-chromatogram baseline. The consistency of a single peak indicates the initial purity of C$_{60}$ and the lack of transformation.

Complete recovery of the added $^{14}$C$_{60}$ was obtained by toluene extraction of the dry soil (Table 6.2), which confirms the dominant presence of untransformed C$_{60}$ and
indicates the absence of irreversibly bound residue. Interestingly, lower $^{14}C_{60}$ recoveries were obtained by toluene extraction of moist soils (Table 6.3), possibly because the soil particles were not fully dispersed in the toluene phase and water-saturated soil pores were not fully accessible to toluene. Hydrophobic $C_{60}$ has a tendency to be sorbed or coated with soil OC [58, 128], and pore water represents a diffusion barrier for the partitioning of $C_{60}$ from soil OC into the toluene phase. In this case, full $^{14}C$ recovery was only achieved by soil combustion in the biological oxidizer (Table 6.3).

### 6.3.2 $C_{60}$ bioaccumulation

Earthworm bioaccumulation patterns under high-dose conditions were compared for three different soils with varying OC. Note that $C_{60}$ was likely present in these experiments both as agglomerated/precipitated powder and molecular $C_{60}$ sorbed by soil OC (calculations are presented below).

The soil sorption capacity for $C_{60}$ was calculated by the following equation:

$$C_{C_{60},S} = K_p \times S = K_{OW} \times f_{oc} \times S \quad (6.3)$$

where $C_{C_{60},S}$ is the soil sorption capacity for $C_{60}$, $K_p$ is the sorption partitioning coefficient (soil/water), $S$ is the $C_{60}$ water solubility (estimated as 8 ng L$^{-1}$ [127]), $K_{OC}$ is the organic carbon-normalized sorption coefficient (estimated as 10$^{7.1}$ L kg$^{-1}$ for $C_{60}$ [137]), and $f_{oc}$ stands for fraction organic carbon.

Accordingly, the soil sorption capacity for $C_{60}$ was calculated as:

- for Lula soil = $10^{7.1}$ L kg$^{-1} \times 0.27\% \times 8 \text{ ng L}^{-1} = 0.27 \text{ mg kg}^{-1}$,
- for GL soil = $10^{7.1}$ L kg$^{-1} \times 0.7\% \times 8 \text{ ng L}^{-1} = 0.71 \text{ mg kg}^{-1}$,
- for FD soil = $10^{7.1}$ L kg$^{-1} \times 5\% \times 8 \text{ ng L}^{-1} = 5.0 \text{ mg kg}^{-1}$.
Thus, BSAF values estimated in these experiments correspond to a heterogeneous C$_{60}$ system, possibly representative of extreme events such as accidental spill of C$_{60}$ powder during transport. Bioaccumulation of carbon nanotubes [85] and hydrocarbons after oil spills [138] have also been studied in similar multi-phase systems where pure, sorbed and possibly dissolved phases are present.

BSAF for earthworms in the high-OC FD soil containing 100 mg-C$_{60}$ kg$^{-1}$ dry soil increased initially to a peak of 0.190 ± 0.047 after 7 d, and then decreased to 0.093 ± 0.011 over the remainder of the 28-d exposure period (Figure 6.2A). A similar bioaccumulation pattern was observed for phenanthrene in GL soil (Figure 6.2B), where the BSAF peaked at 17.75 ± 3.02 on day 7 and decreased to 7.93 ± 0.86 during the remaining time. Dissolved NOM is known to adsorb onto the surface of carbon nanoparticles via van der Waals forces and π-π stacking [139], which helps disperse and stabilize fullerene in water [65, 67]. Thus, the early BSAF peak (Figure 6.2A) might be due to temporarily enhanced C$_{60}$ availability in pore water with dissolved NOM [65, 128], and the subsequent decrease in BSAF may be attributed to sorption of C$_{60}$ by soil-bound organic matter [128], which is conducive to decreased bioavailability. Whether temporary adsorption of C$_{60}$ onto the gut wall (and subsequent excretion) also contributed to the observed peak was not determined.
Figure 6.2. Bioaccumulation factor (BSAF) for (a) C₆₀ and (b) phenanthrene in *E. fetida* as a function of exposure time for different treatments. The relatively high C₆₀ concentrations in these experiments likely resulted from its presence in both aggregated/precipitated powder and absorbed molecular forms. Data curves are simulations using Equation 1 with parameters (and R² values) listed in Table 6.4. Error bars represent ± one standard deviation.
$C_{60}$ bioaccumulation in the two soils with low OC content followed a different pattern, increasing asymptotically over the incubation period to values similar to (statistically undistinguishable) that of FD soil ($0.111 \pm 0.025$ for GL and $0.179 \pm 0.057$ for Lula soils) (Figure 6.3). Therefore, for these high-dose experiments (i.e., heterogeneous $C_{60}$ systems), soil OC content had no significant effect on the extent of $C_{60}$ bioaccumulation in earthworms after 28 d of exposure, although higher OC resulted in faster initial bioaccumulation (Figure 6.2A). The biokinetic model (Equation 6.1) fit the data well ($R^2 = 0.68$ to 0.87), passing the Chi-Square test at the 95% confidence level, with $\lambda = 0.037 \text{ d}^{-1}$ for FD soil and $\lambda = 0$ for GL soil (Table 6.4). This corroborates that $C_{60}$ bioavailability in high-OC FD soil decreased over time after a transitory peak, while it did not decrease in low-OC GL soil.

