

## A critical review of the environmental impacts of manufactured nano-objects on earthworm species

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DOI:

[10.1016/j.envpol.2021.118041](https://doi.org/10.1016/j.envpol.2021.118041)

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*Document Version*

Peer reviewed version

*Citation for published version (Harvard):*

Adeel, M, Shakoor, N, Shafiq, M, Pavlicek, A, Part, F, Zafiu, C, Raza, A, Ahmad, MA, Jilani, G, White, JC, Ehmoser, E-K, Lynch, I, Ming, X & Rui, Y 2021, 'A critical review of the environmental impacts of manufactured nano-objects on earthworm species', *Environmental Pollution*, vol. 290, 118041. <https://doi.org/10.1016/j.envpol.2021.118041>

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1                   **A critical review of the environmental impacts of manufactured**  
2                   **nano-objects on earthworm species**

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# **A critical review of the environmental impact of manufactured nano-objects on earthworm species**

## **Abstract**

The presence of manufactured nano-objects (MNOs) in various consumer or their (future large-scale) use as nanoagrochemical have increased with the rapid development of nanotechnology and therefore, concerns associated with its possible ecotoxicological effects are also arising. MNOs are releasing along the product life cycle, consequently accumulating in soils and other environmental matrices, and potentially leading to adverse effects on soil biota and their associated processes. Earthworms, of the group of Oligochaetes, are an ecologically significant group of organisms and play an important role in soil remediation, as well as acting as a potential vector for trophic transfer of MNOs through the food chain. This review presents a comprehensive and critical overview of toxic effects of MNOs on earthworms in soil system. We reviewed pathways of MNOs in agriculture soil environment with its expected production, release, and bioaccumulation. Furthermore, we thoroughly examined scientific literature from last ten years and critically evaluated the potential ecotoxicity of 16 different metal oxide or carbon-based MNO types. Various adverse effects on the different earthworm life stages have been reported, including reduction in growth rate, changes in biochemical and molecular markers, reproduction and survival rate. Importantly, this literature review reveals the scarcity of long-term toxicological data needed to actually characterize MNOs risks, as well as an understanding of mechanisms causing toxicity to earthworm species. This review sheds light on this knowledge gap as investigating bio-nano interplay in soil environment improves our major understanding for safer applications of MNOs in the agriculture environment.

51 **Keywords:** Earthworms; Nanomaterials; Trophic transfer; Fate and transport; Nano plastic

52 **Graphical Abstract**



53

54 **1. Introduction**

55 Advances in nanotechnology have enabled the development of manufactured nano-sized objects  
56 (MNOs), such as nanoparticles (NPs), nanoplates or nanofibers, which have interesting and useful material  
57 properties. By definition, NPs have dimensions in the size range between 1–100 nm, and importantly, many  
58 materials of relevance for industrial purposes contain toxic elements such as heavy metals. MNOs are widely  
59 applied for medical purposes (Shakib et al., 2014; Wu et al., 2011), and present several positively recognized  
60 features such as enhancing water security (Alvarez et al., 2018), improving agriculture productivity and food  
61 security (Duhan et al., 2017; Kah, 2015a), energy storage (Hussein, 2016), as well as broad applications  
62 related to environmental services and remediation (Saratale et al., 2018), electric and electronic (Contreras  
63 et al., 2017), fire safety (Olawoyin, 2018), industrial and transportation purposes, (Mathew et al., 2019)  
64 among others. With increasing production volumes, the unintentional release of MNOs into the environment  
65 increases, which may occur along the entire life cycle of MNO-containing products (Froggett et al., 2014;  
66 Keller and Lazareva, 2014; Part et al., 2018a). In addition, MNOs that are used in agriculture as pesticides  
67 or fertilizers (nanoagrochemical) are seen as promising solution to safeguard the future food production and  
68 may enter the ecosystem in large quantities (Sun et al., 2020). In the last two decades, numerous research

69 projects have assessed the environmental health and human safety implications (Haase and Lynch, 2018).  
70 MNOs exhibit nanoscale-specific adverse effects due to their small size, asbestos like needle forms, large  
71 surface-to volume area or higher reactivity compared to conventional non-nanoscale (bulk) materials (Du et  
72 al., 2018; Glisovic et al., 2017; Hristozov et al., 2016). However, MNOs also have positive nano-specific  
73 physiochemical properties useful for relevant purposes, such as in agriculture. Novel nanoformulations  
74 combine several surfactants, polymers and inorganic NPs and are referred as nanoagrochemicals, including  
75 nanopesticides and nanofertilizers that aim at increasing solubility of poorly water soluble compounds or  
76 slow/controlled release (Kah, 2015b; Kah et al., 2013). At the nano-bio-interface, MNOs can be used  
77 specifically for plant protection, for example, to dissociate plant viruses, to act as organic based delivery  
78 systems of nutrients or to increase antioxidant enzyme activity (Farooq et al., 2021; Zulfiqar et al., 2019).  
79 Current research focuses on increasing the efficacy of MNOs but also on risk assessment, if MNOs are to be  
80 directly used for large scale agricultural applications. When MNOs and nanoagrochemicals get into the soil,  
81 a change physical, chemical or biological transformation processes started that is strongly dependent on soil  
82 conditions (e.g., pH, electrolyte and pore water composition, natural organic matter (NOM) content, etc.)  
83 (Kah et al., 2013). At the nanobio-interface, biotransformation, heteroaggregation, oxidation-reduction and  
84 dissolution are very likely – e.g. proteins, humic / fulvic acids or other NOM can adsorb onto the MNO's  
85 particle surface (corona formation) and thus can increase the mobility in the environment, whereas  
86 adsorption of electrolytes (e.g.  $\text{Ca}^{2+}$ ) rather leads to a decrease in particle mobility (Markiewicz et al., 2018;  
87 Quigg et al., 2013). For risk assessment including bioassays it is therefore important to consider  
88 transformation processes of MNOs in order to elucidate exposure pathways and possible uptake by the biota.  
89 Especially, in agricultural soils invertebrates play a significant role in the formation and maintenance of soil  
90 structure and fertility through their direct role in a vast number of biological and biochemical processes. Soil  
91 invertebrates are important indicators of soil quality and also play a significant role in the the risk assessment  
92 of potential polluting substances as long-used model species in regulatory ecotoxicology. Literature reviews  
93 on the potential toxic effects of metal- and carbon-based MNOs on the soil environment have indicated that  
94 MNOs affect the entire soil community and may lead to adverse effects on the environment and its biota, as  
95 well as human health via trophic transfer within the food chain (Liné et al., 2017; Rocha et al., 2017). In  
96 addition, earthworms are common prey to other biota and therefore play a key role in the biomagnification  
97 of soil pollutants, often leading to negative consequences for sensitive vertebrate species (Rodríguez-

98 Castellanos and Sanchez-Hernandez, 2007; Roodbergen et al., 2008). In terms of ecosystem services,  
99 earthworms modify soil structure, increase soil carbon stabilization (Adil et al., 2019; Whitehead et al., 2018;  
100 Wiesmeier et al., 2019), and improve crop production (Eisenhauer et al., 2012; van Groenigen et al., 2014)  
101 by increasing the population of beneficial microorganisms (Wurst, 2010). Earthworms also have the potential  
102 to bioaccumulate or biochemically transform organic or inorganic substances and therefore, could be used  
103 for bioremediation. These important species even play a major role in fixing carbon dioxide and thereby  
104 reduce greenhouse gas emissions from agriculture (Angst et al., 2019; Guo et al., 2019).

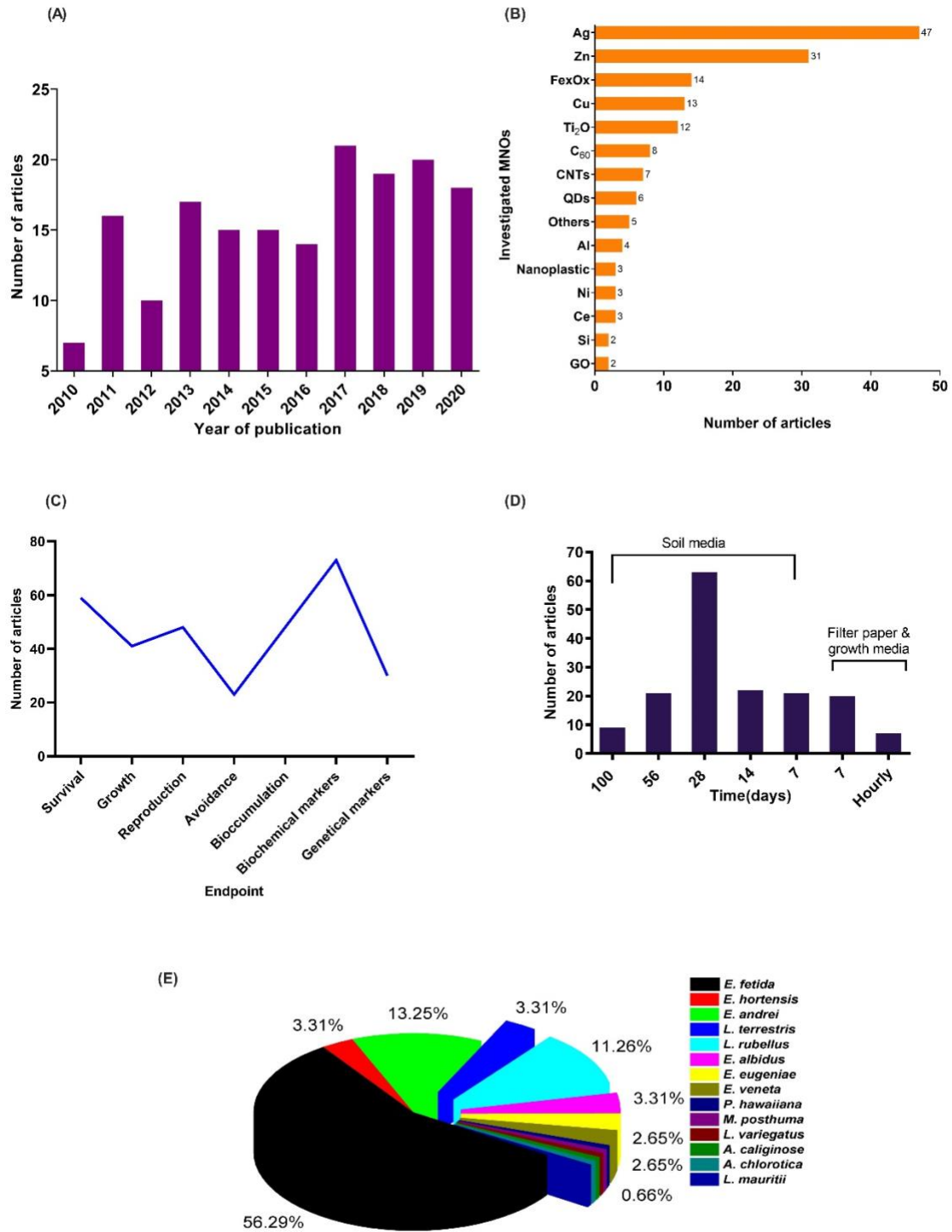
105 Given the important roles of earthworm species, we focus in this review on the potential negative effects  
106 of metal-, metal oxide- and carbon-based MNOs on this group of Oligochaetes. As noted above, these species  
107 are model organisms in soil ecotoxicology and they play a significant role in contaminant fate in soils, are  
108 recommended test organisms according to international standards such as International Organization for  
109 Standardization (ISO) and Organisation for Economic Co-operation and Development (OECD)(ISO 11268-  
110 1:2012; OECD, 2016). Mollusks, mites, isopods and collembolans are also used in terrestrial ecotoxicity  
111 testing in order to derive conclusions on the hazardous properties (persistence, potential bioaccumulation  
112 and toxicity) of MNOs. However, different functional and taxonomic groups have different routes of  
113 exposure, intake and response to MNOs. As such, toxicological data on various MNO types within a specific  
114 taxonomic group like earthworms, belonging to the subclass of Oligochaetes, must first be generated and  
115 then compared with other co-habiting taxonomic groups. In such specific environmental compartment one  
116 can yield a more generalizable statement on the ecotoxicity potential of MNOs. Herein, we specifically  
117 address terrestrial earthworm species of the Oligochaetes group; importantly, these organisms comprise the  
118 majority of invertebrate biomass (>80 %) in terrestrial environments and have over 600 million years of  
119 evolutionary experience as “environmental engineers” (Fierer, 2019). We evaluated 165 peer-reviewed  
120 journal articles that focus on the ecotoxicological effects of MNOs on Oligochaetes. Figure 1 shows that in  
121 the last 10 years, the highest number of studies focused on nano-Ag followed by ZnO, CuO, TiO<sub>2</sub>, Fe-based  
122 NPs (FeO, Fe<sub>2</sub>O<sub>3</sub>, zerovalent Fe<sup>0</sup>), QDs, carbon based materials (nanoplastic, C<sub>60</sub>, graphene oxide (GO), and  
123 carbon nanotubes (CNTs), Al, Ni, Ce, Si, as well as others such as Cr, Yb, La, fullerenes or graphene.  
124 Importantly, research to assess nanotoxicological effects on terrestrial invertebrates is still needed (Johnson  
125 et al., 2018; Mukherjee and Acharya, 2018), particularly given that the chemical transformation of MNOs  
126 in soils presents a high level of complexity and consequently, organic/organo-mineral composition under the

