An investigation into the effects of nano copper oxide to
the soil invertebrates *Eisenia andrei* and *Folsomia*
canadida

by

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Abstract

The fate and effects of nano copper oxide (nCuO) to soil invertebrates in field collected soil and in biosolid amended field soil were investigated in order to understand the risk CuO nanomaterials present to the soil environment. The effects of nCuO were compared to a soluble Cu-salt (i.e., CuSO₄) through sub-lethal toxicity studies with the earthworm *Eisenia andrei* and the springtail *Folsomia candida*, which included survival and reproduction tests for both species and bioaccumulation in *E. andrei*. The fate of both Cu forms was examined in soil through the characterization of Cu, namely total Cu, extractable Cu²⁺ and extractable nano particulate CuO in each exposure scenario. In general, exposure to nCuO compared to CuSO₄ was less toxic for both reproduction and bioaccumulation tests and with or without biosolid amendment. Results demonstrated that the solubilization of and availability of Cu²⁺ in soils could explain the observed effects.

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Table of Contents

Abstract.....	ii
Acknowledgements	iii
Table of Contents	iv
List of Tables	vi
List of Figures.....	vii
List of Appendices.....	viii
Chapter 1: Introduction	1
1.1 Scope	1
1.2 Background.....	2
1.3 Objectives	6
Chapter 2: Sub-lethal toxicity of copper oxide nanoparticles and copper sulphate to Eisenia andrei and Folsomia candida in field soil with and without biosolid amendment	8
2.1 Abstract	8
2.2 Introduction	9
2.3 Material and methods	11
2.3.1 Materials.....	11
2.3.2 Nanomaterial characterization.....	12
2.3.3 Soil Spiking.....	13
2.3.4 Toxicity Tests.....	15
2.3.5 Chemical Measurements	16
2.3.6 Statistics	19
2.4 Results and discussion.....	20
2.4.1 Toxicity Tests.....	20

2.4.2	Extractable Cu ²⁺ and nano Cu	23
2.5	Conclusions	29
Chapter 3: Bioaccumulation and toxicokinetics of nano copper oxide in the earthworm <i>Eisenia andrei</i>		31
3.1	Abstract	31
3.2	Introduction	32
3.3	Materials and Methods	34
3.3.1	Materials.....	34
3.3.2	Soil Spiking.....	35
3.3.3	Bioaccumulation testing.....	36
3.3.4	Soil and Tissue Analysis	37
3.3.5	Statistics	38
3.4	Results and Discussion	40
3.4.1	Soil Analysis	40
3.4.2	Bioaccumulation	43
3.4.3	Earthworm Tissue	46
3.5	Conclusions	50
Chapter 4: Conclusion.....		52
References		57
Appendices.....		70
Appendix A - Chapter 2 Appendix.....		70
Appendix B - Chapter 3 Appendix		76

List of Tables

Table 2-1: Physical and chemical characteristic of test soils. All measurements represent a mean of n=3, except background Cu which is a mean of n=12, ($\pm$ standard deviation)...	12
Table 2-2: SP-ICP-MS operational parameters.	19
Table 2-3: Reproductive inhibitory concentrations (IC) at 10, 25 and 50% effect levels. Estimates produced with logistic model using measured total Cu in soil with and without biosolids for <i>E. andrei</i> and <i>F. candida</i> exposed to nCuO and CuSO ₄ with upper and lower 95% concentration limits in parentheses.....	22
Table 2-4: SP-ICP-MS measurements in soil extracts.....	29
Table 3-1: Bioaccumulation summary including calculated kinetic rates.	44

List of Figures

Figure 2-1: TEM images of nCuO in methanol (A), nanopure water (B) and, soil pore-water (C and D).....	13
Figure 2-2: Dose-response relationships of <i>E. andrei</i> reproductive inhibition based on total measured soil Cu (left) and extractable Cu ²⁺ (right).	23
Figure 2-3: Mean ($\pm$ standard error) log measured extractable Cu ²⁺ concentrations soil extracts relative to total measured Cu in soils with (left) and without (right) biosolid amendment in <i>E. andrei</i> (top) and <i>F. candida</i> (bottom) tests.	25
Figure 2-4: Example of SP-ICP-MS distribution plot from TSPP soil extract spiked with nCuO in biosolids	29
Figure 3-1: Mean ($\pm$ SD) measured total Cu concentration (ug/g) soil (with and without biosolid amendment).....	41
Figure 3-2: Mean ($\pm$ SE) available Cu ²⁺ measured KNO ₃ soil extracts throughout uptake phase.	43
Figure 3-3: Modelled kinetic uptake and elimination curves based on total Cu (solid lines) and extractable Cu ²⁺ (dashed lines) in soil and, measured total Cu in tissues (♦).	46
Figure 3-4: Hyperspectral images of d-21 earthworm tissues (nCuO no biosolid) with, spectra matching nCuO highlighted in red. A – chloragogenous tissue; B – muscle tissue; C – epidermis; D – gut.	49
Figure 3-5: Example of <i>E. andrei</i> section used for enhanced dark-field hyperspectral microscopy, with region of sections marked (A-D) corresponding to the images in Figure 3-4 (A – chloragogenous tissue; B – muscle tissue; C – epidermis; D – gut).	50

List of Appendices

Appendix A – Chapter 2 Appendix

Table A 1: Mean ($\pm$ standard error (SE)) measured total Cu, Cu^{2+} activity, and the pH of the KNO_3 extracts for ISE measurements for the *Eisenia andrei* reproduction assays. NA = not available.....71

Table A 2: Mean ($\pm$ standard error (SE)) measured total Cu, Cu^{2+} activity, and the pH of the KNO_3 extracts for ISE measurements for the *Folsomia candida* reproduction assays. NA = not available.....72

Table A 3: Toxicity endpoints based on measured extractable Cu^{2+} for *E. andrei* and *F. candida* exposed to nCuO and CuSO_4 in soil with and without biosolid amendment. Reproduction inhibitory concentrations (IC) are provided for 10, 25 and 50% effect levels with upper and lower 95% confidence limits in parentheses.....73

Table A 4: Mean ($\pm$ standard error (SE)) adult survival, juvenile production, soil $\text{pH}_{\text{CaCl}_2}$ and moisture content for the *Eisenia andrei* reproduction assays.....74

Table A 5: Mean ($\pm$ standard error (SE)) for adult survival, juvenile production, soil $\text{pH}_{\text{CaCl}_2}$ and moisture content for the *Folsomia candida* reproduction assays.....75

Appendix B - Chapter 3 Appendix

Table B 1: Mean measured Cu^{2+} in KNO_3 soil extracts (log ug/g) and mean pH (SE = standard error).....76

Table B 2: Mean corrected (for background) total Cu measured throughout bioaccumulation tests in soil and tissues. (SE = standard error).....77

1 Chapter 1: Introduction

2 1.1 Scope

3 The fate and effects of nano copper oxide (nCuO) to soil invertebrates in field collected
4 soil and in biosolid amended field soil were investigated to better understand the risk that
5 CuO nanomaterials may present to the soil environment. Since evidence suggests that
6 metallic nanomaterials are likely to transform and solubilize¹⁻³ when exposed to complex
7 natural environments such as soils, the effects were compared with the ionic form of Cu
8 (i.e., Cu²⁺) using copper sulfate (CuSO₄) to examine the influence of solubilized Cu²⁺
9 released from the nanomaterial on toxicity and uptake. The extractable fraction of Cu²⁺
10 available within each soil exposure was in addition to soil invertebrate sub-lethal toxicity
11 endpoints and bioaccumulation potential. Sub-lethal toxicity studies with the earthworm
12 *Eisenia andrei* and the springtail *Folsomia candida* included, survival and reproduction
13 tests for both species and bioaccumulation potential in *E. andrei*. The fate of nCuO was
14 examined in both soils using a suite of analytical techniques aimed to quantitatively
15 characterize Cu exposure, namely total Cu, extractable Cu²⁺ and extractable nano-
16 particulate Cu in each exposure scenario. The fate of nCuO in tissues was briefly explored
17 through qualitative microscopy techniques in earthworms from the bioaccumulation study.
18 The research is presented herein in two sections; the first focused on survival and
19 reproduction tests performed on *E. andrei* and *F. candida*, including the fate of Cu in test
20 soils; the second focused on the bioaccumulation potential in *E. andrei* and includes an

21 investigation of the fate of the nCuO in earthworm tissues. Together these two studies
22 represent the scope of the work completed.

23

24 **1.2 Background**

25 During the past two decades, the production of engineered nanomaterials (ENMs)
26 has increased at a rapid pace, with increased applicability for different ENMs developed
27 every year²⁻⁴. Nanomaterials are defined as having at least one dimension of less than 100
28 nm, but can come in a variety of sizes and shapes (spheres, rods, wires, etc.) within that
29 definition. Additionally, ENM are frequently coated to help reduce aggregation or to
30 functionalize for specific applications; all of these variations are designed to produce
31 ENMs that will interact or react with substrates in different ways. The unique properties
32 and diverse forms and sizes of ENM creates a challenge for risk assessors and
33 ecotoxicologists attempting to determine the fate and effects of ENM in the
34 environment^{2,3,5}. Additionally, traditional metrics for measuring chemicals (e.g., total
35 metal concentrations) in environmental substrates cannot sufficiently characterize ENM
36 (e.g., size, surface area, transformations, etc.). To try to address these challenges, a great
37 deal of research has gone into trying to determine the best methodologies for measuring
38 and characterizing these materials and determining the fate and effects in water, air and
39 soils^{2,6-9}. Despite this, there is still very little understanding of the fate and effects of ENM
40 in the soil environment, which are also diverse and complex, containing a mixture of
41 organics, minerals and colloids that may interact, react with and transform ENM¹⁰. Much
42 of the focus for ENM in soils has been on metallic ENM since they are the highest produced
43 and commercially utilized ENM and, are expected to end up in the soil environment

44 through: deposition from air, sedimentation and agricultural applications⁴. Additionally,
45 research has shown that metallic ENMs can be concentrated in sewage sludge (biosolids)
46 during wastewater treatment^{11,12}. Biosolids are commonly used as a fertilizer on agronomic
47 lands, making their contamination with ENMs a concern from an environmental
48 perspective. Key soil properties that determine the stability of a metallic ENM within a
49 natural soil environment have been identified as pH, organic matter, clay content, and to a
50 lesser extent, cation exchange capacity, carbonates and amorphous Fe^{13,2,14,15,16}.

51 Due to its unique properties, such as anti-fungal, excellent electrical and heat
52 conduction and extreme weather resistance, copper ENMs are used in a large variety of
53 technologies⁴. In 2010, the estimated global production of Cu-based ENMs was
54 approximately 200 tons per year, with the majority of production originating as catalysts,
55 electronics and optics, and coatings, paints and pigments⁴. These uses, along with the
56 incorporation and use of Cu ENMs as a biocide for wood preservation (e.g., fence and deck
57 treatments), and agronomic applications¹⁷ have led to soils being identified as a major sink
58 for Cu ENMs^{4,18,19}. Although copper is an essential trace element for organisms, it can be
59 toxic in higher concentrations^{13,20,21}. At elevated copper concentrations, the formation of
60 reactive oxygen species through the Fenton reaction (i.e., $\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \bullet\text{OH} +$
61 OH^-) can modify the structure of function of essential biomolecules, which can cause acute
62 and chronic toxic effects. For example, the EC50 for reproduction in *E. andrei* in a natural
63 reference soil was previously reported as 130.9 mg Cu/ kg soil¹³. Despite extensive
64 research on the bulk form of Cu, relatively little is known about how soil characteristics
65 affect interactions between Cu ENMs, soil and soil organisms¹⁸. In recent years, there has
66 been some research into the effects of Cu ENMs in the environment with much of the

67 attention given to aquatic organisms. With regards to soil organisms, the majority of studies
68 has been on plants and the soil microbial community and few on soil invertebrates^{10,14}. The
69 research on the effects of Cu ENMs are conflicting, with some studies suggesting nano-
70 specific effects through greater or different effects compared to a bulk Cu counterpart^{22,23},
71 and others attributing the effects observed from Cu ENMs to the release of copper ions
72 through the solubilization of the ENM¹⁸.

73 Soil invertebrates represent an important part of the ecosystem, as primary
74 consumers they play an integral role in organic decomposition and the cycling of nutrients
75 in the soil environment. With regards to the effects Cu ENMs have on soil invertebrate
76 species, Amorim and Scott-Fordsmand²⁴ demonstrated nano-specific effects on
77 reproduction and avoidance response when *Enchytraeus albidus* were exposed to Cu
78 ENMs in soil, as exhibited by little to no dissolution of the ENM, relative to the Cu salt.
79 Further studies on the same species have observed varying response patterns in oxidative
80 stress and differential gene expression between Cu salt and Cu ENMs²⁵, but with no
81 differences in lipid, carbohydrate and protein energy reserves²⁶. In contrast, a study using
82 *Eisenia fetida* demonstrated significant effects on reproduction for CuCl₂ (salt) relative to
83 Cu ENMs, and observed oxidation of the Cu ENM surface, suggestive of dissolution and
84 release of Cu ions as a contributor of toxicity²⁷. Unrine et al.²⁸ reported metallothionein
85 induction upon exposure of *E. fetida* to Cu ENM and CuSO₄ in soil, with greater sensitivity
86 to the Cu salt (i.e., CuSO₄); however, the authors also observed that oxidized Cu ENMs
87 were taken up by the test organisms, which may result in Cu ‘hot spots’ within tissue.
88 Gomes et al.²⁹ also detected partial oxidation of Cu ENM, but with limited dissolution, and
89 found that the Cu salt (CuCl₂) was more toxic than the Cu ENM when *Enchytraeus*

90 *crypticus* were exposed to these substances in a natural soil. Evidence of Cu ENMs taken
91 up into tissue may result in bioaccumulation and trophic transfer, however, studies
92 examining the uptake and elimination rates of metal ENMs in earthworm are rare³⁰. Despite
93 growing interest in the effect of metal ENMs on terrestrial systems, ecotoxicity studies on
94 soil dwelling species remain limited relative to aquatic based studies^{2,31}.