Table 6.4. Biokinetic model parameters (± one standard error) for $C_{60}$ and phenanthrene (phen) bioaccumulation in *E. fetida*. Data correspond to high-dose system where $C_{60}$ was likely present in the soil in both aggregate/precipitate powder and absorbed molecular forms.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$k_s$ (d$^{-1}$)</th>
<th>$k_e$ (d$^{-1}$)</th>
<th>$\lambda$ (d$^{-1}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL soil, $C_{60}$, 60 mg kg$^{-1}$</td>
<td>0.036 ±0.006</td>
<td>0.33 ± 0.07</td>
<td>0</td>
<td>0.86</td>
</tr>
<tr>
<td>GL soil, $C_{60}$, 100 mg kg$^{-1}$</td>
<td>0.024 ± 0.005</td>
<td>0.23 ± 0.06</td>
<td>0</td>
<td>0.85</td>
</tr>
<tr>
<td>GL soil, $C_{60}$, 300 mg kg$^{-1}$</td>
<td>0.057 ± 0.031</td>
<td>0.98 ± 0.56</td>
<td>0</td>
<td>0.68</td>
</tr>
<tr>
<td>FD soil, $C_{60}$, 100 mg kg$^{-1}$</td>
<td>0.12 ± 0.02</td>
<td>0.52 ± 0.13</td>
<td>0.037 ± 0.006</td>
<td>0.87</td>
</tr>
<tr>
<td>FD soil, phen, 50 mg kg$^{-1}$</td>
<td>10.04 ± 3.51</td>
<td>0.07 ± 0.01</td>
<td>0.36 ± 0.05</td>
<td>0.81</td>
</tr>
</tbody>
</table>
Figure 6.3. Bioaccumulation factors (BSAF) for different treatments after 28 d exposure. Soil OC content did not significantly affect BSAF for high-dose (heterogeneous C₆₀) systems ($p > 0.05$), whereas BSAF decreased with increasing soil OC in low-dose (0.25 mg kg⁻¹) systems. Phenanthrene (Phen) had significantly higher ($p < 0.05$) BSAF, despite its lower hydrophobicity. The soil concentration of phenanthrene was lower than that of C₆₀ to avoid acute toxicity. Error bars represent ± one standard deviation.

Interestingly, C₆₀ BSAF values decreased as C₆₀ concentrations increased in high-dose GL soils, stabilizing at 0.126 ± 0.005 for 60 mg kg⁻¹, 0.111 ± 0.025 for 100 mg kg⁻¹, and 0.065 ± 0.039 for 300 mg kg⁻¹ (Figure 6.2A). The same trend was observed for FD soil, with final BSAF values of 0.093 ± 0.011 at 100 mg kg⁻¹ and 0.047 ± 0.007 at 300 mg kg⁻¹ (Figure 6.4). Apparently, as the C₆₀ concentration in soil increases beyond sorption capacity, so does the fraction of C₆₀ precipitates that are more difficult to pass through the gut wall and/or easier to eliminate after ingestion. However, the possibility
for a higher elimination rate due to the toxic effect at high concentrations cannot be excluded [140, 141].

Figure 6.4. Biota-sediment accumulation factors (BSAFs) of C₆₀ by *E. fetida* in FD soil (5% soil OC content). BSAF decreased at higher C₆₀ concentration in soil. BSAF values shown are for high-dose systems where C₆₀ was likely present in the soil in both aggregate/precipitate and sorbed forms. Error bars represent ± one standard deviation.

¹⁴C elimination from worms exposed to FD soil amended with 100 and 300 mg·kg⁻¹ of C₆₀ showed a rapid initial rate (≤ 24 h after initial depuration), indicative of gut clearing (Figure 6.5). Note that the amount of ingested soil remaining after 24 h depuration should be relatively small. Hartenstein *et al.* reported that the gut loading of *E. fetida* is about 0.63 ± 0.022 (dry weight egesta per dry weight worm), and less than 5%
of the gut load remains in the worm after 24-h depuration [142]. Thus the maximum radioactivity that would be contributed by unexcreted $^{14}$C$_{60}$ in FD soil (dosed at 100 mg C$_{60}$ kg$^{-1}$ dry soil, or 8,613 dpm g$^{-1}$) is 209 ± 1 DPM/g dry worm mass. The $^{14}$C$_{60}$ radioactivity measured in worm tissue after 14 d in FD soil (100 mg kg$^{-1}$) was 653 ± 136 DPM/g (Figure 6.5). Thus, under worst case scenario, at least 444 DPM/g (68%) was absorbed in the worm tissue (or adsorbed to the gut wall). These calculations indicate that the measured radioactivity in worm tissue was contributed primarily by $^{14}$C$_{60}$ that had been absorbed (or adsorbed to the gut surface) rather than by soil-associated C$_{60}$.