127 influence of MNOs renders an unpredictable event. Ecotoxicological studies on earthworms are particularly  
128 necessary because these species are relevant indicator organisms with regard to food production and  
129 environmental services. More specifically, MNOs released into the soil can lead to bioaccumulation and  
130 physiological changes in earthworm species, which in turn can have adverse effects on food production, the  
131 environment and finally, MNO release might affect human health.

## 132 **2. Review scope and approach**

133 This study addresses manufactured nano-objects (nanoparticles, nanofibers and nanoplates) which  
134 are abbreviated as “MNOs” and have one, two or three external dimensions in the nanoscale (1–100 nm)  
135 according to [ISO/TS 80004-2:2015](#) (. With regard to ecotoxicity and bioassays, we focused on the taxonomic  
136 subgroup of *Oligochaetes*, as these earthworms are most likely species exposed to nanoagrochemicals or  
137 other MNOs that are unintentionally dispersed in landfills, natural, urban or sludge-treated (agricultural) soil.  
138 Search engines and databases for scientific literature, such as PubMed, Science Direct, Web of Science and  
139 Google Scholar were used to identify peer-reviewed literature from 2010 until August 2020. Primary studies  
140 on earthworms that reported positive, adverse or unidentifiable toxic effects of different types of MNOs were  
141 evaluated. For the literature search, the keywords “bioaccumulation”, “nanoparticles”, “earthworms”, “fate  
142 and transport”, “soil and biota”, “trophic transfer” and the specific types of MNOs were used. We  
143 documented, compiled and interpreted novel as well as recent information about the potential impacts of  
144 MNOs on earthworms. In addition, literature was considered related to MNO production volume,  
145 application, release and environmental behaviour of MNOs so as to present the relevance of MNOs in the  
146 environment from a more holistic perspective. Finally, we summarized the results, possible data gaps, and  
147 challenges to derive future research perspectives from this bibliometric analysis.

148



149

150

151 **Figure 1** (A) Number of peer-reviewed publications (165 in total between 2010–2020) investigating the

152 potential toxic effects of manufactured nano-objects (MNOs) on the earthworm species of the group



153 Oligochaeta obtained from PubMed, Science Direct, Web of Science or Google Scholar (B) Investigated  
154 MNO in the 165 publications (C) endpoints in MNOs studies on earthworms (D) MNOs exposure duration  
155 (E) earthworm species considered in the MNOs studies

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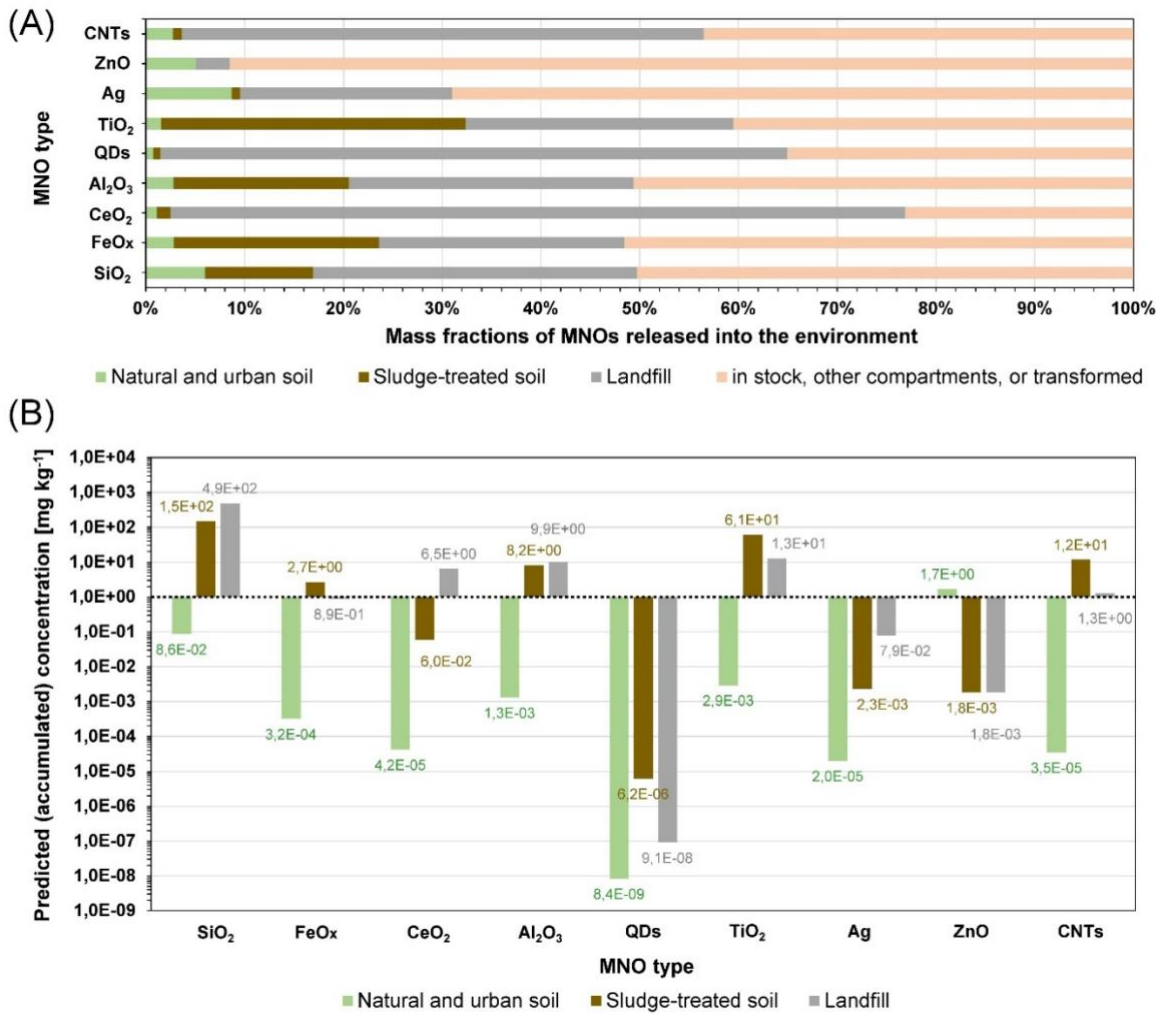
### 157 **3. Occurrence of manufactured nano-objects in soil**

158 Nanotechnology allows for manipulation of material properties through the control of matter, atoms and  
159 molecules. This key emerging technology has enabled a wide range of applications, such as sunscreens,  
160 water-repellent paints, nanocrystalline semiconductors for thin-film solar cells, nanocarriers or  
161 nanotherapeutics for cancer treatment, nanoagrochemicals and many more (Jean, 2020; Kah, 2015a; Lin,  
162 2015; Vance et al., 2015). The total global production of MNOs, such as Ag, Al<sub>2</sub>O<sub>3</sub>, CeO<sub>2</sub>, CNTs, Cu, Fe,  
163 nanoclays, SiO<sub>2</sub>, TiO<sub>2</sub> and ZnO, has been estimated to be approximately 267,000 to 318,300 tons (Future  
164 Markets, 2012). According one market survey, 50% of the MNOs were produced in the USA, followed by  
165 19% in the EU, 12% in China, 6% in Korea, 4% in Japan, 3% in Canada, 2% in Taiwan and 4% in other  
166 countries. A more recent market study estimated the global production volume of MNOs in 2020 to range  
167 from 400,000 to 3,150,000 tons in the case of nano-SiO<sub>2</sub> and from 2 to 4 tons in case of nano-Ag (highest  
168 and lowest value) (Janković and Plata, 2019). Jankovic et al. (Janković and Plata, 2019) highlighted that  
169 MNO production volumes represents only a small share of the total ore production of the mining industry –  
170 for example 1% to 0.000002% of the total Ag or Fe ore production, respectively – and therefore had a minor  
171 influence on entire life cycle of anthropogenic elements in the mining and manufacturing sectors. In  
172 summary, the mass-relevance of MNOs in the global cycle of raw materials or processed materials needs to  
173 be further investigated, particularly given that the currently reported quantities can vary widely and are often  
174 inconsistent.