95 Due to the complex, multi-phase properties of the soil matrix, the quantitative
96 analysis of nanoparticles in soils requires an operationally defined soil extraction procedure
97 that targets the desired soil metal fraction without driving undesirable transformation
98 processes (e.g. dissolution)^{3,16,32}. Commonly, metallic ENM are compared with their ionic
99 or bulk base material as a positive control to try to tease apart effects caused through the
100 dissolution process in environmental matrices versus those caused by the ENM itself (i.e.,
101 small size, large surface area, higher reactivity, etc.). Recently, more emphasis has been
102 put on not simply comparing results to the bulk form, but quantifying the extractable
103 solubilized fraction². In soils, this frequently results in reporting the soluble fraction in soil
104 pore-water or soil extracts^{10,33}. Characterizing the nanomaterial once in the soil is important
105 to understand transformations that may occur in the environment such as homo-
106 aggregation, hetero-aggregation, dissolution, binding or coating with other components of
107 the soil. Sample preparation techniques are required to render the extract suitable for
108 analysis (e.g. removal of micron-sized particulates), thus the soil extraction technique,
109 preparation and analysis are inextricably linked⁹. There is no standard method for
110 measuring ENM in environmental matrices and usually a suite of techniques is required³¹.
111 Each methodology has limitations, which is why typically more than one is utilized and
112 often the selection is based on availability. For example, it is generally agreed that good

113 characterization of nano material(s) should include more than one of the following:
114 electron microscopy techniques (e.g., transmission electron microscope (TEM), scanning
115 electron microscope (SEM), energy dispersive x-ray spectroscopy (EDS)), sizing
116 techniques (e.g., hydrodynamic size via dynamic light scattering (DLS), single particle
117 inductively-coupled plasma – mass spectrometry (SP-ICP-MS)), light spectroscopy
118 techniques (e.g., UV-VIS, enhanced dark-field hyperspectral imaging) or synchrotron
119 techniques (e.g., x-ray absorption fine structure (XAFS), x-ray absorption near edge
120 structure (XANES)).

121

122 **1.3 Objectives**

123 The work presented seeks to address the following four objectives:

- 124 1. Determine if nCuO has an effect on the survival and reproduction of *Eisenia andrei*
125 and *Folsomia candida*
- 126 2. Determine if any observed effects of *E. andrei* and *F. candida* survival and
127 reproduction can be attributed to the extractable (extractable) Cu²⁺ concentrations
128 in soil.
- 129 3. Examine the fate of Cu nanomaterial in soil and biosolid-amended soil.
- 130 4. Assess the bioaccumulation potential of nCuO in *E. andrei* and determine if Cu
131 nanomaterial is taken up into tissue.

132

133 The first three objectives are examined in Chapter 2: Sub-lethal toxicity of copper
134 oxide nanoparticles and copper sulphate to *Eisenia andrei* and *Folsomia candida* in field
135 soil with and without biosolid amendment. Standardized survival and reproduction tests

136 were conducted using *E. andrei* and *F. candida* exposed to nCuO or CuSO₄ in soils with
137 and without biosolid amendment. The results were statistically analyzed based on both total
138 Cu and extractable Cu²⁺ measurements in the test soils to determine effects at the 10th, 25th
139 and 50th percentile levels (i.e., EC10, EC25, and EC50). Sub-samples of soils from the *E.*
140 *andrei* reproduction tests were extracted for single particle inductively coupled mass
141 spectrometry (SP-ICP-MS) analysis to assess the presence and size of nano-particulate Cu.
142 The fourth objective is addressed in Chapter 3: Bioaccumulation and toxicokinetics of
143 nano copper oxide in the earthworm *Eisenia andrei*. The uptake and elimination of both
144 nCuO and CuSO₄ was measured in *E. andrei* in field soil with and without biosolid
145 amendment. Biota-to-soil accumulation factors (BSAF) at steady state, as well as kinetic
146 uptake and elimination rates, are presented for each exposure scenario. Additionally, a
147 qualitative assessment of uptake of intact nCuO particles into earthworm tissue was
148 completed using enhanced dark-field hyperspectral imaging, a light microscopy technique
149 that can match materials based on the spectrum captured by the visible near-infrared (400
150 – 1000nm) spectrophotometer³⁴.
151

152 **Chapter 2: Sub-lethal toxicity of copper oxide nanoparticles and copper**
153 **sulphate to *Eisenia andrei* and *Folsomia candida* in field soil with and**
154 **without biosolid amendment**

155

156 **2.1 Abstract**

157 As engineered nanomaterials continue to find novel applications and places in everyday
158 uses, assessing their potential risk to the environment remains a challenge. In soil
159 ecosystems, this is particularly true due to the complex nature of soil and, the potential
160 interactions between engineered and natural materials. Copper oxide nanomaterials
161 (nCuO) and copper sulfate (CuSO₄) exposures in soil were compared in two
162 environmentally relevant scenarios: field soil with and without biosolid amendment.
163 Subsequent chronic effects were evaluated for two invertebrate soil species within the
164 exposure scenarios: the earthworm *Eisenia andrei* and the collembolan, *Folsomia candida*.
165 The observed results were evaluated based on both total Cu and extractable Cu²⁺
166 concentrations within the test soils to determine if solubilized Cu²⁺ released from nCuO
167 could explain the observed effects. Ionic Cu²⁺ was measured in KNO₃ soil extracts (using
168 a Cu ion selective electrode) to represent the fraction of solubilized Cu²⁺ available to
169 invertebrates in the soil exposures. Exposure to nCuO in soil had a more pronounced
170 adverse effect on *E. andrei* reproduction when compared to *F. candida* reproduction,
171 irrespective of the source of nCuO exposure (i.e., direct application or application via
172 amendment of spiked biosolids). There were no significant differences in toxicity observed
173 between the nCuO and CuSO₄ treatments for either test species. The results demonstrated

174 that the observed inhibition of reproduction, when exposed to nCuO, was likely attributable
175 to the solubilization and availability of Cu²⁺.

176

177 **2.2 Introduction**

178 Within the past decade, the production of engineered nanomaterials (ENMs) has
179 increased at a rapid pace, with numerous applications being developed for different
180 metallic ENMs yearly. Due to unique properties, such as excellent electrical and heat
181 conduction and extreme weather resistance, copper ENMs are used in a large variety of
182 technologies³⁵. In 2010, the global production of Cu-based ENMs was approximately 200
183 metric tons per year, with the majority of production originating as catalysts, electronics,
184 optical devices and, coatings, paints and pigments³⁶. These uses, along with the
185 incorporation and use of Cu NMs as a biocide for wood preservation (e.g., fence and deck
186 treatments), have led to soils being identified as a major sink for Cu ENMs^{18,36}. In addition,
187 there is evidence showing that metallic ENMs may be concentrated in sewage sludge
188 (biosolids) during wastewater treatment processes^{11,12}. Biosolids are used as a fertilizer on
189 agricultural lands in many regions including across Canada, making their contamination
190 with ENMs a concern from an environmental perspective. The evaluation of the risk that
191 metallic ENMs may pose to the soil environment has been presented in the literature as a
192 complex problem that depends on the physical and chemical properties of the ENM and
193 specific soil type^{12,2,29}. With regards to Cu ENMs, two possible risk assessment models
194 apply; that toxicity is primarily the result of dissolution and the release of the Cu²⁺ free ion,
195 making traditional risk assessment applicable, or that there is a nano-specific toxicity (i.e.

196 toxicity not attributed to the Cu²⁺ free ion) that may be significant and therefore traditional
197 risk assessment models are not applicable.

198 Despite extensive toxicological research on the soluble form of Cu, we are only just
199 beginning to understand how different soil and ENM characteristics affect interactions
200 between the ENM, soil and soil organisms¹¹. Griffitt et al.²² and Lee et al.²³ suggested that
201 observed toxicological effects were nano-specific; however, recent studies have shown
202 toxicity to be correlated to the extractable soluble form of Cu (i.e., Cu²⁺)^{27,37–39}. With
203 regards to soil invertebrate species, Amorim and Scott-Fordsmand²⁴ demonstrated nano-
204 specific effects on reproduction and avoidance response when *Enchytraeus albidus* were
205 exposed to Cu ENMs (uncoated Cu nanoparticles) in field soil, as exhibited by little to no
206 dissolution of the ENM, relative to Cu salt (CuCl₂). Further studies on *Enchytraeus spp.*
207 have also shown varying response patterns in oxidative stress and differential gene
208 expression between exposure to Cu salt and Cu ENMs²⁹, but no differences in lipid,
209 carbohydrate and protein energy reserves²⁶. In contrast, a study using *Eisenia fetida*
210 demonstrated significant effects on reproduction for CuCl₂ (salt) relative to Cu
211 nanoparticles (NP), and observed oxidation of the Cu NP surface, suggestive of dissolution
212 and release of Cu ions as a contributor of toxicity²⁷. Unrine et al.⁴⁰ observed
213 metallothionein induction upon exposure of *E. fetida* to Cu NP and CuSO₄ in soil, with
214 greater sensitivity to the Cu salt; however, the authors also observed that the test organisms
215 took up oxidized Cu NPs. Gomes et al.²⁹ also detected partial oxidation of Cu NP, but with
216 limited dissolution (based on XANES analysis for oxidized Cu NP), and found that Cu salt
217 (CuCl₂) was more toxic than the Cu NP in *Enchytraeus crypticus* exposed to these
218 substances in a natural soil. Despite growing interest in the effect of metal ENMs on

219 terrestrial systems, ecotoxicity studies on soil dwelling species remain limited relative to
220 aquatic based studies².

221 The present study evaluated the toxic effects of nano-copper oxide (nCuO) to the
222 invertebrate species, *Eisenia andrei* (earthworm) and *Folsomia candida* (collembola),
223 relative to a soluble form of Cu (CuSO₄). The study incorporated the use of a natural field
224 soil in the presence and absence of biosolids in order to examine different exposure
225 scenarios. The study also incorporated the measurement of ion activity (Cu²⁺) within each
226 exposure scenario to determine the influence of available Cu²⁺ on the observed toxicity,
227 and to help identify instances where organism responses exceeded those predicted by Cu²⁺,
228 indicative of a nano-specific effect. The use of two invertebrate species provides a better
229 understanding of how diverse species may be affected, which in turn can inform
230 environmental risk assessment processes.

231

232 **2.3 Material and methods**

233 **2.3.1 Materials**

234 All tests were performed in a field-collected agricultural sandy loam soil from Vulcan,
235 Alberta, Canada; once collected, the soil was air-dried, sieved (4-mm mesh) and
236 homogenized. The general physico-chemical characteristics of the test soils are described
237 in Table 2-1. The biosolids were obtained from a wastewater treatment plant in St.
238 Hyacinthe (Québec, Canada), as a 20% solids cake. The catchment area of the wastewater
239 treatment plant includes small towns and agriculture lands, but no industrial input. The
240 characterization of the biosolids is described elsewhere⁴¹ in brief, the biosolids had a pH
241 of 7.97; total solids content of 46600 mg/kg; and Cu concentration of 6.89 mg/kg. After

sampling, the biosolids were distributed in packages of 500 g wet weight (wt.) and frozen (-20°C) until required for use. Copper sulphate (CuSO₄) was used to test the readily soluble form of Cu and nano copper oxide (nCuO) as a <50 nm powder, was used to test the nano form; both were purchased from Sigma Aldrich Canada.

Table 2-1: Physical and chemical characteristic of test soils. All measurements represent a mean of n=3, except background Cu which is a mean of n=12, (± standard deviation).

	Soil	Soil with biosolid amendment (10 g/kg)
<i>Clay (%) ($\leq 0.002mm$)</i>	14	14
<i>Silt (%) $0.002\text{—}0.050mm$)</i>	19	19
<i>Sand (%) ($> 0.050mm$)</i>	67	67
<i>Organic matter (OM) (%)</i>	2.0 (±0.10)	3.1 (±0.12)
<i>Cation exchange capacity (CEC) (meq/100g)</i>	8.2 (±0.06)	10.2 (±0.84)
<i>pH CaCl₂</i>	5.6 (±0.12)	7.1 (±0.04)
<i>Water holding capacity (%)</i>	43.9 (±1.7)	46.5 (±1.9)
<i>Cu (mg/kg)</i>	9.2 (± 1.5)	11.3 (±1.3)

2.3.2 Nanomaterial characterization

Transmission electron microscopy (TEM) (FEI Technai G2-F20) with energy-dispersive X-ray spectroscopy (EDS) was used to evaluate the purity, initial size and dispersibility in three different matrices: methanol, nanopure water (18.2Ω) and, soil pore-water. The soil pore-water matrix was prepared by saturating a sub-sample of clean field soil with nanopure water (1:1 on a mass – vol. basis), mixed at 35 rpm for 1 hour, and then centrifuged at 8000xg for 30 min; the resulting supernatant was considered as the soil pore-water extract for subsequent analysis. Samples were dispersed in each respective solution by ultra sonication (bath, 30 min.); a drop was placed onto a nickel TEM grid and air-dried

259 in a nano-enclosure for TEM-EDS analysis. TEM analyses revealed that the nCuO material
260 was highly agglomerated in all three solutions (
261 Figure 2-1). The nCuO particles appeared to be somewhat spherical, with diameters
262 generally ranging from 25 to 60 nm, and in some cases up to 100 nm. The agglomerates/
263 aggregates in the methanol and water matrices appeared to be in the range of 100 to 200
264 nm. In the soil pore-water extract, there were much larger particles and aggregates, which
265 were harder to delineate due to the presence of the organic matter. As the nCuO, powder
266 was not readily dispersible in water, nor soil pore-water, it was dry mixed into the soil to
267 ensure better homogeneity of the Cu-spiked samples.

268

269

270 **Figure 2-1:** TEM images of nCuO in methanol (A), nanopure water (B) and, soil pore-
271 water (C and D).

272

273 2.3.3 Soil Spiking

274 The nominal test concentration range used for all tests was 0, 4, 10, 26, 64, 160,
275 400, 1000 mg/ kg based on the parent compound (see Appendix Table A1 and Table A2).
276 This resulted in nominal Cu test concentrations for the nCuO treatments of 13, 18, 61, 138,
277 329 and 809 mg Cu/ kg soil and nominal Cu test concentration for the CuSO₄ treatments
278 of 44, 70, 116, 195, 335, 579, and 1005 mg Cu/ kg soil. Nominal soil Cu test concentrations
279 for treatments prepared with biosolids were 14, 19, 32, 62, 139, 331, 810 mg Cu/ kg soil

280 and 13, 15, 22, 37, 75, 171, 409 mg Cu/ kg soil for nCuO and CuSO₄ respectively.
281 Concentrations were selected in order to capture environmentally relevant levels (i.e. just
282 above background) on the low end and, to try to achieve 50% effects values on the high
283 end. Hereafter, nominal concentrations refer to the expected soil Cu concentration;
284 however, all results presented are based on measured values.

285 For the nCuO tests without biosolids, powdered nCuO was added directly to the
286 soil and mixed using a rotary mixer for 12 h to create a stock soil equivalent to the highest
287 test concentration (i.e., 860 mg/kg [nominal]). Aliquots of the spiked stock soil were then
288 diluted with clean soil to create the test concentration series. Nanopure water (18.2Ω) was
289 then mixed into the spiked soil samples using an electric mixer to raise the soil moisture
290 content to an optimal content of 20% (i.e., 46.5% of the soil's water holding capacity). To
291 create the CuSO₄ test concentrations, CuSO₄ was added directly to the soil from a stock
292 solution (41 g/L) prepared by dissolving CuSO₄ in nanopure water. Each test also included
293 a control treatment, with no added Cu. The mean total measured Cu in each soil treatment
294 can be found in Appendix A (Table A1 and Table A2).