Additional bioaccumulation experiments were conducted with a lower soil concentration of C$_{60}$ (0.25 mg kg$^{-1}$), at which C$_{60}$ was much more likely to be available in molecular form (sorbed by soil constituents including OC). Higher BSAF values (0.22 to 0.79) were measured compared to high-dose experiments (Figure 6.3), possibly because sorbed/molecular C$_{60}$ is more available for bioaccumulation than the larger precipitates that form when the soil sorption capacity is exceeded. BSAF decreased with increasing OC content (i.e., 0.786 ± 0.078 for Lula soil (0.3% OC), 0.427 ± 0.121 for GL soil (0.7% OC), and 0.217 ± 0.012 for FD soil (5% OC)), and this trend was statistically significant ($p < 0.05$) (Figure 6.3). This is attributed to the lower bioavailability due to greater partitioning into soil with higher OC content. Similar trends have been observed in studies with PAHs [85, 143].
Figure 6.5. Elimination behavior of *E. fetida* after 14-d exposure to FD soil dosed with (A) C₆₀ (100 and 300 mg kg⁻¹) and (B) phenanthrene (phen) (50 mg kg⁻¹). Time zero corresponds to after 24 h depuration, when worms were transferred to clean soil and BSAF was measured. Error bars represent ± one standard deviation.
The BSAF for phenanthrene (added at 50 mg kg\(^{-1}\) in GL soil) on day 28 was 7.93 ± 0.86, which is significantly higher than that of C\(_{60}\) in both the high and low dose scenarios (Figure 6.3). With a higher log \(K_{OW}\) of 6.67 [127], C\(_{60}\) yielded a much lower BSAF than phenanthrene (log \(K_{OW}\) = 4.48 [144]). This seems to be inconsistent with equilibrium partition theory, which suggests that BSAF should be constant for a given system (inherently assuming similar chemical potential for the organic phases in biological and sediment compartments), and thus independent of the \(K_{OW}\) value of the HOC [145]. The lower C\(_{60}\) BSAF may be due to its larger molecular size (which hinders cellular uptake) [95] and/or stronger binding to soil OC. The organic-carbon-normalized partition coefficient (\(K_{OC}\)) of C\(_{60}\) was recently determined as 10\(^{6.2}\) to 10\(^{7.1}\) [137], which is significantly higher than that for phenanthrene (\(K_{OC}\) = 10\(^{4.1}\)) [146] and contributes to its lower bioavailability. Precipitation of larger aggregates likely also hindered C\(_{60}\) bioaccumulation. To date, no studies have shown significant toxicity of pristine C\(_{60}\) to earthworms or microbes [58, 121], and the relatively low BSAF observed here is consistent with low toxicity observations.

When considering the bioaccumulation factor (BAF), which represents the partitioning of a compound between an organism and the water phase (freely dissolved), the BAF of C\(_{60}\) at 0.25 mg kg\(^{-1}\) in GL soil and that of phenanthrene at 50 mg kg\(^{-1}\) in GL soil were calculated as below:

In the Soil-Organism-Water system,

\[
C_s = K_{OC} \times f_{oc} \times C_w \quad (6.4)
\]

where \(C_s\) is organic compound concentration in soil, \(K_{OC}\) is the organic carbon-normalized sorption coefficient (estimated as 10\(^{7.1}\) L kg\(^{-1}\) for C\(_{60}\) [137] and 10\(^{4.1}\) L kg\(^{-1}\) for
phenanthrene [146]), $f_{oc}$ stands for fraction organic carbon, and $C_{w}$ is the aqueous phase concentration of the organic compound, thus $C_{w}$ can be expressed as,

$$C_{w} = \frac{C_{s}}{K_{OC} \times f_{oc}} \quad (6.5)$$

Therefore, BAF can be calculated as,

$$BAF = \frac{C_{\text{org}}}{C_{w}} = \frac{C_{\text{org}}}{C_{s}} \times \frac{C_{s}}{K_{OC} \times f_{oc}} = BSAF \times K_{OC} \times f_{oc} \quad (6)$$

Since 295 ml of water was applied to 1 kg of GL soil, and assuming that $C_{60}$ concentration in water reaches its solubility (8 ng L$^{-1}$), the maximum amount of $C_{60}$ in the aqueous phase is 2.36 ng for 1 kg of soil (dry weight). For phenanthrene (water solubility 1.28 mg L$^{-1}$), the maximum amount in aqueous phase is 0.379 mg. These numbers are very small compared to the total dosed amount (0.25, 60, 100, and 300 mg kg$^{-1}$ soil for $C_{60}$ and 50 mg kg$^{-1}$ for phenanthrene) and can be neglected. Therefore, $C_{s}$ is approximately equal to the total applied organic compound concentration in the soil.

Accordingly, BAFs of $C_{60}$ and phenanthrene were calculated as:

In GL soil, 0.25 mg kg$^{-1}$ $C_{60}$, $BAF = 0.427 \times 10^{7.1}$ L kg$^{-1} \times 0.7\% = 10^{4.58}$ L kg$^{-1}$

In GL soil, 50 mg kg$^{-1}$ phenanthrene, $BAF = 7.93 \times 10^{4.1}$ L kg$^{-1} \times 0.7\% = 10^{2.84}$ L kg$^{-1}$

Therefore, BAF of $C_{60}$ was calculated as $10^{4.58}$ L kg$^{-1}$, much higher than that of phenanthrene, which was calculated as $10^{2.84}$ L kg$^{-1}$. These numbers agree with the values
reported by Arnot and Gobas for the organic compounds with similar $K_{ow}$ [135], and reflect higher BAF with higher $K_{ow}$ as predicted by theory [147].