175 In support of exposure assessment, dynamic material flow models have been applied on the data to  
176 predict the mass flows associated with release along the product life cycle into soil, water or air (Song et al.,  
177 2017a; Sun et al., 2017). Song et al. (2017b) estimated that by 2020, approximately 51% (12,200 tons) of  
178 the total global release of TiO<sub>2</sub>, SiO<sub>2</sub> and FeO<sub>x</sub> occurred during the use of nano-enabled products (e.g.,  
179 construction, packaging, medical products etc.), whereas 43% (9,890 tons) were the result of end-of-life  
180 releases. The remainder of the releases were during manufacturing. Release models for Europe, developed  
181 by Sun et al. (2016a) and Wang and Nowack (2018a), enabled the prediction of environmental concentrations

182 of MNOs that occur in landfills, as well as a natural, urban or sludge-treated soils. Figure 2 summarises the  
183 results obtained from these models and shows that the majority of MNOs remain in the in-use stock or are  
184 transformed during processes such as wastewater treatment or waste incineration. For example, released  
185 nano-Ag either directly attaches to biosolids, dissolves in slightly acidic wastewaters, or transforms to  
186 insoluble  $\text{Ag}_2\text{S}$  or  $\text{AgCl}_2$  species that precipitate or attach to the sewage sludge, which is in turn thermally  
187 treated or used directly for agricultural purposes as a soil amendment (Kim et al., 2010b; Schlich et al.,  
188 2013a). Chemically more stable MNOs such as  $\text{TiO}_2$ ,  $\text{Al}_2\text{O}_3$ ,  $\text{Fe}_x\text{O}_y$  or  $\text{SiO}_2$  also attach to biosolids during  
189 wastewater treatment and may accumulate in measurable quantities in sludge-treated soils, as shown in  
190 Figure 2. Based on the results from the release models for the EU in 2014 (Sun et al., 2016b; Wang and  
191 Nowack, 2018a), we summarise (Figure 2) the predicted environmental concentrations (PEC values) of  
192 MNOs which are most relevant for soil environments and thus, lead to increased risk of exposure to terrestrial  
193 invertebrate species such as earthworms. The lowest MNO concentrations are expected to be ca.  $8.4 \times 10^{-9}$  mg  
194  $\text{kg}^{-1}$  in natural and urban soil (in the case of quantum dots-MNOs), whereas the highest levels are predicted  
195 to occur in landfills in the case of nano- $\text{SiO}_2$  at a concentration at ca.  $4.9 \times 10^2$  mg  $\text{kg}^{-1}$ . For comparison,  
196 Garner et al. simulated ten years of release of nano- $\text{CeO}_2$ , - $\text{CuO}$ , - $\text{TiO}_2$  and - $\text{ZnO}$  in the San Francisco Bay  
197 area (California, U.S.) and found that the highest concentrations and mass fractions of MNOs are predicted  
198 for sewage-treated agricultural soils, as well as freshwater and marine sediments (Garner et al., 2017b).  
199 However, taking account of transformation processes such as de-/sorption, leaching or particle dissolution  
200 in soil pore water, and species sensitivity distributions (obtained from ecotoxicity data), Garner et al. (2017b)  
201 showed that neither nano- $\text{TiO}_2$  nor  $\text{ZnO}$  exceeds the no observed effect concentration (NOEC), even for  
202 sewage-treated agricultural soils. Their predictions also indicate no risks in the case of nano- $\text{CuO}$ , whereas  
203 for nano- $\text{CeO}_2$  no quantitative risk assessment was possible due to the lack of toxicity data for soil organisms.  
204 Based on the currently known production volume of MNOs and nanoagrochemicals, the predicted  
205 environmental concentration of MNOs (apart from quantum dots) in soils is in the range of  $10^2$  to  $10^{-7}$  mg  
206  $\text{kg}^{-1}$ , and in landfills of  $10^3$  to  $10^{-3}$  mg  $\text{kg}^{-1}$  (Garner et al., 2017a; Sun et al., 2016b).

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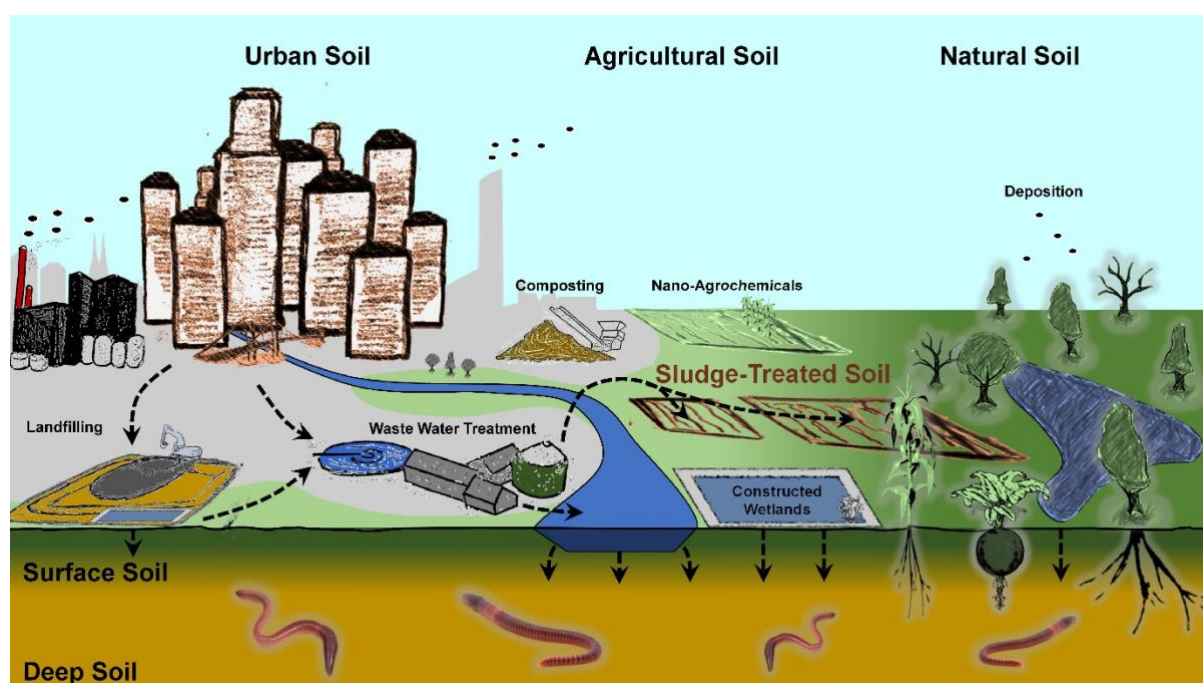
209 **Figure 2** (A) Mass fractions of the total release of MNOs in the EU in 2014, and (B) the predicted  
 210 environmental mass concentrations that accumulated since 1990. All figures are based on the modelling  
 211 results from Sun et al. (2016a) and Wang and Nowack (2018a)

212 Dynamic flow models for exposure assessment also must consider market dynamics and the lifetime  
 213 of nano-enabled products. We highlight that MNOs release pathways depend on the type of application, the  
 214 product's service lifetime, and the waste treatment applied to the product. Natural, urban and sludge-treated  
 215 soils, as well as landfills for solid wastes with high organic content or with bio-covers (used after landfill  
 216 closure), are the most relevant sinks for MNOs and represent an important exposure pathway for terrestrial  
 217 species. With regard to risk assessment and future increases in MNO production volumes, harmful effects  
 218 on terrestrial species in the near future cannot be excluded and release models must therefore be constantly  
 219 updated with relevant data as they provide important guidance for ecotoxicity testing under realistic  
 220 concentration ranges and relevant conditions. For quantitative risk assessment, it is also important to consider

221 possible transformation processes of MNOs in the environment, particularly given that the fate and toxicity  
222 of some transformed particles differs greatly from those of pristine MNOs (Adam et al., 2018; Caballero-  
223 Guzman and Nowack, 2016; Part et al., 2018b; Svendsen et al., 2020). Predictive toxicological approaches  
224 based on high-throughput screening assays or structure activity relationship analysis have revealed that  
225 particle dissolution is not the only driver in nanotoxicity, and that processes such as oxidative stress, redox  
226 activity and production of oxygen species, cationic stress, photoactivation, embryo hatching interference,  
227 and membrane lysis are highly relevant toxicological drivers (Nel et al., 2013). In the last decade, grouping  
228 approaches have been developed for regulatory testing in order to find similarities in toxicological effects  
229 among different MNO types, where the relationship between toxicity and certain physico-chemical  
230 properties will be further investigated (Ha et al., 2018; Hund-Rinke et al., 2018; Lamon et al., 2019; Lynch  
231 et al., 2014). A grouping concept for metals and metal oxide MNOs that is based on ecotoxicity tests using  
232 algae, daphnids and fish embryos showed that for nano-Ag that, not only the solubility, but also the reactivity  
233 of the particle surface, morphology, and shading effects were all relevant and important factors for  
234 ecotoxicity (Hund-Rinke et al., 2018). However, such grouping approaches, particularly regarding  
235 ecotoxicity to terrestrial species, have many limitations as there is often a lack of data on both physico-  
236 chemical properties of a specific MNOs under realistic exposure conditions in the relevant matrices and  
237 comparable results from ecotoxicity tests.

238         Regarding the accumulation of MNOs in soil, Figure 3 shows possible release and exposure  
239 pathways which are relevant for earthworm species. MNOs can either be released unintentionally into soil  
240 or added intentionally when applied as biocides (e.g., Ag-, ZnO- or CuO-NPs) (Zhang et al., 2020c) in  
241 agricultural applications, such as the through the use of nano-pesticides or nano-fertilizers (Kah et al., 2019).  
242 Consequently, broad ecosystem exposure may occur after particle uptake and transfer through trophic levels  
243 from bioaccumulation or biomagnification. For instance, lab-scale experiments showed that amino acid-  
244 conjugated semiconducting quantum dots (QDs) were accumulated by the soil fungi *Penicillium solitum*; in  
245 contrast, no MNO uptake was evident without the conjugated amino acid coating. Hou et al. (2013)  
246 highlighted in their review that invertebrates such as the earthworm *Eisenia fetida* can be used to  
247 quantitatively assess the extent of bioaccumulation by calculating the ratio of MNO tissue concentration to  
248 that in water (bioaccumulation factor or BCF), and biomagnification by the ratio of the MNO concentration  
249 in the predator to that in its prey (biomagnification factor or BMF). Furthermore, the biota-sediment

250 accumulation factor (BSAF) represents the ratio of the MNO concentration in an organism to that in the  
251 sediment (Hou et al., 2013). For example, Hou et al. reported that nanosized  $\text{Al}_2\text{O}_3$  resulted in a lower uptake  
252 than their micro-sized equivalents, and that the uptake of metals (e.g., Ag, Au or Cu) in ionic form was  
253 higher than their nanoparticulate counterparts. Here, when fullerene ( $\text{C}_{60}$ ) and single- or multi-walled carbon  
254 nanotubes (SWCNTs or MWCNTs) were exposed for 28 days to soils at concentrations ranging between ca.  
255 0.3–300 mg MNO  $\text{kg}^{-1}$  dry soils, the BSAF (kg dry soil per kg dry biomass) ranged from 0.0061–0.79 (Hou  
256 et al., 2013). As mentioned above, as the concentration of MNOs in soil environment significantly  
257 increases, as well as it will be important to understand their impact on soil biota.  
258



259  
260 **Figure 3** Summary of possible relevant exposure pathways of MNOs for terrestrial species, such as  
261 earthworms.