295 For tests that incorporated biosolids, the required wet mass of biosolids was
296 calculated based on the biosolid cake containing 20% solids, and an application rate of 10
297 g dry biosolid per kg soil⁴². Frozen biosolids were sub-sampled into polypropylene
298 containers, sealed and incubated for three days in the dark, at room temperature (23°C),
299 allowing the material to thaw prior to Cu additions. In order to spike the biosolids with
300 nCuO, the amount of powdered nCuO required for each treatment was first added to a small
301 portion of soil (equivalent to 50% of dry mass of biosolid) and dry-mixed on a rotary mixer
302 for 12 h. The nCuO-soil carrier was then added to the thawed biosolids along with nanopure

303 water (equal to 50% of biosolid moisture content), and then sealed and shaken vigorously
304 by hand for one minute. In tests to evaluate the effect of CuSO₄ in biosolid-amended soils,
305 a stock solution (19 g/L) of CuSO₄ was proportionally added to the thawed biosolids. Both
306 nCuO and CuSO₄ spiked biosolids were aged for three days in the dark, at room
307 temperature (23°C), and then proportionally added to clean soil. Nanopure water was
308 mixed in to create the same optimal moisture content as for the tests without biosolids.
309 Each test also included a control treatment, with biosolid amendment, but no added Cu.

310

311 **2.3.4 Toxicity Tests**

312 Invertebrate tests began one day after test soils were prepared to allow time for
313 spiked soils to equilibrate. Chronic soil invertebrate tests followed standardized test
314 methods^{43,44} using taxonomically verified in-house cultures (*Folsomia candida* and *Eisenia*
315 *andrei*). For each test, test vessels were incubated at 20 ± 3°C, with a diurnal cycle of 16 h
316 light (> 800 lux) and 8 h dark. Test endpoints included mean adult survival on d 28, and
317 mean number of juveniles produced at the end of the test (i.e., d 28 for *F. candida* and d
318 56 for *E. andrei*). For the *F. candida* test, 30 g (wet wt.) of test soil were allotted to 125-
319 mL glass vessels, and ten 10-12 d old synchronized individuals were added to each test
320 vessel; the control treatment consisted of five replicates, with three replicates per test
321 concentration. Granulated yeast (Fleischmann's Quick-Rise Instant Yeast®) was provided
322 on d 0 and 14, during which the test vessels were aerated and monitored for moisture
323 content. At the end of the test (i.e., 28 d), the soil from each replicate was flooded with
324 water, enabling the adult and juveniles to float to the top for enumeration. For the *E. andrei*
325 test, 300 g (wet wt.) of test soil was allotted to 500-mL glass vessels, and two sexually

326 mature adults were added to each test vessel; each treatment, including the control
327 consisted of 10 replicates. The mean ($\pm$ standard deviation) adult wet wt. of *E. andrei* used
328 was 465 ± 137 mg for tests without biosolids, and 399 ± 88 mg for tests with biosolids.
329 Earthworms were fed 2 mL of Magic Worm Food[®] (Magic Products Inc., USA) on d 0, 14,
330 28 and 42, during which nanopure water was added to maintain the soil's moisture content.
331 On d 28 of the test, adult *E. andrei* were removed, and the cocoons and juveniles were
332 incubated for an additional 28 d, where after, the soils were manually sifted and the
333 juveniles enumerated on d 56.

334

335 **2.3.5 Chemical Measurements**

336 Sub-samples of test soil were taken at the beginning and end of each test for soil
337 moisture content and pH_{CaCl2} analyses. Triplicate samples were also taken for total Cu
338 (beginning of test only) and extractable Cu²⁺ (beginning and end of test). Total copper was
339 measured by acid digestion (ACS grade nitric acid (5mL of 5% solution) and hydrochloric
340 acid (5mL of 5% solution)) of soil samples (~0.5g) followed by inductively coupled
341 plasma-mass spectrometry (ICP-MS) via a commercial laboratory (Maxxam Analytics,
342 Ottawa, Canada⁴⁵); the reportable detection limit was 0.5 μ g/g. Soil extracts for Cu²⁺ ion
343 measurements were prepared by extracting sub-samples of soil with 0.01 M potassium
344 nitrite (KNO₃) in a 1:5 soil to extractant ratio. The slurry was mixed using a rotary mixer
345 at 35 rpm for 2 h, and the samples were then centrifuged at 8000xg for 30 min. The
346 electrochemical potential and pH were measured in the filtered (0.45 μ m nylon)
347 supernatant of each extract. Extractable Cu²⁺ was measured using an IonPlus series model
348 9629 cupric ion selective electrode (ISE) (Orion Research Inc.) attached to a voltmeter

349 (Accument[®] XL50, Fisher Scientific Inc.). The electrochemical potential in samples was
350 related to Cu²⁺ activity via a calibration procedure previously described in Rachou et al⁴⁶.
351 Briefly, a set of iminodiacetic-buffered Cu solutions (0.1 mM) were prepared wherein Cu²⁺
352 activity was varied in each by adding increasing volumes of 0.1 M nitric acid (HNO₃). The
353 measured pH of each Cu calibration solution was then input into the chemical speciation
354 program, Visual MINTEQ⁴⁷, along with the other solution components, to derive the
355 corresponding Cu²⁺ activities in each solution. The relationship between pH_{KNO₃} and Cu²⁺
356 in the calibration solutions was:

$$357 \quad \log Cu^{2+} = -0.09(pH)^2 - 0.245(pH) - 3.586 \quad (1)$$

358 This relationship was then used to calibrate the probe by relating the measured
359 electrochemical potential measured in each solution to the corresponding Cu²⁺ activity.
360 The probe was re-standardized after every six samples, and all measurements were taken
361 at room temperature (21°C). The method detection limit (MDL) for Cu²⁺ measured in the
362 soil extract was determined by producing six replicate control soil extracts, taking the
363 reading interspersed with other samples. The mean of these six replicate control samples
364 plus three standard deviations of that mean was used to determine the MDL. A MDL of -
365 8.15 mol/L (log Cu²⁺) in the extract was determined.

366 The presence of Cu NPs in the test soil was measured by conducting a side
367 experiment wherein the dispersible Cu NP fraction was extracted from nCuO- and CuSO₄-
368 spiked soil samples using the soil dispersing reagent tetra sodium pyrophosphate (TSPP),
369 according the optimized method described by Schwertfeger et al.⁷ and subsequently
370 analyzed by single-particle ICP-MS (SP-ICP-MS). TSPP has been shown to disperse
371 unbound or loosely bound nanoparticles in the soil solution while limiting dissolution⁷. The

test samples for this experiment were prepared with either nCuO (nominal concentration of 800 mg Cu/kg) or CuSO₄ (nominal concentration of 400 mg Cu/Kg), and sets of samples were prepared with and without biosolid amendment, according to the soil spiking procedures described above (section 2.3.3). The prepared test samples were then aged for three weeks at room temperature in aerated containers while the moisture was kept at 70% WHC. After 21 d, the soil samples were air-dried and re-homogenized by sieving to 1 mm. Sub-samples (0.5 g) were weighed into 50-mL centrifuge tubes, to which 50 mL of 2.5 mM TSPP was added. Samples were mixed on a rotary mixer at 30 rpm for 30 min, followed by further dispersion with an ultrasonic probe (60 watts, OMNI International Sonic Ruptor 250 ultrasonic homogenizer, micro-titanium tip, 20 kHz) for 1.5 min. Gravimetric sedimentation (18 h, 20°C) was used to settle out large particles and subsamples were taken between 0.5 to 1.0 cm below the extract surface using a micropipette in order to avoid particles > 1 µm, as estimated using Stoke's law⁴⁸. Subsamples were diluted 100x in Nanopure water and analyzed the same day by SP-ICP-MS using a NexION 300D ICP-MS (PerkinElmerTM) operating in single particle mode with the SyngistixTM Nano-Application module (PerkinElmer Inc., Waltham, MA). Instrumental and data acquisition parameters of the analysis are provided in Table 2-2. Transport efficiency was determined by the particle size method⁴⁹ using a 60 nm Au suspension (NIST Reference Material 8013). Dissolved Cu calibration was performed using solutions ranging from 0.2 to 10 µg/L prepared using ASA standard Cu solution (1% HNO₃). Large dissolved analyte concentrations can interfere with particle analyte intensities; therefore, the analysis followed the dilution series approach described in Schwertfeger et al⁷. While SP-ICP-MS measures the amount of Cu mass in discrete particles, particle size can only be accurately

estimated if geometry and elemental composition (i.e., mass fraction) of the particles are known. Because these parameters are not known for the Cu-containing nanoparticles in the soil extracts, the data were processed using a default spherical shape and Cu mass fraction of 1 to calculate an equivalent mono-metal particle size, denoted with the subscript “eqv” (e.g., size_{eqv}) in order to facilitate understanding the amount of mass detected in the particles. The method detection limit for dissolved Cu was calculated as 0.3 µg/L, which translated to a particle size detection limit of approximately 20 nm.

Table 2-2: SP-ICP-MS operational parameters.

Parameter Group	Parameter	Parameter Value
ICP-MS component	ICP-MS model	Perkin Elmer NexION 300D
	Nebulizer	Low pressure PFA nebulizer
ICP-MS settings	Nebulizer gas flow	1.04 L/min
	Plasma RF power	1600 W
	Pulse Stage Detector Voltage	1049 V
	Sample flow rate	0.270 mL/min
	Analyte monitored	⁶³ Cu
	Sample introduction	Manual, with a 60 s rinse and 20 s read delay
SP Settings	Transport efficiency method	Particle size method, using Au standards 30 nm and 60 nm (NIST)
	Transport efficiency	9% to 10%, determined daily
	Instrument dwell time	50 µs
	Sample analysis time	60 s, or more
	Readings per sample	~ 1.2 x 10 ⁶ (or more)

2.3.6 Statistics

Statistical treatment of data was conducted according to the Environment Canada Guidance Document on Statistical Methods for Environmental Toxicity Tests⁵⁰. Toxicity estimates are reported based on measured total Cu and extractable Cu²⁺ concentrations.

409 Measured total Cu and Cu²⁺ concentrations were log-transformed for statistical analysis.
410 Significant effects were determined with ANOVA where $p > 0.05$ indicated no difference
411 among treatments. Quantitative data (juvenile production) were analyzed by nonlinear
412 regression using SYSTAT 13: of the models assessed, the logistic model yielded the
413 smallest residual mean square error, and was selected as the model of best fit in all cases
414 based on total Cu concentration. When the juvenile production data was assessed based on
415 extractable Cu²⁺ data, either the logistic or gomertz model were selected as model of best
416 fit (Appendix A: Table A3). Assumptions of normality of homoscedascity of residuals were
417 assessed using the Shaprio-Wilks and Levene's tests, respectively. The *E. andrei* data did
418 not meet one or both model assumptions (depending on the test); however, the results from
419 regression analysis were reported regardless, in order to prevent the less preferred linear
420 interpolation method of analysis, whereby only a portion of the data are used to derive the
421 test endpoints⁵¹. Concentrations inhibitory (ICx) to reproduction were determined at 10, 25
422 and 50% effect levels, with upper and lower 95% confidence limits.

423

424 **2.4 Results and discussion**

425 **2.4.1 Toxicity Tests**

426 Despite differences in Cu doses across treatments as described in the section 2.2
427 and Appendix A (Table A1 and Table A2), the recovery of Cu was acceptable with greater
428 recovery attained in soils without biosolid amendment. Mean Cu recovery was 99% (SD =
429 8.3) for CuSO₄ and 101% (SD = 7.9) for nCuO in soils without biosolid amendment. In
430 soils amended with biosolids, the recoveries were 84% (SD = 12.2) and 91% (SD = 12.2)
431 for CuSO₄ and nCuO, respectively. Test validity criteria^{43,44} were met for all endpoints and

both species in all tests. The derived toxicity estimates for inhibition of reproduction, based on measured total Cu is provided in Table 2-3. Both *F. candida* and *E. andrei* adult survival were unaffected by exposure to nCuO ($p > 0.17$), regardless of biosolid amendment (Appendix A: Table A4 and Table A5). The lack of significant effects in adult earthworm survival correspond with other studies conducted with concentrations of nCuO in soil ranging up to 1000 mg Cu/ kg^{27,52,53}. For earthworms exposed to CuSO₄ in soils without biosolid amendment, there was an effect on adult survival at concentrations > 678 mg Cu/ kg only. *E. andrei* reproduction was the most sensitive endpoint overall, with the order of toxicity (from most to least toxic) as follows: CuSO₄ (with biosolids) $>$ CuSO₄ (no biosolids) $\approx$ nCuO (no biosolids) $>$ nCuO (with biosolids) (Figure 2-2). However, given the overlap in 95% confidence limits, the difference in reproductive toxicity between biosolid and non-biosolid exposures was not significant (Table 2-3). Similar trends were evident, concerning species and endpoint sensitivity when analyzed based on Cu²⁺ (Appendix A: Table A3); also with no discernable difference in toxicity between the Cu forms detected. These results suggest that available Cu²⁺ released in the test soils account for the observed toxicity. As such, discussion of the reproductive inhibition in *E. andrei* is addressed in context of extractable Cu²⁺ below in section 2.4.2.

Table 2-3: Reproductive inhibitory concentrations (IC) at 10, 25 and 50% effect levels. Estimates produced with logistic model using measured total Cu in soil with and without biosolids for *E. andrei* and *F. candida* exposed to nCuO and CuSO₄ with upper and lower 95% concentration limits in parentheses.