While $C_{60}$ is relatively stable in the environment [148] and no biochemical or physical transformations were observed here, it could form stable aqueous suspensions upon association with dissolved NOM [67]. The aqueous $C_{60}$ nanoparticles or nC$_{60}$ may be oxidized under certain environmental conditions (e.g., sunlight or UVA irradiation) [149, 150] or harsher conditions during water or wastewater treatment (e.g., irradiation with high UV intensity and/or ozonation) [136, 151], thus becoming more hydrophilic. Such transformations would change the mobility and bioavailability of $C_{60}$, resulting in different bioaccumulation potential(s) than found in this work.

Overall, this study clearly demonstrates that $C_{60}$ bioaccumulates in $E. fetida$, indicating a potential risk of exposure to higher order organisms through food web transfer. This underscores the need for further studies on $C_{60}$, among other engineered nanomaterials, regarding trophic transfer, biomagnification potential, and associated sublethal effects.
CHAPTER 7

BIOCHAR INTERACTIONS WITH EARTHWORMS: ECOTOXICOLOGICAL IMPLICATIONS

7.1. Introduction

Biochar, a form of charcoal produced by pyrolysis of carbon-rich biomass, draws tremendous interest worldwide due to its potential to enhance soil fertility, facilitate soil water management, sequester CO₂, and manage organic waste. Application of biochar to soil has been shown to increase soil water and nutrient retention (e.g., by trapping them in micropores [17]), improve soil fertility, and alter soil structure (e.g., enhanced porosity and soil aeration) [18-20]. The relatively stable nature of biochar and its subsequent long soil residence time make biochar soil amendment a promising approach to enhance plant growth and reduce CO₂ emissions [21]. Biochar is also effective in removing organic contaminants from water [22, 23] and has been demonstrated to be six times more effective in absorbing heavy metals compared to activated carbon [24]. Therefore, it is very likely that biochar will be broadly used in the near future underscoring the need to proactively assess and mitigate any unintended consequences. In particular, the literature has not yet addressed the potential impact of biochar amendment on terrestrial organisms and the organisms' associated response.

Earthworms perform many essential and beneficial functions in soil ecosystems, including decomposition, nutrient mineralization, and soil structure improvement [20], and their ability to perform these functions can be inhibited upon exposure to harmful substances. Many ecotoxicological studies have used earthworms as model system to
assess potentially toxic materials in soils [77-80, 82-84, 87]. Earthworms are useful model organisms because many aspects of their response to environmental perturbations can be assessed and connected to environmental outcomes, including their avoidance behavior, growth rate, enzyme activity level, mortality, and reproduction patterns [88].

The acute earthworm avoidance test was first developed in 1996 [88]. The International Standards Organization (ISO) has established earthworm avoidance test guidelines for rapid screening and evaluation of soil function and influence of contaminants and chemicals on earthworm behavior [90]. Environment Canada has also published a standard earthworm avoidance method to test toxicity of contaminated soil [89]. Avoidance tests have higher sensitivity to contaminants and require less experimental time than other earthworm toxicity tests [77, 88], making them ideal for a rapid screen of emerging substances or materials that may be deliberately or incidentally applied to soils.

In this study, we conducted acute soil avoidance tests and viability assays (e.g., reproduction and weight loss) to assess the potential toxicity of biochar produced by apple wood chips to the model earthworm Eisenia fetida. We also measured lipid oxidation and superoxide dismutase activity in earthworm tissue to investigate whether exposure to biochar induces oxidative stress.

7.2. Materials and methods

7.2.1 Chemicals and reagents

Tris-HCl (molecular grade), EDTA (disodium) solution (2.5% (w/v)), sucrose (99+%), sodium lauryl sulfate, sodium acetate (99+%), acetic acid (99.8%), thiobarbituric acid (99+%), trichloroacetic acid (99+%), 1,1,3,3-tetraethoxypropane (97%), nitro blue
tetrazolium, methionine (99+%), CaCO₃ and riboflavin (98%) were purchased from Fisher Scientific (Pittsburgh, PA).

7.2.2 Organism

_Eisenia fetida_ was purchased from The Worm Farm (Durham, CA). Worms were maintained according to the method described before [152]. Sexually mature earthworms (i.e., clitellated), in a range of 0.3-0.6 g, were selected for all the experiments.

7.2.3 Biochar preparation and characterization

We prepared biochar in a custom-built demonstration-scale batch reactor at Rice University from apple wood sawdust purchased from Allied Kenco, Houston, Texas. The feedstock was composed of wood fragments which were 5-10mm x 0.5-1mm x 0.5-1mm when added to the biochar reactor; the particle size decreased by 25-50% following pyrolysis. The Rice reactor used in this experiment produced approximately 2 kg biochar per 4 hour run. Biomass was sealed within a 20 liter reactor vessel constructed with 304 stainless steel and heated in a propane-fired furnace. Exhaust gases were passively vented to a series of heat exchangers at ambient temperature to remove condensable liquids and bio-oils and prevent their condensation into the biochar. Non-condensable gases were combusted in a secondary (venturi-style) burner to heat the reactor. The initial heating rate was approximately 5°C/minute to a temperature of ~400°C. Thereafter, the heating rate slowed to 1°C per minute, to a maximum temperature of approximately 525°C. Total heating time was approximately 250 minutes (slow pyrolysis, no gasification). No active purging of the atmosphere was involved. The reactor was allowed to cool to ambient temperature (overnight) before removing the
biochar. When removed from the reactor, the biochar was odorless and visually homogenous with no ash formed. The biochar was not rinsed before use.