#### 263 4. Metal and metal oxide nano-object toxicity to earthworm species

##### 264 4.1 Silver

265 Silver nanoparticles (Ag-NPs) are extensively used in industrial and commercial products and  
266 intentionally or unintentionally accumulate in the soil environment (e.g. as applied nanoagrochemical and  
267 fungicide). PEC values of Ag-NPs for soil differ by global region; the PEC value of native soil in Denmark

268 is 13 - 61 ng kg<sup>-1</sup> and agricultural soil contains 6 - 21 ng kg<sup>-1</sup>, (Gottschalk et al., 2015) while across European  
269 soils the predicted levels range between 17.4 - 58.7 ng kg<sup>-1</sup>, and in the USA 6.6 - 29.8 ng kg<sup>-1</sup> of Ag-NPs are  
270 predicted (Gottschalk et al., 2009a).

271 From 2010, 47 studies on the effect of Ag-NPs on earthworm species were published; this is the  
272 highest number for any of the MNO types (Table S1). In general, the studies showed that Ag-NPs were more  
273 toxic than Ag ions, which were typically presented as AgNO<sub>3</sub> salt. Toxicity in general is dependent on several  
274 factors, including particle size, exposure concentration and duration, experimental conditions and Ag-NPs  
275 properties, such as surface stabilization or coating, surface speciation, shape etc. (Bourdineaud et al., 2019;  
276 Mukherjee et al., 2017). A consistent finding was that the toxicity of AgNPs increased with decreasing size  
277 of the NPs; this is likely due to the higher surface to volume ratio of smaller NPs that results in both faster  
278 dissolution and likely greater uptake of discrete particles (Hu et al., 2012). Specifically, Hu et al. reported  
279 that 20 nm NPs were more toxic than 80 nm (500 mg Ag-NPs kg<sup>-1</sup>); the authors speculated that greater  
280 passage of the 20 nm NPs through the cell membranes via membrane proteins (porins) resulted in more  
281 significant interference of metabolic pathways and damage to cellular constituents of *E. fetida*. At the cellular  
282 level, the coelomocytes of earthworms (*E. fetida*) and human cells (THP-1 cells, differentiated THP-1 cells  
283 and peripheral blood mononuclear cells) showed a similar response to Ag-NPs (5.91 µg Ag-NPs mL<sup>-1</sup>)  
284 cytotoxicity after 24 hours. The early molecular responses to Ag-NPs involved an apparent transition from  
285 stress-related to immune-related activation of genes in both cellular models, with a distinct induction of the  
286 metallothionein encoding genes in THP-1 cells (Hayashi et al., 2012). Regarding the lifecycle of earthworms,  
287 several studies reported that the toxicity of Ag-NPs/Ag<sup>+</sup> depended on the developmental stage of the  
288 earthworms at exposure. The strongest effects on avoidance behavior were observed at 12.5 mg kg<sup>-1</sup> (Ag-  
289 NPs/Ag<sup>+</sup>); adverse effects on survival were most significant at 100 mg kg<sup>-1</sup>. Brami et al., (Brami et al., 2017)  
290 indicated that these effects were induced by oxidative stress originating from released Ag<sup>+</sup> ions. In addition,  
291 it was observed that the Ag concentration in the tissue of earthworms increased strongly with dose, which  
292 indicates the bioaccumulation of either Ag ions or nanoparticles or both (Antisari et al., 2016). Exposure  
293 studies conducted for the evaluation of the acute toxicity (1-14 days) have reported accumulation of the  
294 particles or ions in the gut and tissue (Hu et al., 2012; Li et al., 2015; Shoults-Wilson et al., 2011c). However,  
295 Makama et al.(2016) could not find any significant changes in Ag concentration (NPs and ions) in the soil  
296 after exposure and also, no changes on the growth levels and mortality of *E. andrei* during 28 days of AgNO<sub>3</sub>

297 and Ag-NPs exposures ( $EC_{50}$  for 20 nm 66.8 mg  $kg^{-1}$ ). Short duration studies (14 days) at 500 mg  $kg^{-1}$  of  
298 Ag-NPs identified interference with different enzymes and metabolic activity in earthworms, as well as  
299 glutathione reductase, glutathione S-transferase, acid phosphate and ATPase (Das et al., 2018; Gao et al.,  
300 2012; Hu et al., 2012).

301 As with most NPs, it is difficult to separate the toxicity of Ag-NPs from that of Ag ions; as NPs  
302 age in soil, transformation processes such as dissolution and ion release will occur (Lowry et al., 2012),  
303 particularly when the soil matrix conditions (pH, ionic composition, organic matter, etc.) change, and these  
304 dynamic processes (Patricia et al., 2017) can modulate the toxicity of Ag-NPs (Figure 4). In addition,  
305 released ions can subsequently be reduced and agglomerate to form various types of hetero aggregates with  
306 the soil matrix by precipitation and binding to organic matter and clays, or complex with various minerals  
307 (Coutris et al., 2012a) or ligands (e.g. S, Cl). However, a correlation between Ag-NPs adverse effects and  
308 dissolved Ag has been reported (Yang et al., 2012).

309 Additionally, Ag-NPs have been shown to have increased toxicity towards earthworms after aging  
310 in soil. Diez-Ortiz et al. (2015) compared the effect of residence time in soil on the toxicity of Ag-NPs (45-  
311 4395 mg  $kg^{-1}$ ) and  $AgNO_3$  (18-1758 mg  $kg^{-1}$ ) by aging the particles for 52 weeks or 1 week in soil before  
312 initiating earthworm exposure; the  $EC_{50}$  decreased with increasing aging time (1-52 weeks) with exposure  
313 of Ag-NPs (1420-34 mg Ag  $kg^{-1}$  d.w.) as compared to  $AgNO_3$  (49-104 mg Ag  $kg^{-1}$  d.w.). Interestingly,  
314 accumulation of Ag from Ag-NPs in earthworm tissue decreased (64-7  $\mu$ g Ag  $g^{-1}$  d.w.) with increased aging  
315 time as compared to  $AgNO_3$  (16-17  $\mu$ g Ag  $g^{-1}$  d.w.). Another study showed that soil samples containing aged  
316 Ag-NPs was more toxic to earthworms than was non-aged Ag-NPs, which led to the conclusion that the  
317 interaction of the NPs with organic matter increased their toxicity (Coutris et al., 2012b). Aggregation of  
318 Ag-NPs or binding of organic matter to the particle surface are described as the main reasons for changes in  
319 NP toxicity (Gao et al., 2012).

320 We found contradictory reports about impacted physiological endpoints and behavior of Ag-NPs and  
321 ions on earthworms. However, the majority of studies showed that Ag-NPs lead to greater Ag accumulation  
322 in earthworm tissue than Ag ions (Coutris et al., 2012a; Shoults-Wilson et al., 2011a) and that earthworms  
323 showed avoidance behavior from both Ag-NPs/ $Ag^+$  (Shoults-Wilson et al., 2011b). Importantly, the effects  
324 of Ag-NPs on earthworms can be complex and can manifest as growth reduction (van der Ploeg et al., 2014a),  
325 decrease in cocoon production (Schlich et al., 2013b; Shoults-Wilson et al., 2011b), juvenile mortality and

326 reduced reproduction (Heckmann et al., 2011), oxidative stress (Hayashi et al., 2012; Hayashi et al., 2013)  
327 damage to proteins (Tsyusko et al., 2012) and DNA resulting in reduced enzymatic activities (Hu et al.,  
328 2012) and gene expression changes. Despite a relatively large number of studies addressing Ag-NPs toxicity  
329 to earthworm species, there are still significant knowledge gaps. For example, long-term multi-generational  
330 experiments are needed to analyze the impact of Ag-NPs under realistic exposure scenarios. An  
331 understanding of the threshold doses of Ag-NPs in soils that are not expected to affect earthworm growth  
332 and development need to be characterized. In addition, given the complexity of interactions and  
333 transformation processes of Ag-NPs with soil components, an understanding of the role of soil properties in  
334 particle fate and effects relative to earthworm species is needed.

#### 335 **4.2 Zinc oxide**

336 Zinc oxide nanoparticles (ZnO-NPs) are the 3<sup>rd</sup> most frequently produced metal-based NPs  
337 (Merdzan et al., 2014). ZnO-NPs enter the environment via industrial wastewater, domestic sewage, and  
338 application of sewage sludge in agriculture as a fertilizer (Dempsey et al., 2013; Tourinho et al., 2012). In  
339 soil, the mobility and bioavailability of ZnO-NPs are controlled by a series of different physio-chemical  
340 properties (Tourinho et al., 2012), with pH and organic matter having the strongest impact on toxicity  
341 (Romero - Freire et al., 2017). ZnO-NPs form soil complexes retaining the colloidal properties of the NPs,  
342 but may form also larger aggregates, or the NPs may dissolve and release of Zn ions (Tourinho et al., 2012).  
343 Similar to Ag-NPs, the PEC of ZnO-NPs varies regionally. PECs of 0.085 - 0.661  $\mu\text{g kg}^{-1}$  were predicted in  
344 Europe, while values of 0.041 - 0.271  $\mu\text{g kg}^{-1}$  were predicted in soils from the USA (Gottschalk et al.,  
345 2009b). A study from Denmark comparing uncultivated and agricultural soil predicted 0.018 - 0.9 and  
346 0.008 - 0.35  $\mu\text{g kg}^{-1}$ , respectively (Gottschalk et al., 2015).

347 Within the past 10 years, 31 studies were published on the effects of ZnO-NPs on earthworm  
348 species, describing bioaccumulation, toxicity, and oxidative stress (Table S1). Most incubation studies were  
349 conducted for 28 days. A possible mechanism of toxicity involves the release of  $\text{Zn}^{2+}$  from ZnO-NPs by  
350 dissolution, which depends strongly on the surrounding medium. Dispersions of 1000  $\text{mg kg}^{-1}$  ZnO-NPs in  
351 agar medium at different ratios resulted in 100% mortality for *E. fetida* within 4 days. The authors indicated  
352 that the high mortality was caused by a successive loss of antioxidant enzyme protections at this very high  
353 dose, such as superoxide dismutase (SOD). An increased activity of SOD was observed at the lower dose of  
354 50  $\text{mg kg}^{-1}$ , and levels decreased with increasing ZnO NP dose, suggesting that the protective enzyme



355 potential was lost in a dose-dependent fashion and that the worms suffered from excessive oxidative stress  
356 (Li et al., 2011)(figure 4). Laycock et al.,(2015) reported no significant difference between the uptake of two  
357 forms of <sup>86</sup>Zn isotopes (<sup>86</sup>ZnO-NPs and <sup>68</sup>ZnCl<sub>2</sub>) at 5 mg kg<sup>-1</sup> in *L. rubellus*, with the dietary and dermal  
358 pathways accounting for 95% and 5 % uptake, respectively .