IC level (%)	with biosolids			without biosolids		
	50	25	10	50	25	10
<i>E. andrei</i> (µg/g)						
nCuO	149 ^a (111-200)	102 ^a (66-159)	72 ^a (38-138)	119 ^{a,b} (73-193)	73 ^{a,b} (37-145)	47 ^{a,b} (19-120)
CuSO ₄	98 ^{a,b} (59-119)	53 ^{a,b} (27-104)	35 ^{a,b} (14-87)	124 ^{a,b} (85-179)	98 ^{a,b} (56-171)	79 ^{a,b} (34-185)
<i>F. candida</i> (µg/g)						
nCuO	c	c	c	c	c	c
CuSO ₄	c	c	c	490 (380-646)	324 (219-490)	219 (123-398)

^a Data not homogenous ($p < 0.01$)

^b Data not normal ($p < 0.05$)

^c No significant effect ($p > 0.23$)

F. candida was relatively insensitive to copper, with no significant adverse effects observed for any endpoint in nCuO tests with and without biosolids, and reproductive effects evident only at higher concentrations of CuSO₄ in soils without biosolid amendment (i.e., > 678 mg/kg). Very little research is available on the effects of Cu nanomaterials on springtails, even though they are a model organism for soil ecotoxicology studies. Recent research examined the effects of various engineered nanomaterials including nCuO on *F. candida* and found that neither survival nor reproduction were impacted by soil (LUFA 2.2) exposures to a very high nCuO (15-20 nm) concentration (6400 mg/kg) over four generations⁵². In another study, when exposure to nCuO was compared to the soluble Cu form (CuCl₂), the number of juveniles produced was reduced; the concentration of total Cu in pore-water extract was up to 3x higher relative to that observed for the nCuO, suggesting that observed effects were due to soluble Cu⁵². Studies on non-nano forms of Cu have shown that *F. candida* are less sensitive to Cu compared to other soil invertebrates (e.g., *E.*

469 *crypticus*, *E. andrei*)^{54,55}, and that effect levels for survival and reproduction range from
 470 EC50s of 2270 mg Cu/kg⁵⁶ to no effect at concentrations greater than 750 kg/ha⁵⁵. The
 471 difference in the biological response of these two organisms may explain the difference in
 472 sensitivity observed herein. It is known that earthworms will accumulate metals in
 473 chloragocytes, which are then slowly eliminated^{57,58}. However, springtails eliminate metals
 474 from the gut by merocrine and holocrine secretion⁵⁹. The route of exposure to springtails
 475 is primarily through dermal absorption of soil pore water. Springtails regularly moult as
 476 they grow; this shedding of their exoskeleton may also serve as a way to remove toxicants
 477 from their bodies.

478

480 **Figure 2-2:** Dose-response relationships of *E. andrei* reproductive inhibition based on
 481 total measured soil Cu (left) and extractable Cu²⁺ (right). Mean *E. andrei* juvenile
 482 production (relative to control, $\pm$ standard error) exposed to nCuO and CuSO₄ in soil
 483 with and without biosolid amendment.

484

485 2.4.2 Extractable Cu²⁺ and nano Cu

486 The mean extractable Cu²⁺ activity and corresponding pH_{KNO3}, as measured at the
 487 beginning and end of each test are presented in Appendix A (Table A1 (*E. andrei*) and

488 Table A2 (*F. candida*)). Extractable Cu^{2+} was evaluated within this study to determine
489 whether Cu^{2+} activity could explain the observed toxic response between a readily soluble
490 form of Cu (i.e., CuSO_4) and a nano-form (i.e., nCuO) in soil. In soils, it has been suggested
491 that the toxicity to soil organisms may be predicted based on the dissolution, transformation
492 or stability of the metallic nanomaterial^{60,61}. The rate of transformation or dissolution of
493 metallic nanomaterials in soils can be difficult to predict, as there are many variables to
494 consider such as the nanomaterial itself (e.g., size, coating, etc.), as well as the environment
495 (e.g., pH, % OM, cation exchange capacity (CEC), clay content) within which it
496 resides^{12,61}. Moreover, a lack of soil-based studies investigating these variables adds to the
497 difficulty in predicting nanomaterial transformations². For nCuO, dissolution would
498 increase the release and bioavailability of Cu^{2+} , which would elicit Cu^{2+} toxicity when
499 exceeding the tolerable threshold limit for an organism. Extractable Cu^{2+} increased with
500 increasing total Cu concentration at both test start (d 0) and test end (d 28 and d 56 for *F.*
501 *candida* and *E. andrei*, respectively) (Figure 2-3). Based on extractable Cu^{2+} , a difference
502 in Cu^{2+} activity was noted in tests with biosolid amendments, whereby Cu^{2+} concentration
503 increased after at least four weeks (i.e., *F. candida* tests) of aging in test soil (Figure 2-3).
504 In the absence of biosolids, the Cu^{2+} activity remained relatively consistent over the test
505 duration for both the earthworm (56 d – Figure 2-3) and collembolan (28 d –Figure 2-3)
506 tests.
507

Figure 2-3: Mean ($\pm$ standard error) log measured extractable Cu^{2+} concentrations soil extracts relative to total measured Cu in soils with (left) and without (right) biosolid amendment in *E. andrei* (top) and *F. candida* (bottom) tests.

Despite the measured differences in Cu^{2+} activity, no significant difference in toxicity were observed (based on overlapping confidence intervals) between soils with or without biosolid amendment for either species. McShane *et al.*⁶² described an increase in Cu^{2+} measured in nCuO treated soils over 56 d, similar to our biosolid-amended tests, although their soils had no biosolid amendment. McShane *et al.*⁶² correlated the changes in Cu^{2+} activity to soil pH over time, which we also observed here. By d 56, Cu^{2+} concentrations in exposures without biosolids decreased, which corresponded to an increase in soil pH_{KNO_3} (Appendix A: Table A1 and Table A2), suggesting increased Cu^{2+}

522 fixation and subsequent reduction in the extractable Cu pool. Contrastingly, exposures with
523 biosolids showed increased Cu^{2+} concentrations by 56 d, which corresponded to decreases
524 in soil pH_{KNO_3} (Figure 2-3 and Table A1). The reduction in soil pH drives the dissolution
525 of Cu. Samarajeewa et al.⁶³ have shown that soils amended with biosolids show increased
526 microbial activity and decomposition rates, which in turn acidifies the soil. Since Cu has a
527 strong affinity for organic matter, it is likely that much of the added Cu initially bound to
528 the organic residues in the biosolids were then released via microbial decomposition, or
529 via proton exchange from the increased soil acidity. This happened largely in the CuSO_4
530 treatments, which showed a 28-fold increase in Cu^{2+} concentrations by 56 d, compared to
531 the nCuO treatments, which only showed a 12-fold increase (Table A1). It is possible that
532 some of the nCuO was stabilized by the biosolid residues^{12,61}, thus reducing the initial
533 dissolution of the particle and subsequent pool of organically bound Cu^{2+} .

534 Frequently, the toxicity of metallic nanomaterials in the environment can be
535 explained based on the dissolution of the nanomaterial into its base ionic form and/
536 or, a nano-specific effect⁶⁰. Studies examining the effects of nCuO in the soil
537 environment to date are no different, with some concluding that toxicity observed
538 may be attributed to available Cu^{2+} , and others describing a nano-specific effect. A
539 nano-specific effect would result in an effect on test organisms that is either different
540 from an ionic treatment or could not be explained by measured Cu^{2+} activity in the
541 test media. Since *E. andrei* reproduction was the most sensitive endpoint, this data
542 was used to compare the dose response based on extractable Cu^{2+} and total Cu
543 (Figure 2-2) in order to determine if the extractable Cu^{2+} could explain differences in
544 effects between the nCuO and CuSO_4 treatments. Examining the data in this way shifted

the order of toxicity slightly to nCuO (no biosolids) $\approx$ CuSO₄ (biosolids) > CuSO₄ (no biosolids) $\approx$ nCuO (biosolids). However, once the confidence limits were considered, there was no significant difference in toxicity to *E. andrei* between the two forms of Cu when expressed as Cu²⁺ concentrations (Table A3). The modelled IC_x at the 10th, 25th, and 50th percentile based on concentration of extractable Cu²⁺ for both species are provided in Appendix A (Table A3).

Examples of both conclusions (i.e., toxicity due to nano form vs. release of Cu²⁺ ions) are present in the literature for earthworms, particularly for sub-lethal effects. When *E. fetida* was exposed to either CuCl₂ or nCuO (80 nm) in a sandy loam soil, exposure to CuCl₂ exerted a greater effect on reproduction and adult survival, leading to the hypothesis that toxicity may have been due to the release of Cu ions; however, Cu²⁺ activities were not reported²⁷. In another species of earthworm (*Metaphire posthuma*) exposed to either CuSO₄ or nCuO in a natural soil, an immunotoxic effect was observed, indicative of oxidative stress attributed to a significant decrease in coelomocyte counts compared to the controls³⁹. In this instance, the evidence of oxidative stress was observed in both nCuO and CuSO₄ exposures, with greater effects in the CuSO₄, indicative that effects were likely due to Cu²⁺ activity³⁹. Unrine et al.⁵³ did not observe significant sub-lethal toxicity in *E. fetida* exposed to Cu NP concentrations up to 65 mg/kg; however, they did observe Cu²⁺ ions and oxidized Cu NPs accumulated in earthworm tissues at concentrations $\geq$ 20 mg/kg. This could suggest a nano-specific effect whereby nCuO may be taken up into earthworm tissues and act as a source of Cu²⁺ ions; however, in this instance, the authors found no evidence that nCuO in the tissue was releasing Cu ions⁵³. A study, which compared effects of exposure to nCuO to CuCl₂ in the oligochaete *E. cryticus* over its entire life span (approx.

200 d), found that organisms had shorter lifespan, delayed reproduction and, produced fewer juveniles when exposed to nCuO⁶⁴. The authors concluded that since they could not definitively correlate the observed reproduction effects to measured available Cu²⁺ (using Cu ISE) in soil pore-water over 28 d, a nano-specific effect may have been possible⁶⁴.

SP-ICP-MS results from a side experiment, which was conducted to investigate the potential presence of CuNPs in the exposure media, revealed that nano-particulate Cu was detected in all nCuO and CuSO₄ treatments analyzed. The detection of CuNPs in the CuSO₄ treatments not only illustrates the lack of specificity of the SP-ICP-MS analysis for pristine, engineered NPs, but also the degree to which Cu²⁺ transformed into detectable Cu-containing nanoparticles. As extractable Cu²⁺ was also observed in the nCuO treatments (see extractable Cu²⁺ results above), it is likely that dissolved Cu²⁺ precipitated into solid-phase (i.e., particulate) transformation products in both the nCuO treatments, as well as in the readily soluble CuSO₄ treatments. Overall, there was very little dispersible nano-particulate Cu extracted from samples (i.e., <0.2 % of total soil Cu) (Table 2-4), indicating that much of the added nCuO remained (or further) agglomerated/aggregated at the micron (or larger) size, or was oxidized/solubilized and contributed to the dissolved Cu pool. Of the detectable, dispersible Cu-containing nano-particulates in the extracts, the most frequent sizes (i.e., equivalent pure Cu⁰, spherical diameter) were 55 and 52 nm in the nCuO treatments, (without and with biosolids, respectively) and 47 and 45 nm in the CuSO₄ treatments (without and with biosolids, respectively) (Table 2-4). While the presence of biosolids had little effect on the size of particles extracted, it reduced the overall

amount of CuNPs extracted. Extractable CuNPs were reduced by 38% and 46% (on a Cu mass basis) in the nCuO and CuSO₄ treatments, respectively.

Table 2-4: SP-ICP-MS measurements in soil extracts.

Sample	Measured total Cu (mg/kg)	Most frequent size _{eqv} (nm)	Mean size _{eqv} (nm)	CuNP conc. (part./g soil)	CuNP fraction (% NP _{Cu} /Soil _{Cu})
nCuO	870	55	69	6.49E+08	0.12
nCuO with biosolids	723	52	70	3.61E+08	0.07
CuSO ₄	417	47	58	4.54E+08	0.11
CuSO ₄ with biosolids	498	45	54	3.43E+08	0.06

Figure 2-4: Example of SP-ICP-MS distribution plot from TSPP soil extract spiked with nCuO in biosolids

2.5 Conclusions

The study presented herein provides exposure and effect data for standard soil test species exposed to nCuO and CuSO₄ in two environmentally relevant scenarios (i.e., field soil with and without biosolid amendment) and relates the observed results to both total Cu

602 and Cu^{2+} concentrations within the test soils. This approach showed that *E. andrei*
603 reproduction was more sensitive upon exposure to nCuO when compared to *F. candida*
604 survival and reproduction, irrespective of the source of nCuO exposure (i.e., with or
605 without biosolids) but did not significantly affect toxicity results. Comparison of the effects
606 of a metallic nanomaterial to its ionic counterpart is an approach that has been previously
607 used to determine if the nano form poses a greater risk than its soluble counterpart^{26,41}. As
608 current risk assessment models for metals are based on the premise that ionic activity
609 correlates to level of risk, this study sought to evaluate the application of this approach to
610 nCuO. There was no significant difference in toxicity between the Cu forms and the Cu^{2+}
611 activity in the soil exposures indicated that the observed sub-lethal toxicity was most likely
612 due to the extractable Cu^{2+} , rather than a nano-specific effect. However, the pool of
613 extractable Cu^{2+} changed throughout the exposure duration and depended on the soil
614 exposure scenarios (i.e., with or without biosolids). This finding would indicate that current
615 risk assessment models based on the solubilized metal form might be applicable for Cu;
616 however, time for particle dissolution becomes an important factor to consider for nCuO.
617 Given the sub-lethal effects observed on *E. andrei* reproduction, further investigation into
618 the fate of nCuO in these organisms should be completed to examine whether nCuO is
619 taken up, thereby potentially providing a point source of Cu^{2+} ions within the organism.

Chapter 3: Bioaccumulation and toxicokinetics of nano copper oxide in the earthworm *Eisenia andrei*

3.1 Abstract

With the number of novel applications and increase in routine uses for metal and metal-oxide engineered nanomaterials continuing to increase, the understanding of their potential risk and fate in the environment remains a challenge. In soil ecosystems, this is particularly true due to the complex nature of soil and, the potential interactions between engineered and natural materials. In order to assess the potential of nCuO to bioaccumulate, earthworms (*Eisenia andrei*) were exposed to copper oxide nanomaterials (nCuO) and copper sulfate (CuSO₄) in field soil with and without biosolids. Earthworms were exposed during a 21-day uptake phase, then removed to clean field soil for a 21-day elimination with sampling of soil and tissues throughout in order to estimate the uptake and elimination kinetics of Cu and compare between nano (nCuO) and ionic forms (CuSO₄). Both the biota-to-soil accumulation factor (BSAF) and kinetic bioaccumulation factor (BAF_k) were greater in CuSO₄ exposures both with and without biosolid amendment compared to nCuO. However, the elimination and therefore the calculated half-life was much longer in earthworms exposed to nCuO. Qualitative assessment of nCuO in earthworm tissue was completed through enhanced dark-field hyperspectral imaging to look at the fate in the earthworm tissue and, revealed that most nCuO was associated with the gut or epidermis of the worm.