The surface area of this biochar was $8.93 \pm 0.95 \text{ m}^2/\text{g}$, based on the Brunauer-Emmett-Teller method using a Quantachrome Autosorb-3B Surface Analyzer (Quantachrome Instruments, Boynton Beach, FL, USA). Polycyclic aromatic hydrocarbon (PAH) content in biochar was analyzed by a commercial lab (Pace Analytical Services Inc., Lenexa, KS) using EPA method 8270.

7.2.4 Artificial soil preparation and amendment

Artificial soil (AS) was prepared as described by Environment Canada [89]. It consisted of 10% Sphagnum peat moss (previously sieved through 2mm mesh), 20% kaolin clay and 70% quartz sand. The pH of dry soil was adjusted with CaCO$_3$ to the optimal range (6.5-7.5).

We chose biochar amendment levels of 10, 100, and 200 g biochar/kg soil. The background level of charcoal in US agricultural soils ranges from about 1-15 g/kg [153], with larger values typically occurring in prairie soils with a history of wildfire [154, 155]. An application rate of 100 g/kg, or 10% biochar by mass, corresponds to an application rate of 90 ton/ha at a tillage depth of 10 cm or 180 ton/ha at a tillage depth of 20 cm. This is at the high end of application rates currently employed (8 to 116 ton/ha) [156-158]. An application of 200 g/kg (180 ton/ha at a tillage depth of 10 cm, or 360 ton/ha tilled to 20 cm) is at the outer range of maximum plausible application levels [21]. Phenanthrene, a representative polycyclic aromatic hydrocarbon (PAH), was added separately at 0.1 g phenanthrene/kg soil as positive control in the avoidance test. Application of phenanthrene to soil was described previously [152].
7.2.5 Soil Avoidance Bioassays

We conducted soil avoidance bioassays following a modified method developed by Environment Canada [89]. This test was conducted in stainless-steel avoidance wheels with six pie-shaped compartments connected to a circular, center chamber that served as the test arena. Negative control soil (unamended artificial soil) was placed into alternating compartments (3 compartments/ unit, 250 g of soil/ compartment), while test soil was transferred to the remaining compartments. Test soil and control soil were separated by removable aluminum partitions. Soil in each compartment was hydrated with Milli-Q water to 85% of water holding capacity according to the method published by Coleman et al. [99]. Partitions were removed after adding the water. Ten worms were selected for each avoidance wheel. Worms were introduced to the center of the avoidance wheel individually. After the addition of ten worms to an avoidance wheel, a lid was placed over the wheel to prevent escape and time was recorded \((t=0)\). At least five replicates were conducted for each concentration of biochar. The wheels were placed in a dark area at 22°C for 48 h. At the end of 48 h time period, the lid was removed and the partitions were inserted back to the chambers to prevent further worm movement between the compartments. The numbers of worms in each compartment were recorded. If a worm was cut by the partition and separated in two compartments, 0.5 was recorded for both of the compartments. The percentage of worms (10 in total) in control and test soils was then calculated separately.

7.2.6 Growth and reproduction tests

To measure the effects of prolonged exposure of biochar to earthworms on their growth and reproduction, we conducted a 28-d toxicity test according to Environment Canada protocols [89]. After depuration on filter paper hydrated with DI water for 24 h,
Earthworms were transferred to 500-ml glass jars filled with soil (10 worms/200 g soil) containing different concentrations of biochar (0, 10, 100, and 200 g kg\(^{-1}\)) for 28 d. Soils in all treatments were hydrated with Milli-Q water to 85% of water holding capacity. Worm weight was measured and recorded at day 0 and day 28 after depuration. Cocoon numbers in each treatment were counted and recorded. All treatments were prepared in triplicate.

7.2.7 Lipid peroxidation and superoxide dismutase activity measurement

We exposed worms to the highest level of biochar amended soil (200 g kg\(^{-1}\)) for 1, 2, 7, and 14 days. We chose this biochar concentration because it represents an upper limit of what is likely to be applied to agricultural soils. At each time point, 5 worms were picked out randomly and sacrificed for oxidative stress tests. Worm tissue was homogenized on ice in nine parts (w/v) of 50 mM Tris-HCl buffer containing 1 mM EDTA and 0.25 M sucrose (pH 7.6) with a motor-homogenizer (Brinkmann Instruments, Inc., Westbury, NY). The homogenate was centrifuged at 8,000 rpm for 20 min and the supernatant was saved for future tests.