359 Cañas et al. found that ZnO-NPs exposure induced a range of negative impacts on earthworms: for  
360 example, at 10,000 mg kg<sup>-1</sup> ZnO-NPs (unrealistic dose) caused mortality, reduced the cocoon and juvenile  
361 production (*E. fetida*). Toxicity was attributed to dissolution of the NPs to Zn<sup>2+</sup> (Cañas et al., 2011).  
362 Conversely, another study in soil showed little effect on earthworm (*E. andrei*) survival at concentrations up  
363 to 4000 mg kg<sup>-1</sup>, (EC<sub>50</sub> estimated as 1020 mg kg<sup>-1</sup>). However, reproduction and the number of juvenile  
364 offspring were significantly affected (Alves et al., 2019). Another study was published that reports long-  
365 term exposure (140 days) of ZnO-NPs to earthworms in different soil types (entisol and tropical artificial  
366 soil). The reproductive endpoints for *E. andrei* were in accordance with the short-term exposure studies (28  
367 days); the reproductive rate was reduced to 45% at 500 and 1000 mg kg<sup>-1</sup>, but no significant effect was found  
368 on growth and survival (Romero - Freire et al., 2017). In an interesting co-contaminant study, combined  
369 exposure of ZnO-NPs with chlorpyrifos (CPF) (125/40 mg kg<sup>-1</sup>) for 28 days showed no effect on survival  
370 but decreases (21-43%) in growth and fertility were reported. Furthermore, the enzymatic activities of CAT,  
371 GST and MDA were not affected. However, acetylcholinesterase (AChE) activity was diminished as  
372 compared to control and the extent of inhibition increased with increasing concentration of chlorpyrifos  
373 (García-Gómez et al., 2019; Uwizeyimana et al., 2017). A similar study conducted for 56 days evaluated the  
374 combined effects of ZnO-NPs with CPF on 2<sup>nd</sup> generation earthworms after exposure. The results showed  
375 that the growth of the 2<sup>nd</sup> generation was affected by the parental exposure and that this progeny had lower  
376 body weights, produced fewer cocoons and consequently, fewer (33.2 %) juvenile earthworms. GST and  
377 CAT were upregulated in organisms simultaneously exposed to a mixture of ZnO and CPF as compared to  
378 controls. Other biomarkers such as AChE activity were inhibited more strongly in the 1<sup>st</sup> generation  
379 compared to the 2<sup>nd</sup> generation (Lončarić et al., 2020). Stress responsive gene expression analysis following  
380 dietary exposure to ZnO-NPs in *E. fetida* showed that Zn induced expression of SOD (increase 3.35 fold),  
381 CAT (3.03 fold) and MT (7,68 fold) and the heat shock protein 70 (5.05 fold) following exposure to 500 mg  
382 kg<sup>-1</sup> ZnO for 15 days (Xiong et al., 2012). However, a detailed molecular mechanism of ZnO-NPs response  
383 was not described. Li et al showed that exposure of earthworms to ZnO-NPs (10, 50 and 250 mg kg<sup>-1</sup>) caused

384 a dose-dependent upregulation of antioxidant biomarkers, including ROS, SOD, and MDA (Li et al., 2019).  
385 Across the 31 studies, contradictory lethal doses of ZnO-NPs were reported, and the reason for this  
386 variability remains unknown. Given this and the potential for bioaccumulation and long-term effects of ZnO-  
387 NPs, additional study is needed. In addition, many of the knowledge gaps noted above for Ag-NPs also exist  
388 for ZnO-NPs. In addition, the literature on ZnO-NPs highlights the importance of additional mechanistic  
389 work on co-contaminant exposure and interaction studies, as well as on the development of important  
390 biomarkers for exposure and response.

391

### 392 **4.3 Copper**

393 Copper oxide nanoparticles (CuO NPs) are utilized in a range of industries, including as  
394 nanoagrochemicals and antimicrobial products, which has led to increased release into terrestrial and aquatic  
395 ecosystems (Dugal and Mascarenhas, 2015; Kim et al., 2010c). The annual CuO-NPs consumption is  
396 approximately 79,000 tons in North America, which covers 50% of the global market (Amorim and Scott-  
397 Fordsmand, 2012). More than 300 tons of CuO-NPs were manufactured in the United States during 2014  
398 (Mashock et al., 2016). Several studies have documented that CuO-NPs impact the growth and reproduction  
399 of earthworm species under different conditions. From 2010 to 2020, 13 studies related to CuO-NPs and  
400 different earthworm species were published (Table S1).

401 A comparative study of CuO-NPs and CuCl<sub>2</sub> reported that CuO-NPs were 2-8 fold more toxic to  
402 *Enchytraeus albidus* than was CuCl<sub>2</sub> as measured by reproductive output (EC<sub>50</sub> 95 mg kg<sup>-1</sup>) and induced  
403 avoidance behavior (EC<sub>50</sub> 241 mg kg<sup>-1</sup>) (Amorim and Scott-Fordsmand, 2012). Similarly, a comparative  
404 study exploring gene expression through microarray analysis of *E. albidus* grown in soil amended with 400-  
405 1000 mg kg<sup>-1</sup> Cu-NPs or CuO-Cl<sub>2</sub> for 48 h reported significantly different gene responses (increase/decrease)  
406 and that CuO-NPs effects on soil ecotoxicology and ecotoxicogenomic parameters were likely caused by the  
407 NPs themselves and not by released ions (Gomes et al., 2012b). Furthermore, both nanoscale and ionic Cu  
408 induced oxidative stress, with overt differences evident between the two copper forms (Gomes et al., 2012a).  
409 CuO-NPs showed no effects on mortality or the growth rate of *Capitella teleta* under sediment exposure at  
410 250 mg CuO g<sup>-1</sup>, whereas the ionic form caused a 26% increase in mortality, demonstrating that Cu in the  
411 ionic form is more toxic than the nanoscale form (Dai et al., 2015). Gomes et al. concluded that both  
412 nanoscale and ionic Cu induced oxidative stress, with overt differences evident between the two copper

413 forms (Gomes et al., 2012a). At 1000 mg kg<sup>-1</sup> of CuO-NPs, a significant reduction was reported in *Metaphire*  
414 *posthuma* SOD levels, total coelomocyte count (with maximum depletion as 15.45 and 12.5 cells mL<sup>-1</sup>), and  
415 population density as compared to copper sulphate (Gautam et al., 2018). Mudunkotuwa et al. demonstrated  
416 that organic acids in soils and natural environments highly affect the mobility and aggregation behaviour of  
417 Cu-NPs and CuO-NPs (1.0 g L<sup>-1</sup>) through ligand promoted dissolution (Mudunkotuwa et al., 2012).  
418 Furthermore, organic acid adsorption to NP surface drives dissolution and the release of metal ions (Schrand  
419 et al., 2010). Cu bioavailability determines toxicity in the environment as demonstrated by the fact that: 1)  
420 small CuO-NPs are more toxic than larger particles; 2) NPs toxicity is enhanced due to positive charges  
421 facilitating interactions between cells and NPs; and 3) CuO-NPs dissolution and toxicity depends heavily on  
422 pH and temperature of the solution (Chang et al., 2012).

423 Unrine et al. (2010) found that oxidized Cu NPs can enter food chains through the soil, but that  
424 direct toxicity to earthworms might likely only occurs at high concentrations, e.g., >65 mg Cu kg<sup>-1</sup> soil  
425 (Unrine et al., 2010). Trophic transfer (TTF) of CuO NPs and dissolved Cu from earthworms (*Tubifex*  
426 *tubifex*) to fish (*Gasterosteus aculeatus*) occurred for both forms of Cu. Interestingly, the transfer of CuO  
427 NPs from *T. tubifex* to the fish was more limited compared to that of dissolved Cu (Lammel et al., 2019).  
428 Given the limited number of published studies and the significant potential of nanoscale Cu in nano-enabled  
429 agriculture, many unanswered questions related to fate and effects on earthworm species need to be  
430 addressed. Many of these knowledge gaps are similar to those for Ag-NPs and ZnO-NPs. In addition, future  
431 studies are needed to assess the fundamental mechanisms controlling toxicity and accumulation in complex  
432 multi-species exposure scenarios. Also, given the potential agricultural applications, an understanding of  
433 interactions with other analytes of concern is needed.

#### 434 **4.4 Zero-valent iron and iron oxide**

435 The most common forms of FeO-NPs are magnetite (Fe<sub>3</sub>O<sub>4</sub>), maghemite (γ-Fe<sub>2</sub>O<sub>3</sub>), and hematite (α-  
436 Fe<sub>2</sub>O<sub>3</sub>) (Ali et al., 2016). Nano-zerovalent iron (Fe<sup>0</sup>, nZVI) is known as a cost-efficient agent used for the  
437 degradation of several environmental pollutants (Yang et al., 2010). As such, there has been interest in the  
438 toxicity of nZVI to earthworm species. Between 2010-2020, 12 studies were published that report on  
439 ecotoxicity of Fe-based NPs to earthworms (Table S1). Due to this modest number of publications and the  
440 complexity of Fe chemistry in soils, the influence of nanoscale Fe based NPs on bioavailability and toxicity  
441 is not well understood.

442 Liang et al (2017) reported that exposure to nZVI (~50 to 100 nm) coated with 1-nm iron oxide  
443 shells at 500 and 1000 mg kg<sup>-1</sup> for 7, 14, 21, or 28 days increased avoidance response and inhibited the  
444 growth and respiration in *E. fetida* (Liang et al., 2017). Interestingly, the inhibitory effects of nZVI at 1000  
445 mg kg<sup>-1</sup> appeared to be both time and dose dependent, with growth reduction and avoidance behaviour at 15  
446 and 40%, respectively, at the highest nZVI concentration (Liang et al., 2017). This kind of inhibition  
447 behaviour was attributed to the depletion of glycogen and lipid content, as well as decreased in protein  
448 content. After incubation of nZVI with earthworms for 28 days, the SOD activity was significantly inhibited  
449 in worms exposed to low (100 mg kg<sup>-1</sup> nZVI) and high (1000 mg kg<sup>-1</sup> nZVI) concentrations (Liang et al.,  
450 2017). Liang et al. (2017) proposed that nZVI induced oxidative stress by upregulating antioxidant enzyme  
451 activities of SOD and CAT in response to the accumulation of reactive oxygen species (ROS) in the tissues.  
452 However, despite the high nZVI concentration (1000 mg kg<sup>-1</sup>), no changes in the body weight or  
453 reproduction of *E. fetida* were observed (Yoon et al., 2018). Similarly, the survival of the *E. fetida* was not  
454 affected by nZVI even at concentrations as high as 3000 mg kg<sup>-1</sup>. However, DNA damage and lipid oxidation  
455 were reported (Yirsaw et al., 2016). Another study showed negative effects of nZVI at 500 and 1000 mg  
456 kg<sup>-1</sup> on earthworm dermal tissues, such as disorganized texture, symptoms of dehydration, and lacerations  
457 in the intersegmental furrows or setae (Liang et al., 2017). Liang et al. suggested that this damage originated  
458 from reactive oxygen species (ROS) induced after nZVI exposure (Liang et al., 2017). Valerio-Rodríguez et  
459 al. (2018) reported that Fe<sub>2</sub>O<sub>3</sub>-NPs above 150 mg kg<sup>-1</sup> were highly inhibitory to *E. fetida* reproduction but  
460 that no effects were observed on reproduction at doses lower than 150 mg kg<sup>-1</sup> (Ali et al., 2016). Samrot et  
461 al. (2017) reported that synthesized magnetite Fe-NPs (17 and 28 nm) in aqueous study easily penetrated *E.*  
462 *eugeniae* epithelium, causing the deposition of lipofuscin in the circular muscle (fibrosis), erosion of the  
463 epithelium, and gut disintegration at 0.2 and 0.4 mg in 0.01 L<sup>-1</sup>.