642 3.2 Introduction

643 The global use of engineered nanomaterials (ENM) continues to increase. While
644 there is a wide range of ENM produced, metallic ENM represent the largest amount
645 produced worldwide⁴. Soils are predicted to be a significant environmental sink for metallic
646 ENM^{4,65}, either through land applications of biosolids contaminated with ENM from the
647 waste water stream¹¹, deposition from the air or, direct contamination via agricultural
648 applications; however, studies examining the fate, toxicity and bioaccumulation of ENM
649 in soils remain relatively rare^{2,14}. Copper ENM have a wide variety of applications
650 including in conductors and semi-conductors, catalysts in the manufacturing of
651 electronics³⁵, fungicides in agriculture, and as wood preservatives⁶⁶. Most recently, the
652 lethal and chronic toxicity of Cu ENM in soils has been investigated for plants⁶⁷, microbial
653 communities and soil invertebrates⁶⁸, although much of the work completed to date has
654 been in artificial substrates^{2,6}, and the need for studies in natural soil is widely recognized⁶⁸.
655 For those studies that have been conducted in natural soil environment, most have
656 presented evidence that Cu ENM are generally less toxic than their bulk or ionic
657 counterparts^{27,39,52,69} (Chapter 2; though there is no consensus on this^{68, 24,69}. Regardless,
658 it is clear that understanding the fate of the Cu ENM in both the soil and exposed organism
659 is needed to understand or assess the risk an ENM may pose in the soil environment.

660 Two proposed modes of ecotoxicity by Cu ENM are: (a) that the ENM interacts
661 with the environmental substrate and through transformation processes, solubilize,
662 releasing Cu²⁺ ions, thus resulting in toxicity similar to that observed from bulk or soluble
663 Cu materials; or (b) that the Cu ENM can be taken up (due to their small size) through
664 regular cell transport processes, and once internalized, release Cu²⁺ ions⁶ (this is sometimes

referred to as the Trojan horse effect³⁰). Either, or, a combination of both fates may be the cause of observed effects on survival, growth and reproduction in soil organisms. Generally, this is tested by comparing the effects of a nano form with a soluble metal salt (e.g. CuSO₄, Cu(NO₃)₂, CuCl₂). In some cases, the amount of dissolved Cu²⁺ is measured in soil pore-water or a soil extract to represent the fraction of Cu²⁺ available to soil organisms⁶⁸. Unfortunately, due to the variety of approaches used to compare between ionic and non-ionic (i.e., nano) effects, it is difficult for risk assessors to compare across studies for the assessment of the potential risk of Cu ENM in the soil environment^{30,68}. A better understanding might be attained if comparisons of the effects of ionic Cu²⁺ and studies on the fate of the ENM in the environment (i.e., soil) is combined with an examination of the fate of Cu ENM in the test organism. Bioaccumulation testing can provide evidence for the potential uptake and elimination of test materials, including the kinetic rates associated with these parameters indicating the potential of trophic transfer. However, with regards to Cu ENM, very few studies have incorporated the use of bioaccumulation with soil invertebrates^{30,70}. Urine et al.⁵³ presented evidence that Cu ENM could be taken up into earthworm tissue when exposed in artificial soil; however, they did not evaluate elimination kinetics. Earthworms are known to internalize metals from their environment⁷¹, and are able to regulate the accumulation of metals to some extent^{72,73}.

The research presented herein examined the uptake and elimination kinetics of nano Cu oxide (nCuO) in the earthworm *Eisenia andrei* when exposed to the substance in a natural soil. Measurements of extractable Cu²⁺ in spiked field soil and in spiked biosolid amended into field soil were compared between nCuO and ionic Cu (as CuSO₄) throughout the metal exposure, or uptake phase, of testing. Models suggest that biosolids are a likely

route of exposure to the soil environment through the waste water stream for metallic ENM including nCuO⁷⁴. The relationship between the BSAFs and modeled kinetic uptake rates between different exposures, were evaluated to determine if extractable Cu²⁺ measurements can predict rates of uptake. Through, enhanced dark-field hyperspectral imaging of earthworm tissues after exposure to nCuO, a qualitative assessment of the fate of nCuO in earthworm tissue was also explored.

3.3 Materials and Methods

3.3.1 Materials

Nano copper oxide (nCuO) and copper sulphate (CuSO₄) were purchased from Sigma Aldrich Canada. Nano CuO was purchased as < 50 nm dry powder and in-house characterization was completed to confirm size, size distribution and aggregation state in water, soil pore-water and biosolid extracts via transmission electron microscope (TEM). Test soil was collected from Vulcan, Alberta, Canada and characterized as a sandy loam (**Error! Reference source not found.**). The collected field soil was air-dried, sieved (< 4 m m), homogenized, and then distributed into 20-L pails and stored at room temperature (~20°) until use. Biosolids were collected from a wastewater treatment facility in St. Hyacinthe, Quebec, Canada which has no industrial inputs (primarily agriculture and residential). The biosolids were collected as a 20% solids cake and are described elsewhere⁷⁵; in brief, the biosolids had a pH of 7.97; total solids content of 46600 mg/kg; and Cu concentration of 6.89 mg/kg. Freshly collected biosolids were distributed into

709 freezer bags and frozen (-20°C) until use. The physical and chemical characteristics of the
710 soil and biosolid-amended soil are described in Table 2-1.

711

712 **3.3.2 Soil Spiking**

713 The nominal test concentration consisted of sub-lethal concentrations (based on
714 previous toxicity experiments) of 6 and 11 mg Cu/ kg soil for CuSO₄ and nCuO tests,
715 respectively. To spike nCuO into soils without biosolids, powdered nCuO was added
716 directly to pre-weighed, air-dried soil and, mixed using a rotary mixer for 12 hours. To
717 create the CuSO₄ spiked soil with no biosolid; 100 mL of a 1.12 g/L stock solution of
718 CuSO₄ in nanopure water (18.2 Ω) was added directly to the soil. For both nCuO and
719 CuSO₄ treatments, nanopure water was then mixed into the spiked soil using an electric
720 mixer to raise the soil moisture content to an optimal content of 20% (i.e., 46.5% of the
721 soil's water holding capacity) and to ensure sample homogeneity. Each test also included
722 control treatments, with no added Cu.

723 For tests that incorporated biosolids, the required wet mass of biosolids was
724 calculated based on the biosolid cake containing 20% solids, and an application rate of 10
725 g dry biosolid per kg soil⁴². Frozen biosolids were sub-sampled into polypropylene
726 containers, sealed and incubated for three days in the dark, at room temperature (23°C),
727 allowing the material to thaw prior to Cu addition. In order to spike the biosolids with
728 nCuO, the amount of powdered nCuO required was first added to a small portion of soil
729 (equivalent to 50% of dry mass of biosolid) and dry-mixed on a rotary mixer for 12 h. The
730 nCuO-soil carrier was then added to the thawed biosolids along with nanopure water (equal
731 to 50% of biosolid moisture content), and then sealed and shaken vigorously by hand for

one minute. In tests to evaluate the effect of CuSO₄ in biosolid-amended soils, 100 mL of a 1.59 g/L solution of CuSO₄ was added to the thawed biosolids. Both nCuO and CuSO₄ spiked biosolids were aged for three days in the dark, at room temperature (23°C), and then added to clean soil. Nanopure water was mixed in to create the same optimal moisture content as for the tests without biosolids. Each test also included control treatments, with biosolid amendment, but no added Cu.

3.3.3 Bioaccumulation testing

Bioaccumulation tests followed the methodology described in the OECD guideline 317⁷⁶. *Eisenia andrei* from taxonomically verified in-house cultures were exposed to contaminated test soil for a 21-day uptake period, and then moved to clean test soil for a 21-day elimination phase. Soils (spiked for the uptake phase and clean for the elimination phase and controls) were prepared as described above (section 3.3.2) and, 100 g (wet wt.) were distributed into 125-mL glass jars. One adult *E. andrei* was added to each test vessel and fed magic worm food (Magic Products Inc., USA) on day 0 and 14 of each uptake and elimination phase. The mean initial wet mass of *E. andrei* was 0.4439 (SD = 0.1194). Test vessels were destructively sampled on days 0, 1, 2, 3, 7, 10, 14, 17 and, 21 in triplicate for soil and tissue analysis in each phase of the bioaccumulation testing. Control treatments were created with soil or soil amended with biosolids (as applicable) for soil and tissue analysis at days 0 and 21 (4 replicates each) for both uptake and elimination phases.

752 Moisture content and $\text{pH}_{\text{CaCl}_2}$ of test soils were also measured on days 0 and 21 for both
753 uptake and elimination phases.

754

755 **3.3.4 Soil and Tissue Analysis**

756 Soil samples were air-dried and then sieved (<1 mm) and homogenized before
757 being divided for either total Cu or extractable Cu^{2+} analysis. Total Cu in soils was
758 measured by acid digestion (ACS grade nitric acid (5 mL of 5% solution) and hydrochloric
759 acid (5 mL of 5% solution)) of soil samples (~ 0.5 g), followed by inductively coupled
760 plasma-mass spectrometry (ICP-MS) via a commercial laboratory (Maxxam Analytics,
761 Ottawa, Canada); the reportable detection limit was $0.5 \mu\text{g/g}$. Soil extracts for Cu^{2+} ion
762 measurements were prepared by extracting sub-samples of soil with 0.01 M potassium nitrite
763 (KNO_3) in a 1:5 soil to extractant ratio. The slurry was mixed using a rotary mixer at 35 rpm
764 for 2 h, and the samples were then centrifuged at $8000\times g$ for 30 min. The electrochemical
765 potential and pH were measured in the filtered ($0.45 \mu\text{m}$ nylon) supernatant of each extract.
766 Extractable Cu^{2+} was measured using an IonPlus series model 9629 cupric ion selective
767 electrode (ISE) (Orion Research Inc.) attached to a voltmeter (Accument[®] XL50, Fisher
768 Scientific Inc.) as described in section 2.3.5.

769 Earthworms sampled during bioaccumulation testing were depurated for 24 h in the
770 dark on a moistened filter paper, then individually frozen at -20°C before being lyophilized
771 (Labconco FreeZone) in preparation for total Cu analysis in tissues. Total Cu in tissues was
772 performed in-house by acid digestion and analysis via ICP-MS. The masses of lyophilized
773 worms were recorded (to 0.001 g), 2 mL of HNO_3 was added to each tube containing a
774 worm and, heated to 110°C for 5 h. The digested tissue was then cooled and the diluted to

15 mL with nanopure water. Analysis for total Cu in earthworm tissue was performed using a Perkin Elmer NexION ICP-MS. Additional replicates were added at the end of the uptake phase (d 21) for earthworm samples to be processed for imaging via enhanced dark-field hyper-spectrophotometry. Earthworms were depurated for 24 h in the dark on moistened filter paper, and then fixed with a 2% formaldehyde in 200 mM phosphate buffer (pH 7.2) solution. Three millimetre sections from the posterior of each preserved earthworm were sampled for processing using a tissue processor (Leica EM TP automatic tissue processor). Briefly, the fixed tissue sections were processed as follows: 70% ethanol (1 h); 95% ethanol (1 h); 4 x ethanol (increasing holding time from 1 – 2 h); 2 x xylene (1 h each); 2 x paraffin wax (1 h each). Processed tissues were embedded in paraffin blocks and sectioned to 5 μ m using a Leica UC7 ultra-microtome. Sections were stained using hematoxylin and eosin, and mounted on polystyrene slides with a glass cover slip. Prepared sections were analyzed with CytoViva enhanced dark-field hyperspectral imaging. A spectral library was created using clean nCuO material and control earthworm sections (i.e., not exposed to nCuO), such that spectrum could be matched to the nCuO material used in testing with the spectra of earthworm tissue being removed as background. This method was used to qualitatively determine if nCuO could be observed in earthworm tissues.

3.3.5 Statistics

All statistical analysis was performed using SYSTAT (version 13). Statistically significant changes ($p \leq 0.05$) in earthworm mass, total Cu concentration in tissues and soils as well as the biota-to-soil accumulation factor (BSAF), during the uptake and elimination phases, were determined using single factor analysis of variance (ANOVA)

798 with a Fisher's Least Significant Difference (LSD) post hoc test or; in the event that the
 799 data did not meet assumptions of homogeneity and normality, the Kruskal-Wallis test was
 800 used. Earthworm growth rates were determined by linear regression of the natural
 801 logarithm (ln) of fresh mass versus time (in days). The BSAF (g dry soil g⁻¹ dry tissue) was
 802 determined at each sampling date throughout the uptake phase as the ratio of Cu measured
 803 in tissue and soil:

$$804 \quad \text{BSAF} = C_w(t) / C_s(t) \quad (2)$$

805 Where C_w is the total Cu concentration in the tissue (µg g⁻¹ dry tissue) at time t (d),
 806 and C_s is the total Cu concentration in soil (µg g⁻¹ dry soil) at time t (d). Steady state
 807 (BSAF_{ss}) was considered achieved when there were no significant changes in total tissue
 808 Cu concentration over three consecutive sampling points. The BSAF_{ss} was calculated as
 809 the mean BSAF across the non-significantly different consecutive sampling points from
 810 the time steady state was achieved. The kinetic bioaccumulation factor (BAF_k) was
 811 calculated using a first-order one-compartment kinetic model (equation 3) to estimate the
 812 kinetic uptake and elimination rates, and then using equation 4 to determine the BAF_k
 813 constant (µg g⁻¹ dry tissue d⁻¹)

$$814 \quad C_w(t) = [k_u/k_e \times C_s(0)] \times [1 - e^{-k_e \times t}] \quad (3)$$

$$815 \quad \text{BAF}_k = k_u / k_e \quad (4)$$

816 Where k_u is the kinetic uptake rate constant (µg g⁻¹ dry tissue d⁻¹), k_e is the kinetic
 817 elimination rate constant (d⁻¹), and $C_s(0)$ is the initial chemical concentration in the soil
 818 (i.e., day 0). If a significant change in the mass of earthworms was observed, the resulting
 819 growth rate was added to equation 2, resulting in the following:

$$820 \quad C_w(t) = [(k_u \times C_s(0)) / (k_e + g)] \times [1 - e^{-(k_e + g) \times t}] \quad (5)$$

821 Where g is the growth rate (d^{-1}), calculated as the rate of change in mass over time.

822 The earthworm tissue data for the elimination phase were fit to a first-order one-
823 compartment kinetic elimination model:

$$824 \quad C_w(t) = C_w(0) \times e^{-k_d \times t} \quad (6)$$

825 Where $C_w(0)$ is the chemical concentration within earthworm tissue at the start of the
826 elimination phase and k_d is the depuration rate constant (d^{-1}). The depuration half-life of
827 the chemical substances in the earthworms ($t_{1/2w}$; day) was then calculated using:

$$828 \quad t_{1/2w} = \ln(2)/k_d \quad (7)$$

829

830 **3.4 Results and Discussion**

831 **3.4.1 Soil Analysis**

832 Total Cu analysis confirmed a consistent homogenous ($p \geq 0.05$) exposure of Cu
833 throughout the 21-day uptake phase for all treatments (Figure 3-1). Background level only
834 concentrations of total Cu were measured and found to be consistent throughout the 21-
835 day elimination phase. $CuSO_4$ (no biosolids) was the only exception, where there was
836 significant variation in soil total Cu concentration over time ($p < 0.04$), the elimination of
837 outliers from this data did not significantly change the variation. The variation observed in
838 the elimination phase of the $CuSO_4$ (no biosolids) test included a significant increase in
839 total Cu measured in soils after elimination on day 7. This may be due to the elimination
840 of Cu from earthworms into soil⁷³ (Figure 3-3). Though this phenomenon was not observed
841 in any of the other treatments, the $CuSO_4$ (no biosolids) treatment had the fastest
842 elimination rate with an estimated half-life of 6.5 days (Table 3-1), which may suggest that
843 the *E. andrei* are able to regulate Cu in this exposure scenario. All bioaccumulation and

kinetic rate calculations were completed using data that was corrected for background concentrations and with outliers removed (ANOVA $p < 0.05$).