Protein concentration of the worm homogenate was determined following the Bradford method [159] using bovine serum albumin as standard. Oxidative stress damage in the form of lipid peroxidation was assessed by malondialdehyde (MDA) formation using the method described by Hannam et al. [160] with modifications. Briefly, worm tissue homogenate (200 µl) was added to a 5-ml glass test tube containing 200 µl sodium lauryl sulfate (SDS) solution (8.1% w/v sodium lauryl sulfate), 1.5 ml of 0.2 M NaAc-HAc buffer solution (pH 3.6), 750 µl TBA solution (1% w/v thiobarbituric acid), 750 µl TCA solution (10% w/v trichloroacetic acid), and 1 ml of Milli-Q water. The cocktail was incubated in water bath at 90°C for 60 min, cooled down to room temperature, and
centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant was measured using an Ultrospec 2100 pro spectrophotometer (GE Healthcare, Piscataway, NJ) at 530 nm. A blank was prepared by substituting 200 µl of worm homogenate with 200 µl of Milli-Q water. The MDA concentration was determined against the standard curve with 1,1,3,3-tetraethoxypropane. Results were expressed as nmol MDA per mg protein.

The activity of superoxide dismutase (SOD), an enzyme that protects organisms against oxidative stress from superoxide (a type of ROS), was measured by using the nitroblue tetrazolium chloride (NBT) method described by Song et al. [161]. One unit of SOD activity (U) was defined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction (measured as 50% of the absorbance of the SOD-free control), and the result was expressed as U per mg protein.

7.2.8 Statistical analysis

Whether differences between two sets of treatments were statistically significant was determined using Student’s t-test at the 95% confidence level.

7.3. Results and discussion

7.3.1 Earthworms response to the presence of biochar in soil

We assessed the effects of biochar on the earthworms’ avoidance behavior, growth rate, and reproduction. Earthworms did not avoid soil containing 10 g kg⁻¹ biochar (Figure 7.1), a level within the upper limits of natural soil biochar concentrations. However, a statistically significant earthworm avoidance effect was observed with soils amended with dry biochar at higher concentrations (100 and 200 g kg⁻¹). Phenanthrene, which is a commonly studied PAH and can be formed during biomass pyrolysis, was used as a positive control. Phenanthrene elicited a more significant avoidance effect at
concentrations as low as 0.1 g/kg, three orders of magnitude lower than our highest soil biochar concentrations (Figure 7.1).

Figure 7.1. Percentage of worms recovered in amended versus unamended soil compartments after 48-h avoidance tests. Worms showed a significant avoidance effect in soils amended with 100 g/kg and 200 g/kg biochar ($p < 0.05$). Phenanthrene (phen), the positive control, also showed a significant avoidance effect at 0.1 g/kg. * indicates a significant difference from controls at the 95% confidence level. Error bars represent ± one standard error (n = 5)
In a separate test, earthworm weight loss was assessed after 28-day incubation in soil amended with different concentrations of dry biochar (10, 100, and 200 g kg\(^{-1}\)). Because earthworms were not fed during the exposure period, they experienced weight loss even in the control soil. The weight loss for earthworms collected from soils containing 100 and 200 g kg\(^{-1}\) biochar was 37.1±1.7% and 40.3±2.5%, respectively, which is significantly higher \(p < 0.05\) than the weight loss for worms in the control soil (32.1±1.0%) (Figure 7.2A). Soil containing 10 g kg\(^{-1}\) dry biochar did not significantly affect weight loss. No significant effect on earthworm reproduction was observed for all tested biochar concentrations (Figure 7.2B).
Figure 7.2. Worm weight loss (A) and cocoon production (B) after 28-d incubation in unamended soil or soils amended with dry biochar. Significantly higher weight loss was observed in both treatments with 100 and 200 g/kg dry biochar amended soils. Biochar did not have a significant effect on the earthworms' cocoon production. * indicates significant difference from controls at the 95% confidence level. Error bars represent ± one standard error (n = 3).
7.3.2 Three hypotheses for avoidance response

We pose three hypotheses that could explain the observed avoidance response: 1) the response was driven by nutrient scarcity, since biochar is non-nutritive and its addition in large amounts to soils would dilute the total amount of nutrients available in the soil; 2) biochar had a high water-holding capacity and dry biochar may desiccate earthworms; and 3) contaminants such as PAHs, which could be formed during biochar production by pyrolysis [162], were toxic and avoided by the earthworms.

To test the first hypothesis, we conducted an avoidance test with soil amended with 200 g kg\(^{-1}\) sand instead of biochar. Sand contains no earthworm nutrients and similarly to this biochar it would dilute those present in the artificial soil. Figure 2 shows that earthworms did not differentiate between the control soil and the reduced-nutrient soil containing extra sand, indicating that nutrient deficiency did not drive the avoidance behavior. Nutrient deficiency due to nutrient sorption by biochar was unlikely given the experimental design. The only possible source of nutrients in the tested artificial soil was one of the soil ingredients, peat moss (about 2 mm diameter), which cannot penetrate into the much smaller pores of biochar (on the order of tens of microns). Furthermore, the short duration of exposure (2 days) in the absence of water flow makes significant leaching of nutrients from peat moss into the biochar highly improbable.