464 El-Temsah and Joner (2012) exposed *E. fetida* and *L. rubellus* to nZVI that had been aged in non-  
465 saturated soil for 30 d prior to earthworm addition. Earthworm reproduction was negatively affected at 100  
466 mg kg<sup>-1</sup>, but nZVI toxicity was significantly reduced when compared to non-aged soils. High nZVI  
467 concentrations (≥500 mg nZVI kg<sup>-1</sup> soil) induced avoidance, weight loss, and mortality (79% and 89%  
468 mortality was observed for *E. fetida* and *L. rubellus* in sandy loam soil, while no mortality was observed at  
469 the same concentration with LUFA 2.2 soil). More specifically, the LC<sub>50</sub> values for acute toxicity (14 days  
470 exposure) were 399 and 447 mg kg<sup>-1</sup> of soil-aged nZVI for *E. fetida* and *L. rubellus*, respectively. In addition,

471 experiments conducted under the OECD test guideline yielded acute LC<sub>50</sub> value of 866 mg kg<sup>-1</sup> nZVI for *L.*  
472 *rubellus* after 14 days in soil. This study showed that avoidance behaviour was quite similar at 511-582 mg  
473 kg<sup>-1</sup> for both types of soil (sandy loam and LUFA 2.2 soil) and earthworm species (El-Temsah and Joner,  
474 2012).

475 Importantly, TTF of Fe-based NPs has been studied in different terrestrial and aquatic species  
476 (Baker, 2017; Hyseni, 2016; Tangaa et al., 2016) but no studies have investigated these phenomena in  
477 earthworms. Therefore, Fe-NPs bioaccumulation and toxicity mechanisms in different trophic levels are still  
478 unclear and need to be characterized. In addition, few studies have evaluated the impacts of particle  
479 weathering and transformation on bioavailability and toxicity to earthworm species. Similar to other particle  
480 types, the long-term potential ecotoxicological effects of chronic low dose exposure need to be evaluated,  
481 potentially with the use of detailed or even 'omic biomarker responses.

#### 482 **4.5 Titanium oxide**

483 TiO<sub>2</sub>-NPs have a wide range of uses, mostly in commercial products (Hu et al., 2010). Nano-TiO<sub>2</sub>  
484 can also be used as a nanoagrochemical (Kah et al., 2013). The PEC value of TiO<sub>2</sub>-NPs in Europe has been  
485 estimated at 1.01-4.45 µg kg<sup>-1</sup>, 70.6-310 µg kg<sup>-1</sup>, 100-433 mg kg<sup>-1</sup> in soil, sludge-treated soil and sewage  
486 sludge, respectively (Gottschalk et al., 2015; Gottschalk et al., 2009a); this suggests that sewage sludge poses  
487 the greatest source of TiO<sub>2</sub>-NPs hazard for earthworms. Importantly, even soils that are treated with such  
488 TiO<sub>2</sub>-NP contaminated sewage sludge exhibit concentrations that are orders of magnitude lower than the  
489 concentrations used in many publications (≥1000 mg kg<sup>-1</sup>) (Hu et al., 2010). Between 2010-2020, there were  
490 14 published reports on titanium dioxide nanoparticle (TiO<sub>2</sub>-NPs) toxicity to earthworms (Table S1),  
491 consisting of 9 full life cycle (28-58 days) studies and 5 shorter term studies (7-14 days).

492 As with other particles, the toxicity of TiO<sub>2</sub>-NPs can depend on particle size, purity, surface coating,  
493 crystallinity, shape, and solubility (Di Virgilio et al., 2010; Shah et al., 2017). McShane et al. (2012)  
494 investigated the reproduction, juvenile growth and avoidance behaviour of earthworms (*E. fetida* and *E.*  
495 *Andrei*) in soils spiked with TiO<sub>2</sub>-NPs by two methods: 1) liquid dispersion and 2) dry powder mixing. Both  
496 amendment protocols yielded similar findings; low (200 mg kg<sup>-1</sup>) and high (10,000 mg kg<sup>-1</sup>) concentrations  
497 of pure TiO<sub>2</sub>-NPs (5, 10 and 21 nm) showed no adverse effects on *E. andrei* and *E. fetida* on any parameters,  
498 including avoidance behaviour, juvenile survival and growth, adult earthworm survival, cocoon production,  
499 cocoon viability and total number of juveniles hatched from cocoons. Heckmann et al. (2011) used uncoated

500 TiO<sub>2</sub>-NPs that were characterized for particle size, surface charge, agglomeration, purity and chemical  
501 composition; the authors reported a 49% reduction in the number of juveniles upon exposure to 1,000 mg  
502 kg<sup>-1</sup> crystallite sized spherical, multi-faced and elongated TiO<sub>2</sub>-NPs (nominal particle size: 21 nm) under  
503 similar incubation conditions. Similar to other NPs, aggregation, agglomeration and surface area of TiO<sub>2</sub>-  
504 NPs are factors governing the relationship between earthworm reproduction and avoidance behaviour.  
505 Conversely, no negative effects were reported on the number of adults, reproduction, juvenile growth,  
506 number of cocoons, and number of shells of *E. fetida* cocoons in a long term study (over 90 days) at lower  
507 doses of 150 or 300 mg kg<sup>-1</sup> TiO<sub>2</sub>-NMs (Sánchez-López et al., 2019).

508 In soils amended with 200 and 10,000 mg kg<sup>-1</sup> TiO<sub>2</sub>-NPs, the reproductive rate of *E. andrei* was up  
509 to twice as high as that of *E. fetida* but did vary with experimental conditions, such as artificial and natural  
510 soil exposure (McShane et al., 2012). This highlights the importance of species-specific responses to NPs  
511 exposure in general (Domínguez et al., 2005). Lapied et al. (2011) reported that no lethal effects for *L.*  
512 *terrestris* at 100 mg kg<sup>-1</sup> TiO<sub>2</sub>-NPs. In the same study even at high concentrations (1000 mg kg<sup>-1</sup>), an aqueous  
513 suspension TiSiO<sub>4</sub>-NPs of < 50 nm caused no toxicity to *E. andrei*. However, TiO<sub>2</sub>-NPs induced  
514 mitochondrial injury at 5000 mg kg<sup>-1</sup> in *E. fetida* (Hu et al., 2010); interestingly, the authors indicate that  
515 TiO<sub>2</sub>-NPs toxicity was probably due to changes the crystal structure. TiO<sub>2</sub>-NPs are known to cause oxidative  
516 stress originating from reactive oxygen species (ROS) generation (Khalil, 2015); histopathology has shown  
517 this leads to cuticle loss from the body wall and muscle damage by breakage and shrinkage of cells (Priyanka  
518 et al., 2018). Moreover, Ti bioaccumulation in *E. fetida* was significantly increased when soil was amended  
519 with 150 mg TiO<sub>2</sub>-NPs kg<sup>-1</sup> compared to the control treatment (0 mg kg<sup>-1</sup> NPs) (Valerio-Rodríguez et al.,  
520 2020), although no toxic effects on growth parameters were found (Zhu et al., 2020).

521 At the biochemical level, environmentally relevant concentrations (1-100 mg kg<sup>-1</sup>) of TiO<sub>2</sub>-NPs  
522 caused significant impairment of key processes such as reducing *Pheretima hawaiiiana* acetylcholinesterase  
523 (AChE) by 70%, SOD activity by 130%, CAT activity by 161%, glycogen content by 6%, total lipid by 92%  
524 and total soluble proteins by 82% (Khalil, 2015). Similarly, Lapied et al. (2011) reported that exposure of *L.*  
525 *terrestris* to 15 mg kg<sup>-1</sup> TiO<sub>2</sub>-NPs in the soil for four weeks significantly increased apoptotic frequency in  
526 the cuticle and intestinal epithelium.

527 Importantly, the findings of the long-term lab-based studies with TiO<sub>2</sub>-NPs do not align well with  
528 the shorter-term studies. An explanation for this difference may be found in the fate of the TiO<sub>2</sub>-NPs. For

529 example, when nanoscale TiO<sub>2</sub> is exposed to clay particles, the materials can heteroaggregate over time,  
530 effectively minimizing accumulation by and exposure to earthworm species (Sánchez-López et al., 2019;  
531 Shi et al., 2017). This highlights that both particle properties and environmental conditions control fate of  
532 TiO<sub>2</sub>-NPs, and can explain unexpected variation in toxicological data (Peijnenburg et al., 2015; Wagner et  
533 al., 2014). Additional studies are needed that address the effects of TiO<sub>2</sub>-NPs on earthworms at the molecular  
534 level, including the use of critical biomarkers to enable understanding of the mechanisms of response.

535

#### 536 **4.6 Aluminum oxide**

537 Aluminium oxide NPs are produced in the form of nanocomposites with other metals and have  
538 demonstrated efficiency in the removal of fluoride pollutants for environmental applications (Liu et al.,  
539 2015). There are 5 studies investigating Al<sub>2</sub>O<sub>3</sub>-NPs toxicity to earthworm species from 2010-2020 (Table  
540 S1).