Figure 3-1: Mean ($\pm$ SD) measured total Cu concentration (ug/g) soil (with and without biosolid amendment).

The extractable fraction of Cu was measured in KNO_3 soil extracts to represent the extractable pool of Cu^{2+} solubilized from the nCuO and CuSO_4 . In general, for treatments in which Cu was added directly to the soil (i.e., no biosolids), extractable Cu^{2+} was consistent over the 21-d uptake period with no significant differences observed for either nCuO or CuSO_4 treatments ($p > 0.07$) (Table B 1). However, when Cu was spiked into biosolids, significant differences were observed for both forms of Cu ($p < 0.01$), with greater variability observed in the nCuO treatments (Figure 3-2). If only start and end times are considered (as is frequently the case in other studies, wherein the available ionic form is measured^{16,77,78}), the addition of biosolids reduced the bioavailability of both nCuO and CuSO_4 , likely due to interactions with organic matter in the soil preferentially binding the Cu^{2+} making it less available³³. However, by looking at the extractable Cu^{2+} throughout the

uptake phase, when nCuO was added to the soil via spiked biosolids, we observed that the extractable Cu^{2+} increased by day 7, but then decreased by day 21, becoming equivalent to the amount of extractable Cu^{2+} from nCuO in the soil (no biosolids) (Figure 3-2). Sekine et al.¹⁶ used x-ray absorption near edge spectroscopy (XANES) to show that over the short term (i.e., 0 - 5 days), the transformation of nCuO and Cu salt ($\text{Cu}(\text{NO}_3)_2$) in soils was dependent on the initial form of Cu and soil properties (e.g., pH, SOM), but after four months, none of these factors influenced the form of Cu observed, since the spectra of both nano and bulk Cu in several soil types looked nearly identical. Similarly, we observed differences in the measured amount of soluble extractable Cu^{2+} from the various treatments (Figure 3-2) at the beginning of the uptake phase, which may be due to differences in solubility between nCuO and CuSO_4 , changes in soluble organic matter (SOM) from the addition of biosolids but, for nCuO at least, the amount of Cu^{2+} extracted from the soil was nearly the same between treatments by d21. However, for the CuSO_4 treatments the measured extractable Cu^{2+} remained consistent throughout the uptake phase, with more Cu^{2+} available in treatments without biosolid amendment, indicating that 21 days may not be time for the soluble salt equilibrate with the increased SOM associated with being spiked into biosolids. If the accumulation of Cu in tissue can be predicted based on available Cu^{2+} in the soil matrix, it would be expected that the CuSO_4 added directly to the soil (i.e., no biosolids) would produce the greatest accumulation rate and, the greatest amount of extractable Cu^{2+} (Figure 3-2) was measured here. As of the end of the uptake phase, based on extractable Cu^{2+} measurements, the prediction of Cu accumulation in earthworms was

883 CuSO_4 (no biosolids) > nCuO (no biosolids) $\approx$ nCuO in biosolids > CuSO_4 in biosolids
 884 (Figure 3-2).

886
 887 **Figure 3-2:** Mean ($\pm$ SE) available Cu^{2+} measured KNO_3 soil extracts throughout uptake
 888 phase.

890 3.4.2 Bioaccumulation

891 Based on total Cu measurements in tissues and soils, there was less Cu accumulated
 892 in treatments where worms were exposed to nCuO compared to CuSO_4 with the following
 893 BSAF_{SS} : CuSO_4 in biosolids (2.25) > CuSO_4 (1.33) > nCuO (0.74) > nCuO in biosolids
 894 (0.62) (Table 3-1). The Cu in earthworm tissues reached steady state as early as day 4 when
 895 exposed to CuSO_4 without biosolids and, as late as day 17 when exposed to CuSO_4 with
 896 biosolids; accumulated Cu reached steady state by day 14 in both nCuO treatments (Table
 897 3-1). An evaluation of published data to 2013 demonstrated log BSAF values for
 898 earthworms exposed to Cu ENMs ranging from -0.33 to 0.27³⁰; our results with the nCuO
 899 exposure scenarios fall within range with log BSAF_{SS} values of -0.21 to 0.35. Typically, a

BSAF value greater than one (log 0) is indicative that a substance is bioaccumulative, as it demonstrates a greater amount of substance in the animal (i.e., tissue) relative to the exposure environment (i.e., soil), however, this value does not consider the kinetic uptake and elimination rates.

Table 3-1: Bioaccumulation summary including calculated kinetic rates.

Treatment	Steady State (day)	BSAF _{ss} ¹ (SE)	K _u ² (LCL – UCL) ⁷ (µg Cu /g dry tissue/ day)	K _e ³ (LCL – UCL) ⁷ (/day)	BAF _k ⁴ (g dry soil/ g dry tissue)	K _d ⁵ (LCL – UCL) ⁷ (/day)	T _{1/2} ⁶ (day)
nCuO in biosolids	14	0.62 (0.06)	0.15 (0.04-0.39)	0.19 (-0.03 – 0.54)	0.80	0.003 (-0.02 – 0.03)	231.05
nCuO	17	0.74 (0.00)	0.08 (0.02 – 0.14)	0.10 (-0.04 – 0.24)	0.81	0.03 (0.02-0.05)	21
CuSO ₄ in biosolids	14	2.25 (0.53)	1.90 (-0.57 – 4.37)	0.82 (-0.6 – 2.24)	2.31	*	*
CuSO ₄	4	1.33 (0.49)	2.15 (-5.35 – 9.66)	1.92 (-4.99 – 8.83)	1.12	0.11 (0.04 – 0.17)	6.48

* Values could not be reliably calculated due to no significant depuration of Cu from tissue.

¹ Biota-to-soil accumulation factor at steady state (BSAF_{ss})

² Kinetic uptake rate (K_u)

³ Kinetic elimination rate (K_e)

⁴ Kinetic bioaccumulation factor (BAF_k)

⁵ Depuration rate constant (K_d)

⁶ Half life of Cu in tissue (T_{1/2})

⁷ 95% lower confidence limits (LCL) and upper confidence limits (UCL)

The calculated kinetic bioaccumulation factors (which takes into consideration uptake and elimination rates) produced the same pattern as the BSAF_{ss} with the CuSO₄ treatments demonstrating greater bioaccumulation potential and, the two nCuO treatments demonstrating a lower, but similar, BAF_k (Table 3-1). The kinetic uptake rate of Cu to earthworm tissue was greatest in CuSO₄ without biosolids (K_u = 2.15), as might be predicted by the measured available Cu²⁺ in KNO₃ soil extracts (see section 3.4.1). The

uptake and elimination rates observed here fall within previously reported (0 – 0.191 $\mu\text{g/g/day}$) values for bulk copper in a variety of soils⁷². Uptake and elimination rates did not seem to be affected by the presence of biosolids (Table 3-1), however, when the kinetic uptake curves are estimated based on extractable Cu^{2+} measurements, less Cu is accumulated when biosolids are the route of exposure (Figure 3-3). Unfortunately, as Cu^{2+} could not be measured in earthworm tissues, elimination rates and therefore BAF_k or BSAF could not be reliably calculated based on Cu^{2+} concentrations. Based on kinetic uptake and elimination rates, the elimination of Cu from tissue was greater than the uptake rate when organisms were exposed to nCuO; and, the elimination rates were lower relative to uptake for the CuSO_4 treatments (Table 3-1, Figure 3-3) resulting in bioaccumulation of Cu in earthworms exposed to CuSO_4 (i.e., $\text{BAF}_k > 1$). Based on the available Cu^{2+} , there was also a lower uptake rate in earthworms exposed to nCuO compared to CuSO_4 (Figure 3-3). These results seem to support the hypothesis that the greater the amount of soluble Cu^{2+} in the soil solution (assuming that CuSO_4 represents a completely soluble form), the greater the rate of uptake by earthworms. Since CuSO_4 is meant to represent the soluble form of Cu within the test media, the observation of the greatest uptake rate and BAF_k is as expected. However, based on the measured available Cu^{2+} (Figure 3-2), CuSO_4 in biosolids should have the lowest available Cu for uptake thus having the lowest extractable Cu^{2+} . Therefore, the total Cu measurements in uptake soil was a better predictor of total Cu in tissue and hence, bioaccumulation (Figure 3-1 and Figure 3-3). In earthworms however, the exposure route can be both through soluble forms in (e.g., dermal absorption through

soil pore water), as the extractable Cu^{2+} represents or, through ingestion of soil and food containing contaminants (i.e., nCuO particulates).

944

945

946

Figure 3-3: Modelled kinetic uptake and elimination curves based on total Cu (solid lines) and extractable Cu^{2+} (dashed lines) in soil and, measured total Cu in tissues (♦).

949

950 3.4.3 Earthworm Tissue

Based on the results of the bioaccumulation tests, earthworms exposed to nCuO in soil with or without biosolids did not bioaccumulate Cu to a significant extent. When the elimination rates are examined, Cu was eliminated from earthworms exposed to the nCuO treatments at a much slower rate relative to CuSO_4 , with half-lives ($t_{1/2}$) of 231 (with

955 biosolids) and 21 (without biosolids) days. Other authors have presented evidence that
956 despite lower or no difference in the BSAF of metallic ENM compared to their bulk
957 counterparts, there may be uptake of the ENM into soil invertebrate tissues³⁰. If ENM are
958 within tissue, it may result in a slow release of the base material (i.e., Cu²⁺) and prolong or
959 delay the potential for toxicity, sometimes referred to as the Trojan horse effect².

960 To determine if nCuO was taken up into earthworm tissue throughout the uptake
961 phase of this study, tissues from day 21 of the nCuO treatment without biosolid amendment
962 were preserved and processed for analysis with enhanced dark field hyperspectral imaging.
963 Through this method, contaminated tissue samples were compared with control tissues and
964 with the nCuO material on its own (i.e., pristine), resulting in images in which only areas
965 that contain the spectrum matching nCuO are highlighted (Figure 3-4). The resulting
966 images provide evidence that nCuO was taken into *E. andrei* tissue intact, with nearly all
967 associated with the gut or epidermis (i.e., C and D - Figure 3-3 and Figure 3-4) when
968 compared to muscle (Figure 3-3 and Figure 3-4 – B) or chloragogenous tissues (Figure 3-3
969 Figure 3-4 – A), these areas were looked on as they are the most likely tissues to accumulate
970 metals. Copper ENM have previously been shown to associate with epidermis cells in the
971 earthworm *E. fetida* when exposed to nCuO in a standard reference field soil (Lufa 2.2)⁷⁸.
972 Despite depurating, the earthworms for 24 h, processing the tissue for thin sectioning and
973 embedding in paraffin wax particles of soil and nCuO were still observed in the gut of the
974 worm in the study presented here. It has previously been demonstrated that 24 h should be
975 sufficient to depurate Cu from contaminated *E. andrei*, but that there is no way to
976 completely eliminate all soil particles (and therefore, associated nCuO) from the animals⁷³.
977 If the nCuO were accumulating in earthworm tissue through the gut, it would likely be

978 associated with the chloragogenous tissue as this area has been shown to accumulate metals
979 from the environment⁷¹. Although several sections ($n \geq 3$) of each worm (i.e., 2 replicates)
980 sampled were examined, with similar findings, unfortunately, additional samples (e.g.
981 other time points, treatments, etc.) were not examined due to time limitations on the
982 instrument. Despite the small sample size, there were matches to the nCuO spectral library
983 in the muscle and chloragogenous tissues (Figure 3-3), which suggests that a small fraction
984 of the nCuO may be accumulated within internal earthworm tissues. Others have also
985 presented evidence that metallic ENM can be taken up into earthworm tissue^{30,79}. Unrine
986 et al.⁴⁰ found evidence of Cu nano-particles in earthworm gut and muscle tissue after
987 bioaccumulation exposures of 20, 40 and < 100nm Cu in artificial soil via scanning x-ray
988 fluorescence micrographs. Another study that looked at the effects of aging and coatings
989 on nCuO bioaccumulation in earthworms found nCuO particles in the gut and epidermis in
990 histology samples⁷⁸. The uptake of other metallic ENM in soil invertebrates have been
991 investigated to some extent^{30,79}, however there is a lack of understanding of the potential
992 risk of trophic transfer in general, particularly in different soil conditions.
993

Figure 3-4: Hyperspectral images of d-21 earthworm tissues (nCuO no biosolid) with, spectra matching nCuO highlighted in red. A – chloragogenous tissue; B – muscle tissue; C – epidermis; D – gut.

1000

1001 **Figure 3-5:** Example of *E. andrei* section used for enhanced dark-field hyperspectral
 1002 microscopy, with region of sections marked (A-D) corresponding to the images in Figure
 1003 3-4 (A – chloragogenous tissue; B – muscle tissue; C – epidermis; D – gut).

1004

1005 **3.5 Conclusions**

1006 By examining the bioaccumulation of nCuO in a field soil through two
 1007 environmentally relevant exposure scenarios (i.e., with and without biosolids) and
 1008 comparing with a soluble Cu salt (i.e., CuSO₄) this study demonstrates that the risk for
 1009 nCuO to bioaccumulate is not greater than bulk or ionic form of Cu. It was hypothesized
 1010 that extractable Cu²⁺ could predict the amount of Cu taken up by earthworms and hence

1011 the biota-to-soil accumulation factor (BSAF), however this was found not to be true.
1012 Though the presence of biosolids did not significantly affect the estimated BSAF or kinetic
1013 bioaccumulation rates (BAF_k) for either nCuO or CuSO₄, it did influence the stability and
1014 concentration of measured Cu²⁺ in uptake phase soil extracts. Earthworms are known to be
1015 able to regulate copper⁷² and evidence of regulation is seen here in the nCuO treatments
1016 through higher kinetic elimination rates compared to uptake rates and the ability to
1017 maintain low levels of internal body Cu concentration. Despite the low (< 1) BAF_k
1018 estimates for nCuO in this study, evidence of nCuO particles taken up into earthworm
1019 tissues was observed by d21, the majority of which was associated with the epidermis or
1020 gut of the earthworm, but a small amount was also seen in muscle and chloragogenous
1021 tissue. This indicates that nano-particles taken up from soils are translocated in *E. andrei*
1022 for storage (over the long-term); unfortunately, the enhanced dark-field hyperspectral
1023 imaging technique used to identify nCuO particles in earthworm tissues is not quantitative.
1024 Future work is required to better understand the effects metallic ENM have once taken up
1025 into tissue and see if these materials pose a risk for trophic transfer, over a longer time-
1026 period or if they are regulated similarly to bulk Cu in earthworms.