To investigate the effects of biochar's water-holding capacity, we repeated the 100 g/kg experiment with biochar wetted to field capacity. The field capacity of this biochar was more than four times greater than that of the artificial soil (2.2 vs 0.47 ml water per g char/soil). Extra water (beside the standard amount of water added to reach 85% of the
artificial soil’s water holding capacity) was added to every 250 g of amended soil (100 g kg⁻¹ biochar) to saturate the biochar to its field capacity. With this extra moisture, the avoidance effect was no longer statistically significant relative to the unamended control (p > 0.05) (Figure 7.1), indicating that moisture was likely a key factor affecting earthworm behavior in biochar-amended soil. Accordingly, desiccation may also explain the significantly higher weight loss in earthworms exposed to dry biochar for 28 d (Figure 7.2A). However, additional (as yet undetermined) factors besides desiccation may have also contributed to the observed behavior since decreased avoidance upon wetting was significant relative to unamended controls but not relative to dry biochar (p > 0.05) (Figure 7.1).

Potential toxins in biochar, such as PAHs produced during pyrolysis, may be assimilated by the earthworms. Out of the 16 PAHs regulated by the EPA, only three were detected within the biochar prior to its addition to the soil: fluorene (25.9 µg/kg), naphthalene (3,290 µg/kg), and phenanthrene (102 µg/kg). These values are below cleanup action levels for PAH-contaminated soil [163], which are 2,300 mg/kg for fluorene, 120 mg/kg for naphthalene, and 1,700 mg/kg for phenanthrene. Thus, there is no amendment levels at which it would be possible to exceed legally acceptable PAH limits with this biochar. Nevertheless, PAH could induce the intracellular production of reactive oxygen species (ROS) [164], which in turn cause oxidative stress to cellular components [165] and induce a detectable biochemical response in the organisms. Specifically, the common oxidative stress biomarker MDA, which is an oxidation product of ROS interaction with polyunsaturated fatty acids [161], could be produced. To assess the possibility that worms experienced oxidative stress, we monitored total
superoxide dismutase (SOD) activity and lipid peroxidation (per MDA formation) in earthworm cells during exposure to control soil and soil amended with 200 g kg\(^{-1}\) biochar. No discernable differences or increase in either SOD or MDA levels was observed during the 14-day test for earthworms incubated in control versus biochar-amended soils, (Figure 7.3). Therefore, exposure to this biochar did not cause lipid peroxidation or induce antioxidant defense in the earthworms, indicating that the avoidance response was not likely the result of chemical (e.g., PAH) toxicity.
Figure 7.3. Malondialdehyde (MDA) (A) and superoxide dismutase (SOD) (B) measurements in worm tissue in control soil and soil amended with 200 g/kg biochar during 28-day exposure. Error bars represent ± one standard error (n = 5).

Previous studies have shown different response of earthworms to biochar in soil. Chan et al. (2008) found that earthworms preferred soil amended with biochar produced
from poultry litter pyrolyzed at 450°C. However, this effect was not reproducible with biochar from the same feedstock pyrolyzed at 550°C [75]. Field capacity of biochar increases with increasing pyrolysis temperature up to about 500°C, (Kinney et al., in preparation), suggesting that the earthworm behavior observed by Chan et al. (2008) may also be a function of biochar water retention properties. In another study with biochar derived from papermill waste, earthworms exhibited a slight but statistically indiscernible attraction to biochar [76]. However, the underlying mechanism(s) for earthworm attraction (or avoidance) have not been discerned. This may require considering how biochar affects the nutritional and water activity properties of the soil, which can potentially affect earthworm behavior. We showed that although soil amendment with dry biochar could desiccate earthworms, this negative effect can be overcome by pre-wetting the biochar. Furthermore, with a much higher field capacity than soil (2.2 versus 0.47 g water per g biochar/soil for the biochar used here), wet biochar may buffer the soil from large fluctuations in soil moisture (unpublished data showed that biochar slows soil water loss).

7.4. Summary

Earthworms avoided soils amended with high concentrations (≥ 100 mg kg⁻¹) of dry biochar (produced from apple wood chips), and experienced significant weight loss after 28-day exposure. The avoidance response was likely to avert desiccation rather than to avoid potential toxicants (i.e., PAHs formed during biochar production by pyrolysis) or nutrient scarcity. By wetting the biochar to field capacity before exposing the worms, we found that this adverse effect could be completely mitigated in application levels as high as 100 g/kg (90 ton/ha). Therefore, depending on site-specific conditions and irrigation or
rainfall patterns, wetting biochar either before or immediately after soil application may be needed to prevent desiccation of earthworms and enable their beneficial effects on plants.
8.1 Conclusions

This research has investigated the potential impact of two emerging carbonaceous materials, C$_{60}$ and biochar, to terrestrial organisms. The following points highlight the primary conclusions of this study connected to specific objectives:

- **Evaluate the potential terrestrial ecotoxicity of C$_{60}$ including its antimicrobial activity, and discern the effect of natural organic matter on bioavailability and impact.**
  
  - The antibacterial activity of nC$_{60}$ can be mitigated by the presence of NOM as a soil constituent or dissolved in the water column. Sorption to soil might decrease the bioavailability of nC$_{60}$ and thus its toxicity to bacteria, and this mitigating effect likely increases with the organic content and surface area of the soil. Aqueous organic matter also may mitigate nC$_{60}$ toxicity, by coating nC$_{60}$, hindering direct contact of with cells, and possibly altering nC$_{60}$ surface chemistry through an undetermined mechanism.
  