541 When nanoscale and microparticles of Al<sub>2</sub>O<sub>3</sub> were introduced into soil containing adult  
542 *Dendrobaena veneta*, a strong correlation was found between particle size and the levels of water-soluble  
543 and EDTA-extractable aluminium. Importantly, earthworm soil activities such as digestion, excretion and  
544 repeated ingestion substantially affect the speciation of Al and can result in reduced bioavailability of the  
545 metal. Bystrzejewska-Piotrowska et al. (2012) reported the bioaccumulation of Al in earthworm tissues in  
546 both nanoscale and microparticles (MPs) form, although uptake was 95% higher for the smaller particles.  
547 Al<sub>2</sub>O<sub>3</sub> NPs at 3.69 mg kg<sup>-1</sup> increased the Al content in *D. veneta* as compared to Al<sub>2</sub>O<sub>3</sub> MPs (1.89 mg kg<sup>-1</sup>)  
548 after depuration. However, Al<sub>2</sub>O<sub>3</sub>-NPs in soil eluates and *D. veneta* tissues were determined after 1 and 10  
549 days before and after gut cleansing (depuration) but no accumulation of Al was found. Furthermore, the  
550 presence of Al<sub>2</sub>O<sub>3</sub>-NPs in soils can significantly influence the bioavailability and toxicity of metals *D. veneta*  
551 (Bystrzejewska-Piotrowska et al., 2012). Interestingly, disk-shaped Al<sub>2</sub>O<sub>3</sub> NPs were not toxic to *D. veneta*  
552 after a 10 day exposure even at the unrealistically high concentration of 10 g NPs kg<sup>-1</sup> of soil (Bystrzejewska-  
553 Piotrowska et al., 2012). However, high NPs concentrations in soil greatly affect soil-metal equilibrium and  
554 metal extractability. Reproduction of *E. fetida* was negatively affected at 3,000 mg kg<sup>-1</sup> of large sized Al<sub>2</sub>O<sub>3</sub>  
555 (50-200 µm), but agglomerated nanometric Al<sub>2</sub>O<sub>3</sub> (11 nm) was found to be non-toxic. However, exposure  
556 to high concentrations (3,000-5,000 mg kg<sup>-1</sup>) of both micro- and nanometric Al<sub>2</sub>O<sub>3</sub> induced avoidance  
557 behaviour of *E. fetida* (Coleman et al., 2010). Importantly, these high concentrations are highly unrealistic

558 in the soil environment. Similarly, no mortality or negative influence on the reproductive behaviour of *E.*  
559 *fetida* were observed upon exposure to 1000 mg kg<sup>-1</sup> Al<sub>2</sub>O<sub>3</sub>-NPs (Heckmann et al., 2011). At 3,000 mg kg<sup>-1</sup>,  
560 a significant reduction in earthworm enzyme activities, reproduction, and survival was observed. These  
561 two studies also highlight contradictory findings as a function of dose on reproduction. The SOD and CAT  
562 activities in *E. fetida* decreased following exposure to Al<sub>2</sub>O<sub>3</sub>-NPs at 3,000 mg kg<sup>-1</sup> (Yausheva et al., 2017).  
563 Drawing further conclusions on Al<sub>2</sub>O<sub>3</sub>-NPs is difficult given the limited existing literature and as such, there  
564 is clearly much work to be done involving multiple dosing and exposure regimes and involving a broad  
565 range of biochemical, physiological and molecular endpoints.

#### 566 **4.7 Nickel oxide**

567 Nickel oxide nanoparticles (NiO-NPs) have unique properties that have led to use in a number of  
568 commercial applications in the electronic industry; however, the estimated production of NiO- NPs is still  
569 modest at approximately 20 tons per year in the United States (Gomes et al., 2019). From 2010-2020, there  
570 were three published reports on NiO-NPs toxicity to earthworms (Table S1). It is noted that no exposure  
571 study could be found in which PEC values for NiO-NPs were determined (as of July 2021).

572 Adeel et al reported that spherical NiO-NPs (30 nm size) at 5, 50 and 200 mg kg<sup>-1</sup> had no impact on  
573 the survival, reproduction and growth rate of adult *E. fetida* (Adeel et al., 2019b). However, reproduction  
574 was reduced by 50 - 70% at 500 and 1000 mg kg<sup>-1</sup>. Ultrastructural and histological observations of  
575 earthworm tissues exposed to 500 - 1000 mg kg<sup>-1</sup> NiO-NPs (30 nm) for 28 days showed abnormalities in the  
576 epithelial layer, microvilli, and mitochondria, including underlying pathologies of the epidermis and  
577 muscles, as well as adverse effects on the gut barrier (Adeel et al., 2019b). Similar results were reported in  
578 a separate study with *Enchytraeus crypticus* exposed to NiO-NPs (40 nm cubic); here an EC<sub>50</sub> of 870 mg  
579 kg<sup>-1</sup> was reported and negative effects were evident on embryo development, yielding a reduced number of  
580 juveniles in later life stages (Santos et al., 2017).

581 Another study found that exposure of 250 mg kg<sup>-1</sup> NiO-NPs in vegetable residues resulted in Ni  
582 retained in earthworm gut for over four weeks (Antisari et al., 2015). Furthermore, exposure over 7 and 28  
583 days to Ni-NPs at higher concentrations (>500 mg kg<sup>-1</sup>) caused oxidative stress which led to DNA damage  
584 and increased of proteolysis, apoptosis and inflammatory response, as well as interference with the nervous  
585 system (Gomes et al., 2019). Importantly, nothing is known about the TTF of NiO-NPs, as longer-term  
586 studies has thus far only focussed on understanding the impacts on behavior, as well as biochemical and



587 molecular responses of the exposed species. There are several significant knowledge gaps related to NiO-  
588 NPs which need to be addressed for thorough and accurate risk assessment: 1) Fate and transport mechanism  
589 of NiO-NPs in the presence of earthworm species; 2) longer term exposure experiments at environmentally  
590 relevant doses, and 3) NiO-NPs effects on gene expression and key biomarkers (SOD, CAT, etc.).

#### 591 **4.8 Cerium oxide**

592 Ce is widely used as CeO<sub>2</sub>-NPs as catalyst, a fuel additive (Auffan et al., 2014a) and also in optics,  
593 which has raised production levels of the nanoscale form in global market up to 1000 metric tons/year.  
594 Cerium (Ce) covers approximately 0.0046% of the earth's crust by weight (Johnson and Park, 2012), and  
595 exists in three possible oxidation states; Ce are Ce<sup>2+</sup>, Ce<sup>3+</sup>, and Ce<sup>4+</sup>. The most common and stable valence  
596 for Ce is Ce (IV) oxide (CeO<sub>2</sub>), followed by cerium (III) oxide (Ce<sub>2</sub>O<sub>3</sub>) (Adeel et al., 2019a).

597 Limited data is available concerning the PEC of CeO<sub>2</sub>-NPs; Denmark's uncultivated and agricultural  
598 soil PECs are estimated at 24-1500 ng kg<sup>-1</sup> and 10-530 ng kg<sup>-1</sup>, respectively (Gottschalk et al., 2015). Data  
599 on CeO<sub>2</sub>-NPs toxicity to terrestrial invertebrates is generally scarce; there are 3 published studies addressing  
600 toxicity, and as such, drawing conclusions is difficult as there are still large knowledge gaps. The EC<sub>50</sub> and  
601 LC<sub>50</sub> values for *E. fetida* after 28-days of exposure were 294.6 and 317.8 mg Ce kg<sup>-1</sup>, respectively (Lahive  
602 et al., 2014). Some researchers have focused on direct effects of Ce-NPs to earthworms as described in  
603 Table S1. For example, Lahive et al reported that *E. fetida* exposure to CeO<sub>2</sub>-NPs at 41-10,000 mg kg<sup>-1</sup> for  
604 28 days had no effect on survival or reproduction, whereas Ce in a salt form (ammonium cerium nitrate)  
605 negatively affected both reproduction and survival at 10,000 mg Ce kg<sup>-1</sup> (Lahive et al., 2014). However,  
606 histological analysis revealed potential toxicity by cuticle loss from body wall and some loss of integrity of  
607 the gut epithelium exposed to CeO<sub>2</sub>-NPs. This suggests that the earthworms are negatively affected by  
608 exposure to lower doses, although proper endpoint selection will be an important factor in accurately  
609 assessing toxicity and risk.

610 A complex link between NP accumulation and toxicity has been reported (Auffan et al., 2014b). Ce  
611 accumulation in *L. rubellus* tissues (5.3 mg kg<sup>-1</sup>) and feces (49 mg kg<sup>-1</sup>) was evident after 7 days of exposure  
612 to CeO<sub>2</sub>-NPs amended soil (at 5000 mg kg<sup>-1</sup>) (Antisari et al., 2012). No accumulation of Ce was evident *E.*  
613 *fetida* as the NPs were rapidly removed (excreted) when worms were moved to clean soil (Carbone et al.,  
614 2016). In addition, no studies have been done to assess the TTF of CeO<sub>2</sub>-NPs from earthworm species to

615 their predators. Clearly additional work is needed to understand the risk of TTF to the food web and  
616 potentially to human health.



617  
618 **Figure 4** Potential MNOs toxic effects and mechanisms reported for earthworms at the organism, organ,  
619 cellular, biochemical and genetic level. At the organism level, MNOs exposure can cause changes in  
620 endpoints such as the avoidance, survival, growth and locomotion. At the organ level, dermal and intestinal  
621 barrier and pathway abnormalities have been reported by histopathological observation. At the biochemical  
622 level, impacts on different stress pathways have been reported, including the general stress and oxidative  
623 stress. These responses could activate signalling cascades that modulate key genetic markers.

624

#### 625 **4.9 Lanthanum**

626 Lanthanum (La) is often applied as manufactured NPs mainly exist in lanthanum hydroxide and  
627 lanthanum oxide. Lanthanum nanoparticles (La-NPs) are white spherical metal particles typically 40 nm in  
628 size with surface area range 130-150 m<sup>2</sup>g<sup>-1</sup> (Brabu et al., 2015). China is one of the leading countries in  
629 production of La and total production of La exceeds 30,000 metric tons worldwide (Reilly, 2019). Recently,  
630 Adeel et al exposed *E. fetida* to various concentration level (25, 50, 100, 200, 500 and 1000 mg kg<sup>-1</sup>) of both  
631 forms (nanoscale and bulk) of La<sub>2</sub>O<sub>3</sub>, concluded that at 100 mg kg<sup>-1</sup>, nanoscale and bulk La<sub>2</sub>O<sub>3</sub> induced

632 earthworm mortality by 33-35% and reduced reproduction by 10-32% respectively. Ultrastructural  
633 observations revealed that nanoscale and bulk REOs at higher doses (500 and 1000 mg kg<sup>-1</sup>) induced  
634 abnormalities in the internal organelles, including mitochondria, Golgi apparatus and chloragosomes.  
635 Nanoscale La<sub>2</sub>O<sub>3</sub> significantly reduced the earthworm digestive (glutathione S-transferase, total glutathione,  
636 glutathione reductase, glutathione peroxidase) and cast enzymes (SOD, POD, CAT and MDA) by 20-80%  
637 at medium and higher concentrations as compared to bulk La<sub>2</sub>O<sub>3</sub>. Results suggest that beta-glucosidase and  
638 alkaline phosphatase were the most sensitive and lipase was the least sensitive digestive enzyme to La<sub>2</sub>O<sub>3</sub>-  
639 NPs toxicity. Interestingly, study reported that earthworms provided a protective role to minimize the toxic  
640 effects of nanoscale and bulk La<sub>2</sub>O<sub>3</sub> on the microbial biomass carbon and soil enzymes at 100-200 mg kg<sup>-1</sup>  
641 (Adeel et al., 2021c). Overall, long-term field studies are needed to enhance the understanding of processes  
642 and bioaccumulation of rare earth oxides (REOs) in *E. fetida*, and to elucidate the risk and recovery potential  
643 of earthworms in the presence of agricultural plants.