1027 **Chapter 4: Conclusion**

1028 The research presented herein aimed to address the following four objectives:

- 1029 1. Determine if nCuO has an effect on the survival and reproduction of *Eisenia*
1030 *andrei* and *Folsomia candida*
- 1031 2. Determine if any observed effects of *E. andrei* and *F. candida* survival and
1032 reproduction can be attributed to the extractable (extractable) Cu²⁺
1033 concentrations in soil.
- 1034 3. Examine the fate of Cu nanomaterial in soil and biosolid-amended soil.
- 1035 4. Assess the bioaccumulation potential of nCuO in *E. andrei* and determine
1036 if Cu nanomaterial is taken up into tissue.

1037

1038 The first three objectives were addressed in chapter 2: “Sub-lethal toxicity of
1039 copper oxide nanoparticles and copper sulphate to *Eisenia andrei* and *Folsomia candida*
1040 in field soil with and without biosolid amendment”. The results found that, although *E.*
1041 *andrei* were more sensitive to nCuO compared to *F. candida*, the effects observed on
1042 reproduction for both species were less than those observed for the bulk form of Cu (i.e.,
1043 CuSO₄). The route of exposure (i.e., spiked directly into test soil or spiked biosolid
1044 amended to test soils) did not significantly change the effects observed on survival or
1045 reproduction for either species. Measurements of extractable Cu²⁺ were completed in
1046 KNO₃ soil extracts at the start and end of each toxicity tests. When levels of effects (i.e.
1047 EC10, EC25, EC50) were looked at relative to the measured Cu²⁺ concentrations, no
1048 difference was observed between the nano and bulk form of Cu indicating that, the soluble
1049 Cu²⁺ is likely a major contributor to the observed sub-lethal toxicity. The Cu²⁺

1050 measurements in combination with measurements of particulate Cu in (TSPP) soil extracts
1051 was used to try to characterize the fate of the nCuO in test soils. Single-particle ICP-MS
1052 was used to measure the mean Cu particle size, size distribution and proportion of Cu nano-
1053 particle to dissolved Cu in soil extracts. The combination of these analyses indicate that
1054 most of the added nCuO is solubilized in the test soils with biosolids having very little
1055 influence in general. This agrees with other authors who have found Cu ENM rapidly
1056 solubilizes in soils⁷⁸; however, a more in-depth study on the rate of dissolution in both
1057 biosolids and field soils would be useful in understanding the long-term fate of Cu ENM
1058 in the environment since nano-particulate Cu was detected in soil extracts after three weeks
1059 of aging.

1060 The fourth objective was addressed in chapter 3: “Bioaccumulation and
1061 toxicokinetics of nano copper oxide to the earthworm *Eisenia andrei*”. Copper was slightly
1062 bioaccumulative to earthworms exposed to CuSO₄ in soils with and without biosolids (i.e.,
1063 BAF_k > 1) and not when exposed to nCuO (either with or without biosolids). Though the
1064 presence of biosolids did not significantly (based on overlapping confidence limits) effect
1065 the uptake of Cu from soil into tissue, the elimination was slower in earthworms exposed
1066 to both nCuO and CuSO₄ via biosolids. Elimination rates of Cu were slower in earthworms
1067 exposed to nCuO, however they were still greater than the uptake rate, which may indicate
1068 that *E. andrei* is able to regulate nano Cu as it does with bulk metals. Both toxicity and
1069 bioaccumulation tests results indicated that *E. andrei* took up Cu from nCuO and although
1070 it was likely mostly solubilized Cu²⁺, the solubilized fraction did not account for all of the
1071 observed effects, suggesting that the nCuO may have been taken-up untransformed. In
1072 order to determine if particles matching the virgin nCuO test material were present in *E.*

1073 *andrei* tissue; enhanced dark field hyperspectral imaging was performed. The results of
1074 this technique indicated that there was indeed material matching the spectrum of the nCuO
1075 and that is was primarily associated with the gut and epidermis of the worm. Objective
1076 three was also addressed in chapter 3 through the measurement of extractable Cu²⁺
1077 throughout the uptake phase of bioaccumulation testing. The use of measured Cu²⁺ data
1078 enabled the modeling of the kinetic uptake of Cu based on the soluble form, which was
1079 compared to modeled uptake based on total Cu (which would include solubilized as well
1080 as bound and particulate Cu forms). The comparison of modelled kinetic uptake curves
1081 showed that exposure through biosolid amendment reduced the uptake of Cu for both nano
1082 and bulk form. When soils were spiked directly, no difference was observed in the uptake
1083 of Cu in CuSO₄ exposures between extractable Cu²⁺ and total Cu measurements. However,
1084 there was less uptake when only solubilized Cu²⁺ is considered compared to total Cu for
1085 nCuO (with no biosolids), indicating that not all of the nCuO is dissolved to available Cu²⁺,
1086 as seems to be the case with CuSO₄.

1087 Though other researchers have noted differences in solubility and availability based
1088 on soil characteristics (e.g., pH, SOM, clay content), there were no other studies found that
1089 have looked at the effects biosolids have as an exposure route for Cu ENM ^{2,10,16,29,30,62}.
1090 Exposure to Cu (either nCuO or CuSO₄) via spiked biosolids did not significantly change
1091 the survival, reproduction or bioaccumulation potential in *E. andrei*. The presence of
1092 biosolids may have buffered against toxicity for the springtails, but in the end, the Cu
1093 (regardless of form) was not toxic to *F. candida*. Nevertheless, the presence of biosolids
1094 did affect the measured available Cu²⁺ in soil extracts from both sets of experiments. For
1095 example, in the toxicity tests there was a greater change in extractable Cu²⁺ between the

1096 start and end of tests (in both *E. andrei* and *F. candida* tests) when Cu was spiked into
1097 biosolids. For the bioaccumulation tests, there was greater variability in the measured Cu^{2+}
1098 through out the uptake phase (particularly for nCuO) when biosolids were present. The
1099 addition of biosolids did not change the percent organic matter in the test soil very much
1100 (i.e., 2.0 % without biosolids and 3.1% with biosolid amendment), and increased the pH
1101 (i.e., from 5.6 to 7.1). Nevertheless, the data presented here suggests that the ability to use
1102 extractable Cu^{2+} measurements to represent the available soluble form of Cu and to predict
1103 toxicity or bioaccumulation potential is likely tied to the soil conditions.

1104 Soil invertebrates represent an important part of the ecosystem, as primary
1105 consumers they play an integral role in organic decomposition and the cycling of nutrients
1106 in the soil environment. As such, soil invertebrates are frequently used in standardized
1107 toxicity studies to evaluate the health of a soil environment or the risk of a substance to the
1108 soil environment. Risk assessors rely on the data produced from these studies when making
1109 regulatory decisions. Comparison of the effects of a metallic nanomaterial to its ionic
1110 counterpart is an approach that has been previously used to determine if a nano form poses
1111 a greater risk than its soluble counterpart^{26,41}. As current risk assessment models for metals
1112 are based on the premise that ionic activity correlates to level of risk, this study sought to
1113 evaluate the application of this approach to nCuO. The toxicity results suggest that the
1114 ionic activity of Cu from nCuO was able to predict the risk to earthworm and springtail
1115 reproductive success. However, the ionic activity could not predict the risk of
1116 bioaccumulation of Cu from nCuO in earthworms. Earthworms are known to be able to
1117 regulate copper⁷² and evidence of regulation is presented here. Future work is required to
1118 better understand the if earthworms regulate metallic ENM in the same way as their bulk

1119 counter-parts, and if metallic ENM taken up can translocate within tissue and if they
1120 represent a risk via trophic transfer.

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1406 **Appendices**

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1408 **Appendix A - Chapter 2 Appendix**

1409 **Table A 1:** Mean ($\pm$ standard error (SE)) measured total Cu, Cu²⁺ activity, and the pH of
1410 the KNO₃ extracts for ISE measurements for the *Eisenia andrei* reproduction assays. NA
1411 = not available

		Measured Concentration									
Nominal Concentration (mg/kg)	Cu dose (mg/kg)	Total Cu (mg/kg)		Test Start (d 0)				Test End (d 56)			
				Ionic Cu (nM)		pH _{KNO3}		Ionic Cu (nM)		pH _{KNO3}	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
nCuO, no biosolids											
0	9.8	10.3	0.4	1.606	0.282	5.76	0.05	0.950	0.252	6.14	0.01
4	13	13.7	0.3	0.867	0.043	5.83	0.02	0.572	0.045	6.12	0.01
10	18	17.0	0.0	2.834	0.200	5.83	0.01	1.336	0.495	6.28	0.04
26	31	31.3	0.3	3.880	0.284	5.79	0.01	0.456	0.018	6.29	0.02
64	61	61.3	0.9	22.925	1.830	5.87	0.02	3.979	0.241	6.09	0.01
160	138	140.0	0.0	138.676	2.990	5.94	0.03	18.811	1.671	6.32	0.04
400	329	340.0	10.0	539.973	35.717	6.14	0.05	253.211	29.436	6.40	0.06
1000	809	830.0	23.1	1216.381	49.252	6.29	0.06	374.737	7.612	6.74	0.05
nCuO, with biosolids											
0	11.3	11.0	0.6	0.481	0.199	6.34	0.02	0.868	0.412	5.99	0.02
4	14	14.3	0.3	0.049	0.009	6.56	0.00	0.497	0.053	5.99	0.00
10	19	19.0	0.0	0.044	0.004	6.64	0.01	1.131	0.218	5.94	0.01
26	32	32.3	0.9	0.063	0.006	6.61	0.03	1.056	0.157	6.10	0.03
64	62	63.3	1.2	3.890	0.012	6.22	0.01	12.987	0.472	5.93	0.03
160	138	143.3	3.3	3.154	0.244	6.63	0.01	55.411	3.218	6.01	0.01
400	331	316.7	3.3	13.497	1.046	6.68	0.01	139.327	4.293	6.27	0.02
1000	810	776.7	31.8	53.071	3.182	6.61	0.01	468.286	80.781	6.33	0.01
CuSO ₄ , no biosolids											
0	9.8	8.8	0.2	2.0	NA	5.87	NA	1.374	1.052	5.86	0.02
87	44	47.3	3.0	15	NA	5.93	0.01	2.696	0.193	6.00	0.01
152	70	69.7	0.9	45	NA	5.75	NA	17.817	1.919	5.88	0.01
267	116	116.7	3.3	82	NA	5.87	0.01	29.007	1.147	5.88	0.02
466	195	210.0	0.0	292	NA	5.75	0.02	90.304	0.726	5.97	0.01
816	335	336.7	16.7	860	NA	5.69	0.03	43.022	2.644	5.80	NA
1429	579	676.7	8.8	4961	NA	5.44	0.01	NA	NA	5.60	NA
2500	1005	990.0	10.0	9621	NA	5.21	0.02	15445.152	318.69	5.45	0.01
CuSO ₄ , with biosolids											
0	11.3	11.0	0.4	0.100	0.027	6.70	0.02	0.544	0.025	6.03	0.02
4	13	11.3	0.3	0.035	0.002	6.85	0.02	0.718	0.049	5.80	0.03
10	15	13.3	0.3	0.046	0.003	6.84	0.01	0.711	0.140	6.09	0.02
26	21	19.3	0.3	0.032	0.006	6.81	0.09	1.006	0.149	5.92	0.04
64	37	31.0	1.5	0.064	0.003	6.68	0.01	3.256	0.431	6.03	0.02
160	75	53.7	1.3	0.233	0.072	6.61	0.02	5.516	0.932	6.19	0.04
400	170	120.0	0.0	2.279	0.198	6.59	0.02	97.742	13.518	5.96	0.02
1000	409	220.0	0.0	58.387	10.519	6.36	0.02	1189.974	132.009	5.87	0.02

1412

1413 **Table A 2:** Mean ($\pm$ standard error (SE)) measured total Cu, Cu²⁺ activity, and the pH of
1414 the KNO₃ extracts for ISE measurements for the *Folsomia candida* reproduction assays.
1415 NA = not available

		Measured Concentration									
Nominal Concentration (mg/kg)	Cu dose (mg/kg)	Total Cu		Test Start (d 0)				Test End (d 28)			
		(mg/kg)		Ionic Cu (nM)		pH _{KNO3}		Ionic Cu (nM)		pH _{KNO3}	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
nCuO, no biosolids											
0	9.8	9.1	0.2	0.941	0.252	6.03	0.01	3.685	1.349	5.72	0.05
4	13	12.5	0.5	1.519	0.356	5.94	0.01	0.734	0.088	5.86	0.02
10	18	18.7	0.3	2.374	0.960	5.89	0.03	1.747	0.686	5.78	0.06
26	31	29.0	0.6	3.426	0.370	5.91	0.03	2.201	0.086	5.85	0.01
64	61	63.7	1.5	22.511	3.350	5.94	0.01	12.592	1.213	5.85	0.01
160	138	146.7	3.3	115.712	8.092	5.92	0.01	128.394	40.642	5.83	0.07
400	329	330.0	10.0	561.784	11.152	6.03	0.07	582.260	14.139	6.06	0.04
1000	809	856.7	27.3	1030.469	37.863	6.26	0.04	1175.801	35.433	6.30	0.03
nCuO, with biosolids											
0	11.3	12.3	1.2	0.013	0.003	6.65	0.02	0.090	0.009	6.24	0.03
4	14	14.0	0.6	0.010	0.000	6.72	0.01	0.011	0.002	6.59	0.01
10	19	16.3	0.3	0.032	0.004	6.64	0.02	0.014	0.003	6.91	0.08
26	32	30.0	0.0	0.014	0.001	6.72	0.01	0.009	0.002	6.93	0.06
64	62	52.7	1.9	0.102	0.002	6.69	0.00	0.125	0.018	6.84	0.03
160	138	84.0	2.1	0.218	0.025	6.67	0.03	0.222	0.016	7.08	0.02
400	331	263.3	3.3	0.738	0.085	6.68	0.04	974.226	82.175	5.70	0.03
1000	810	673.3	28.5	0.920	0.142	6.65	0.03	3362.438	101.321	5.78	0.04
CuSO ₄ , no biosolids											
0	9.8	13.5	0.5	1.957	NA	5.87	NA	NA	NA	5.87	NA
87	44	47.3	3.0	7.736	0.479	5.96	0.02	9.501	1.131	5.72	0.02
152	70	69.7	0.9	23.356	1.351	5.93	0.04	28.287	1.884	5.64	0.01
267	116	116.7	3.3	66.701	4.038	5.85	0.02	54.189	0.842	5.87	0.02
466	195	210.0	0.0	248.029	7.527	5.87	0.00	216.228	NA	5.77	NA
816	335	336.7	16.7	1065.015	14.734	5.66	0.02	895.515	NA	5.68	NA
1429	579	676.7	8.8	5564.773	35.284	5.49	0.01	6348.992	NA	5.43	NA
2500	1005	990.0	10.0	35412.14 2	NA	5.12	NA	34439.4 49	NA	5.21	NA
CuSO ₄ , with biosolids											
0	11.3	10.3	0.4	0.003	0.001	6.91	0.03	0.415	0.119	5.48	0.06
4	13	14.0	0.6	0.001	0.000	6.87	0.02	0.388	0.010	5.39	0.02
10	15	16.3	0.3	0.007	0.001	6.84	0.04	1.397	0.765	5.41	0.01
26	21	30.0	NA	0.003	0.000	6.87	0.04	1.036	0.062	5.38	0.02
64	37	52.7	1.9	0.002	0.000	6.86	0.02	9.032	1.186	5.42	0.07
160	75	84.0	2.1	0.004	0.001	6.82	0.00	26.136	1.129	5.39	0.00
400	170	263.3	3.3	0.547	0.129	6.64	0.04	31.693	8.203	5.78	0.01
1000	409	673.3	28.5	43.253	4.418	6.38	0.02	35.053	5.809	6.52	0.08

Table A 3: Toxicity endpoints based on measured extractable Cu²⁺ for *E. andrei* and *F. candida* exposed to nCuO and CuSO₄ in soil with and without biosolid amendment. Reproduction inhibitory concentrations (IC) are provided for 10, 25 and 50% effect levels with upper and lower 95% confidence limits in parentheses.