  - Pristine C$_{60}$ exerts reproduction toxicity to earthworms, but no negative effects on either behavior or growth. The reproduction toxicity of C$_{60}$ may have a significant effect to the population of the earthworms and thus impact the terrestrial food web and the wider environment.

- **Assess the uptake and bioaccumulation of C$_{60}$ by soil invertebrates (i.e., earthworms) and the factors affecting its bioavailability.**
C₆₀ nanoparticles were rapidly bioaccumulated by earthworms, although to a lower extent than smaller phenanthrene molecules that are more hydrophobic; thus, the large molecular size of C₆₀ hinders its bioaccumulation. Less bioaccumulation occurred at higher C₆₀ concentration in soil, which is counterintuitive and reflects that higher C₆₀ concentrations that exceed the soil sorption capacity exist as larger precipitates that are less bioavailable. C₆₀ was not transformed during the process of bioaccumulation. Overall, the relatively low extent but rapid bioaccumulation of C₆₀ in E. fetida suggests the need for further studies on the potential for trophic transfer and biomagnification.

- Investigate the potential toxicity of biochar to earthworms.

- Earthworms avoided soils amended with high concentrations of dry biochar, and experienced significant weight loss after 28-days exposure. The avoidance response was likely to avert desiccation rather than to avoid potential toxicants (i.e., PAHs formed during biochar production by pyrolysis) or nutrient scarcity. By wetting the biochar to field capacity before exposing the worms, we found that this adverse effect can be completely mitigated in application levels as high as 100 g/kg (90 ton/ha). Therefore, depending on site-specific conditions and irrigation or rainfall patterns, wetting biochar either before or immediately after soil application may be needed to prevent desiccation of earthworms and enable their beneficial effects on plants.

C₆₀ and biochar are both emerging carbonaceous materials having great potential of wide applications in the near future. Both of these materials showed negative effects to earthworms only at high concentrations that are rarely achieved in the
environment. C\textsubscript{60} and biochar both may affect the population of earthworms and thus the food web in a certain area, although by different mechanisms.

- C\textsubscript{60} decreased earthworm’s cocoon production, while biochar showed negative effect to earthworm’s growth and behavior. The toxicity mechanism of C\textsubscript{60} is still unclear, while for biochar, the adverse effect is likely the result of desiccation. With a smaller particle size, C\textsubscript{60} can be readily accumulated by earthworms and may be transferred to higher trophic level, while for biochar, although bioaccumulation of the charcoal itself is not a concern, the toxic compounds produced during pyrolysis may be introduced into the soil and accumulated by organisms.

### 8.2 Engineering significance

The main objective of this project is to understand the potential environmental impact of two emerging carbonaceous materials, C\textsubscript{60} and biochar. The engineering significance of this research is illustrated as below:

- The antibacterial activity of nC\textsubscript{60} can be mitigated by the presence of NOM and soil sorption, indicating the behavior of nC\textsubscript{60} in complex environmental systems may be different from the well-defined lab conditions. This provides a foundation of ecological risk assessment to inform the decisions of policy makers.

- The avoidance effect of dry biochar to earthworms found in this study gave an alert of the potential environmental impact of biochar application to soils. This information is very important to the fast developing industry of biochar. We
suggested wetting biochar either before or immediately after soil application to prevent desiccation of earthworms and enable their beneficial effects on plants.

- The overall method established in this research can be used to evaluate the potential environmental impacts of other emerging materials.

### 8.3 Recommendations

Both C₆₀ and biochar have a potential of extensive use in the near future. This research provides a basis for ecological risk assessment to inform the decisions of policy makers and to set up a method by which emerging materials can be evaluated for their environmental impacts. The observed toxic effects of C₆₀ and biochar to terrestrial organisms call for in-time regulations for appropriate use and disposal to avoid health and environmental disasters. Although a specific safe disposal level is not available based on this research, waste containing high concentrations of C₆₀ (e.g., biosolids from wastewater treatment plants) is not recommended to be directly disposed of in the field. Long-term toxicological studies with earthworms and other organisms are recommended for future research. This research also underscores the need for further studies on C₆₀, among other engineered nanomaterials, regarding trophic transfer, biomagnification potential, and associated sub-lethal effects. For biochar application, we recommend wetting biochar either before or immediately after soil application. Trial studies are also recommended before large scale field application in order to find out the appropriate application rate of biochar without compromising other soil functions and the wider environment. Long-term field studies are recommended for a better understanding of the potential impacts of biochar to ecosystem services, including nutrient cycling, biodegradation of pollutants (e.g., pesticides), and primary productivity.
Appendix A. Data for figures.

Table A.1. Rate of CO₂ production by *E. coli* (µmoles/min) (Figure 4.3A).

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Table A.3. Cumulative CO₂ production of *E.coli* (μmoles) (Figure 4.4 & 4.5)

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Table A.4. C<sub>60</sub> BASF in the earthworms during 28-day incubation (Figure 6.2).

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Table A.5. C$_{60}$/phenanthrene (phen) concentration (mg/kg) in earthworms during 7-day elimination after 14-d exposure to FD soil dosed with C$_{60}$ (100 and 300 mg kg$^{-1}$) and phen (50 mg kg$^{-1}$) (Figure 6.5).

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References


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