<i>IC level (%)</i>	with biosolids			without biosolids		
	50	25	10	50	25	10
<i>E. andrei (nM)</i>						
nCuO	54 ^{a,c} (34-86)	32 ^{a,c} (15-65)	20 ^{a,c} (7-52)	15 ^{a,b,c} (6-37)	7 ^{a,b,c} (2-20)	4 ^{a,b,c} (1-13)
CuSO₄	21 ^{a,b,c} (6-69)	7 ^{a,b,c} (2-21)	4 ^{a,b,c} (1-18)	41 ^{a,b,d} (18-93)	27 ^{a,b,d} (8-95)	18 ^{a,b,d} (3-98)
<i>F. candida (nM)</i>						
nCuO	e	e	e	e	e	e
CuSO₄	e	e	e	1.32 ^c (0.16-2.49)	0.34 ^c (0-0.83)	0.09 ^c (0-0.28)

^a Data not homogenous

^b Data not normal

^c Logistic model

^b Gompertz model

^e No significant effect (p > 0.23)

Table A 4: Mean ($\pm$ standard error (SE)) adult survival, juvenile production, soil pH_{CaCl2} and moisture content for the *Eisenia andrei* reproduction assays.

Nominal Concentration (mg/kg)	Adult Survival (%)		No. of Juveniles Produced		Soil pH _{CaCl2}		Soil Moisture (%)	
	Mean	SE	Mean	SE	Day 0	Day 56	Day 0	Day 56
nCuO, no biosolids								
0	100.0	0.0	50.8	6.8	5.62	5.97	20	23
4	100.0	0.0	57.6	9.4	5.57	5.83	21	18
10	100.0	0.0	50.9	10.4	5.56	5.83	20	18
26	105.0	5.0	46.3	6.4	5.54	5.66	20	17
64	100.0	0.0	34.9	10.1	5.59	5.62	46	21
160	100.0	0.0	24.4	5.8	5.60	5.73	21	22
400	110.0	10.0	2.3	1.5	5.72	5.85	21	21
1000	65.0	13.0	0.0	0.0	5.85	6.02	21	6
nCuO, with biosolids								
0	100.0	0.0	52.9	6.0	6.26	5.82	23	20
4	100.0	0.0	63.4	8.0	6.35	5.72	24	22
10	100.0	0.0	52.0	7.4	6.44	5.74	25	22
26	110.0	10.0	79.4	7.1	6.43	5.70	24	23
64	100.0	0.0	61.6	7.9	6.38	5.64	24	23
160	100.0	0.0	30.0	6.4	6.51	5.61	24	23
400	100.0	0.0	11.6	4.5	6.49	5.70	24	23
1000	100.0	0.0	1.8	0.9	6.50	5.61	24	24
CuSO ₄ , no biosolids								
0	100.0	0.0	17.5	5.5	5.44	5.38	21	17
87	100.0	0.0	13.4	3.3	5.30	5.41	21	18
152	100.0	0.0	14.6	4.1	5.40	5.36	19	16
267	100.0	0.0	8.3	2.6	5.41	5.37	20	20
466	100.0	0.0	2.0	1.0	5.16	5.58	22	20
816	65.0	10.7	0.0	0.0	4.95	5.43	22	18
1429	5.0	5.0	0.0	0.0	4.95	4.74	20	18
2500	5.0	5.0	0.0	0.0	4.79	5.25	20	15
CuSO ₄ , with biosolids								
0	100.0	0.0	55.6	8.4	6.60	6.02	21	24
4	100.0	10.0	62.5	8.0	6.63	6.04	20	24
10	100.0	0.0	38.3	7.7	6.65	6.06	20	20
26	100.0	0.0	43.5	9.4	6.65	6.00	21	21
64	90.0	10.0	62.5	11.3	6.54	6.10	22	23
160	100.0	0.0	32.6	4.9	6.42	5.87	22	24
400	100.0	0.0	23.1	3.7	6.32	6.07	23	25
1000	100.0	0.0	2.2	2.0	6.11	6.00	23	22

Table A 5: Mean ($\pm$ standard error (SE)) for adult survival, juvenile production, soil pH_{CaCl2} and moisture content for the *Folsomia candida* reproduction assays.

Nominal Concentration (mg/kg)	Adult Survival (%)		No. of Juveniles Produced		Soil pH _{CaCl2}		Soil Moisture (%)	
	Mean	SE	Mean	SE	Day 0	Day 28	Day 0	Day 28
nCuO, no biosolids								
0	8.0	0.7	228.2	50.0	5.66	5.60	19	19
4	9.0	1.0	237.3	35.2	5.63	5.56	10	15
10	9.7	0.3	249.7	59.7	5.63	5.48	19	17
26	10.0	0.0	298.0	45.7	5.72	5.44	20	16
64	8.3	0.7	252.7	4.8	5.65	5.46	19	15
160	9.0	0.6	290.3	30.8	5.62	5.52	20	18
400	8.7	1.3	217.3	24.9	5.65	5.60	20	18
1000	7.7	0.7	207.0	38.7	5.77	5.70	20	17
nCuO, with biosolids								
0	9.4	0.6	198.5	34.9	6.38	5.74	26	27
4	8.0	0.6	126.0	35.5	6.33	6.29	25	24
10	8.0	1.5	195.3	20.8	6.40	6.45	24	23
26	8.7	0.3	154.7	42.9	6.32	6.53	26	25
64	8.3	0.9	199.0	9.2	6.35	6.55	26	23
160	10.0	1.0	209.7	4.7	6.33	6.66	25	24
400	8.7	0.3	221.5	9.4	6.33	5.95	25	17
1000	9.3	0.3	146.5	14.3	6.30	5.64	24	23
CuSO ₄ , no biosolids								
0	8.2	1.2	369.4	51.7	5.24	5.13	21	13
87	9.3	0.3	411.7	38.9	5.50	5.03	20	15
152	9.3	0.7	371.0	31.6	5.59	4.94	21	14
267	9.0	0.6	393.0	37.1	5.21	5.08	20	11
466	8.3	0.3	370.7	12.1	5.18	5.07	21	16
816	10.0	0.0	298.5	29.0	5.06	5.15	21	15
1429	9.3	0.3	150.0	22.2	4.79	5.03	21	17
2500	8.0	0.0	76.0	9.7	4.76	4.88	21	16
CuSO ₄ , with biosolids								
0	9.6	0.4	301.2	13.0	6.73	5.40	24	NA
4	7.7	1.2	248.0	27.9	6.66	5.22	24	20
10	9.3	0.3	307.0	100.2	6.65	5.13	24	18
26	10.0	0.0	295.0	49.0	6.64	5.09	24	16
64	8.7	0.3	382.7	47.6	6.63	5.07	25	21
160	9.3	0.3	310.3	84.1	6.62	5.03	24	20
400	8.0	1.2	221.3	24.5	6.37	5.16	23	18
1000	12.0	3.5	319.0	64.6	6.04	5.95	25	25

1433 **Appendix B - Chapter 3 Appendix**

1434

1435 **Table B 1:** Mean measured Cu²⁺ in KNO₃ soil extracts (log µg/g) and mean pH (SE = standard error)

CuSO ₄ biosolids					CuSO ₄				nCuO biosolids				nCuO			
Control soils																
Day	Mean (log ug/g)	SE	Mean pH	SE	Mean (log ug/g)	SE	Mean pH	SE	Mean (log ug/g)	SE	Mean pH	SE	Mean (log ug/g)	SE	Mean pH	SE
0	-4.52	0.02	6.32	0.02	-4.17	0.03	5.89	0.01	-4.84	0.12	6.65	0.01	-4.64	0.11	6.10	0.03
21	-4.45	0.04	6.16	0.07	-4.35	0.13	5.98	0.08	-4.71	0	6.72	0	-4.56	0.05	6.11	0.01
42	-4.68	0.02	6.39	0.02	-4.21	0.07	5.85	0.05	-4.37	0.04	6.34	0.04	-4.69	0.03	6.24	0.04
Test soils																
0	-4.9	0.07	6.88	0.01	-3.64	0.12	6.06	0.03	-5.42	0.05	7.00	0.01	-3.81	0.03	6.01	0.02
1	-4.9	0.01	6.91	0.02	-3.92	0.06	5.96	0.03	-4.71	0.52	7.07	0.39	-3.93	0.02	6.17	0.00
2	-4.8	0.05	6.91	0.03	-3.97	0.01	6.03	0.02	-4.04	0.20	6.56	0.14	-3.86	0.02	6.15	0.03
4	-4.8	0.03	6.93	0.01	-3.97	0.03	6.13	0.05	-4.12	0.02	6.75	0.02	-3.93	0.02	6.16	0.03
7	-4.7	0.05	6.86	0.05	-3.98	0.05	6.05	0.02	-2.88	0.02	5.60	0.03	-4.32	0.09	6.42	0.22
10	-4.9	0.05	6.81	0.02	-4.09	0.03	6.17	0.03	-4.02	0.02	6.75	0.01	-4.23	0.20	6.38	0.12
14	-4.8	0.23	6.41	0.20	-3.90	0.04	5.99	0.03	-3.58	0.25	6.38	0.25	-3.96	0.11	6.19	0.07
17	-4.6	0.04	5.89	0.02	-4.03	0.07	5.90	0.03	-3.83	0.05	6.58	0.05	-4.00	0.28	6.34	0.33
21	-5.3	0.07	6.29	0.02	-4.17	0.05	6.25	0.09	-4.76	0.19	6.39	0.06	-4.6		6.12	0.06

1436

1437 **Table B 2:** Mean corrected (for background) total Cu measured throughout
1438 bioaccumulation tests in soil and tissues. (SE = standard error)

nCuO					nCuO biosolids				CuSO ₄				CuSO ₄ biosolids			
Day	Mean Corr Soil	SE	Mean Corr tissue	SE	Mean Corr Soil	SE	Mean Corr tissue	SE	Mean Corr Soil	SE	Mean Corr tissue	SE	Mean Corr Soil	SE	Mean Corr tissue	SE
0	11.44	0.56	0.00	0.00	11.45	0.53	0.00	0	6.15	0.03	0.00	0.00	6.60	0.27	0.00	0.00
1	10.07	1.36	0.58	0.58	12.99	0.33	4.15	1.1	7.24	0.38	6.48	6.48	5.81	0.58	3.43	1.68
2	12.94	1.12	0.47	0.47	13.02	0.55	1.17	2.55	6.20	0.09	4.72	2.37	5.86	0.21	15.69	1.64
4	11.82	1.41	2.91	0.99	12.14	0.43	2.82	1.88	6.88	0.60	9.60	4.98	6.98	0.81	16.90	3.08
7	11.80	0.16	6.26	1.18	13.52	0.12	4.70	1.69	6.10	0.11	3.52	1.77	5.27	0.17	6.43	1.03
10	11.69	0.07	5.91	2.67	12.63	0.49	9.01	1.53	6.83	0.15	12.67	2.37	6.06	1.85	20.47	4.03
14	11.34	0.43	5.90	2.74	13.50	0.23	8.31	0.87	8.50	0.62	8.46	2.09	6.11	2.04	11.11	1.61
17	10.99	0.70	8.10	0.70	13.87	0.84	4.73	0.45	8.00	0.65	5.68	3.25	5.04	1.12	7.55	2.18
21	11.93	0.10	8.63	0.00	14.65	0.80	9.68	3.15	10.39	0.13	3.20	1.60	5.94	0.55	7.37	0.00
22	0.00	0.00	1.36	0.73	0.00	0.34	4.40	2.21	0.00	0.00	0.00	0.00	0.41	0.21	1.41	1.41
23	0.00	0.00	6.94	2.77	0.08	0.22	2.95	0.4	0.00	0.00	0.98	0.98	0.06	0.18	0.00	0.00
25	0.00	0.00	5.72	0.67	0.00	0.20	2.82	0.72	0.00	0.00	0.00	0.00	0.06	0.18	0.00	0.00
28	0.00	0.00	1.46	1.08	0.05	0.31	4.06	0.74	0.00	0.00	0.01	0.01	0.06	0.06	2.23	0.56
31	0.00	0.00	1.90	0.40	0.17	0.26	4.43	0.82	0.00	0.00	0.00	0.00	0.00	0.06	3.45	1.66
35	0.00	0.00	2.22	0.15	0.00	0.30	4.29	0.56	1.85	0.00	0.00	0.00	0.00	0.06	1.47	1.20
38	0.00	0.00	3.59	1.89	0.00	0.23	3.50	2.46	3.51	0.00	0.00	0.00	0.00	0.00	1.09	0.55
42	0.00	0.00	2.09	1.36	0.40	0.41	2.36	0.97	7.79	1.85	0.00	0.00	0.00	0.00	5.64	2.15

1439