#### 644 **4.10 Ytterbium**

645 Ytterbium nanoparticles (Yb-NPs) is a unique material, has been growing rapidly and applied in  
646 diverse fields, such as nano electronic devices including light emitting diodes, solid-state lasers, fertilizer  
647 industry and environmental remediation (Venkata Krishnaiah et al., 2013; Zhu et al., 2014). Nowadays,  
648 China is one of the leading country containing REOs reservoirs of about 44 million metric tons (Adeel et al.,  
649 2019a). Global demand of Yb<sub>2</sub>O<sub>3</sub> was estimated in 2017 to be 5,000 to 7,000 tons according to Mineral  
650 commodity summaries 2018, Reston, Virginia, USA (Ober, 2018). Recently, Adeel et al., (2021) reported  
651 that nanoscale and bulk Yb<sub>2</sub>O<sub>3</sub> induced earthworm mortality by 13-15%, and reduced reproduction by 10-  
652 12%, at 100 mg kg<sup>-1</sup>. In another study Adeel et al. determined the biochemical, genetic, and histopathological  
653 effects on *E. fetida* exposed to Yb<sub>2</sub>O<sub>3</sub> at 50, 100, 200, 500 and 1000 mg kg<sup>-1</sup>. This study reported that Yb<sub>2</sub>O<sub>3</sub>  
654 treatment induced neurotoxicity in earthworm by inhibiting acetylcholinesterase by 22-36% at 500 and 1000  
655 mg kg<sup>-1</sup>. Additionally, Yb<sub>2</sub>O<sub>3</sub> at 100 mg kg<sup>-1</sup> significantly down-regulated the expression of annetocin  
656 mRNA in the parental and progeny earthworms by 20%, which is crucial for earthworm reproduction.  
657 Similarly, expression level of heat shock protein 70 (HSP70) and metallothionein was significantly  
658 upregulated in both generations at medium exposure level (Adeel et al., 2021b; Adeel et al., 2021c). In  
659 future, long term field experiments should be conducted to understand the underlying processes and potential

660 risks associated with Yb containing fertilizers. Additional long-term research is needed to understand the  
661 underlying processes and potential risks that nanoscale fertilizers could pose to soil ecology.

#### 662 **4.11 Silica**

663 Silica (Si) NPs are highly crystalline materials (Tchalala et al., 2018) primarily used in agricultural  
664 and commercial products (Kah et al., 2013) (Buchman et al., 2019). For example, porous hollow SiO<sub>2</sub>-NPs  
665 can be used as carriers to control the release and shield from UV of neurotoxins or antibiotics (Li et al., 2007;  
666 Li et al., 2006). In 2016, annual production of Si-NPs was approximately 93,300 tons worldwide which is  
667 3<sup>rd</sup> largest production followed by nTiO<sub>2</sub> and nFeOx and increased gradually year by year (Song et al.,  
668 2017c). The PEC value of SiO<sub>2</sub>-NPs can range from 86-150000 µg kg<sup>-1</sup> in natural, urban or sludge-treated  
669 soils (Wang and Nowack, 2018b). However, their detrimental effects on soil biota is not well understood.  
670 A recent study evaluated the avoidance behavior of five soil species, including *E. fetida*, in SiO<sub>2</sub>-NPs-  
671 contaminated soil. At 100 mg kg<sup>-1</sup>, SiO<sub>2</sub>-NPs induced 50% avoidance behavior (Santos et al., 2020), clearly  
672 highlighting the need for further studies to understand toxicity mechanisms in soil environment. Similarly,  
673 Di Marzio et al (2018) investigated the effect of surface charge of Si -NPs on coelomic cells from *E. fetida*;  
674 the data suggested a strong genotoxic effect at 1 µg m L<sup>-1</sup> with LC<sub>50</sub> values of 73.9 µg m L<sup>-1</sup> (negative charge)  
675 and 116.9 µg mL<sup>-1</sup> (positive charge). The observation that negatively charged NPs were twice as toxic as  
676 positively charged particles highlights the need to understand how material properties control nanoscale fate  
677 and effects (Di Marzio et al., 2018). With only two published studies, clearly more research is needed to  
678 understand not only the mechanisms of toxicity to key species, but also the role of material properties and  
679 environmental factors controlling toxicity, TTF, and risk.

#### 680 **4.12 Quantum Dots**

681 Semiconducting quantum dots (QDs) have attracted significant attention for use in energy,  
682 electronics, solar cells and for biomedical applications. These fluorescent nanocrystals have a size that varies  
683 depending on the material composition and synthesis method, with most types being approximately 1 to 10  
684 nm (Liu et al., 2020). In 2012, global production of QDs was approximately 135 tons and increased gradually  
685 year by year (Future Markets, 2012). QDs are used at laboratory scale as fluorescent markers in nanosafety  
686 research (Murray et al., 2018), but they are currently not used as nanoagrochemical. Although QDs are  
687 already used for micro-LEDs, TV screens and solar cells or as fluorescent markers in medicine and

688 environmental research, only limited data are available on possible negative environmental and health risks  
689 of QDs. The PEC value for QDs was predicted to be very low at ca. 8 pg kg<sup>-1</sup> in natural and urban soils in  
690 the EU (Wang and Nowack, 2018b), but it must be stressed that available exposure model results are  
691 currently very inaccurate, as there is a lack of data on production volume of QDs in particular.

692 Over the last 10 years, 6 studies have been published on the effects of QDs on earthworm species,  
693 describing both bioaccumulation and toxicity (Table S1). From an ecotoxicological perspective, QD uptake  
694 can clearly lead to the liberation of dissolved metal components (e.g., Cd<sup>2+</sup>, Zn<sup>2+</sup> etc.) to lead to toxic effects  
695 (Rocha et al., 2017). For example, QDs, passing the biological barriers, can be taken up into the  
696 intracellularly via endocytosis, leading to the production of reactive oxygen species (ROS) that can induce  
697 oxidative stress and bimolecular damage. Likewise, QDs can penetrate cell membranes and might have a  
698 growth-inhibiting effect on the cell or on the organism (Barroso, 2011). Interestingly, earthworms have the  
699 ability to biosynthesize QDs. *L. rubellus* were exposed to soil amended with CdCl<sub>2</sub> and Na<sub>2</sub>TeO<sub>3</sub> for 11 days  
700 and metal detoxification processes in the earthworm gut (chloragogen cells) resulted in the production of  
701 biocompatible CdTe QDs (Tilley and Cheong, 2013). The earthworms did not seem to be physiologically  
702 affected by the presence of CdTe QDs in their systems, possibly because the generated QDs are coated with  
703 a passivating layer that stabilizes the QDs in biological media. This highlights an interesting metal  
704 detoxication strategy; however, the presence of Cd-containing QDs could still pose a risk to organisms within  
705 the food chain that feed on these earthworm species (Tilley and Cheong, 2013).

706 Kominkova et al., (Kominkova et al., 2014) also studied the biosynthesis of QDs in *E. fetida*.  
707 Earthworms exposed to CdTe QDs showed oxidative stress and antioxidant activity, but earthworms exposed  
708 to CdTe were less negatively affected than with dissolved Cd<sup>2+</sup>. The expression of metallothionein was partly  
709 responsible for the ability of organisms to cope with heavy metals exposure. Stürzenbaum et al., studied the  
710 biosynthesis of QDs in *L. rubellus*. Earthworms were exposed to soil amended with CdCl<sub>2</sub> and Na<sub>2</sub>TeO<sub>3</sub> for  
711 11 days, resulting in the biosynthesis of CdTe QDs that showed optical characteristics typical of quantum-  
712 confined semiconductor materials useful for cell imaging applications (Stürzenbaum et al., 2013). The  
713 bioaccumulation potential and toxic effects of CdTe QDs to *E. fetida* was studied by Tatsi et al (2020)  
714 according to procedures outlined in OECD TG 222. The earthworms were exposed to 50, 500 and 2000 mg  
715 CdTe QD kg<sup>-1</sup> (dry weight) for 28 days, as well as in aged soil (Tatsi et al., 2020). The aged soil was the  
716 same soil used in the initial fresh soil experiments but the earthworms were added only after 6 months of

717 incubation. No effects on survival were noted, but some reductions of growth were observed at the higher  
718 doses, with juvenile production being the most sensitive endpoint. The nominal EC<sub>50</sub> of values were > 2000,  
719 108, 65, 96 mg CdTe kg<sup>-1</sup> for bulk, PEG-, COOH<sup>-</sup> and NH<sub>4</sub><sup>+</sup>-coated CdTe QDs in fresh soil (Tatsi et al.,  
720 2020), again highlighting the significance of particle (surface) chemistry to overall toxicity. The  
721 accumulation of QDs from six-month aged soil was higher, leading to reduced growth and survival of the  
722 adult worms relative to unaged soil. The nominal EC<sub>50</sub> values for juvenile production in the aged soil were  
723 165, 88, 78 and 63 mg CdTe kg<sup>-1</sup> for microscale material, PEG-, COOH- and NH<sub>4</sub><sup>+</sup>-coated CdTe QDs,  
724 respectively (Tatsi et al., 2020). Thus, exposure to nanoscale CdTe QDs, regardless of coating, caused greater  
725 toxicity than the microscale CdTe materials, and toxicity increased after aging of the materials in soil (Tatsi  
726 et al., 2020). Apart from toxicity to individual cells or organisms, QDs could also enter higher levels of the  
727 food chain by TTF. This poses a risk not only to the organisms themselves, but also to the food chain and  
728 potentially human health; as such, additional work in this area is needed.

## 729 **5. Carbon-based nano-objects**

### 730 ***5.1 Carbon nanotubes***

731 Carbon nanotubes (CNTs) are a carbon allotrope with cylindrical nanostructure. Two principal types  
732 of CNTs can be produced, which are single-walled carbon nanotubes (SWCNTs) with single sheet of  
733 graphene and multi-walled carbon nanotubes (MWCNTs) with 2-50 graphene cylinders (Liné et al., 2017).  
734 Due to wide application of CNTs in the electronic industry and in other environmental engineering  
735 applications, there has been concern about exposure of CNTs in environment (Kim et al., 2010a). The  
736 global demand for CNTs was projected to be around U.S.\$4.5 billion, suggesting an  
737 expected increase of more than US\$ 10-15 billion in 2026 (Statista, 2020). CNTs could  
738 be applied as nanoagrochemical, for example, to inhibit virus replication and movement (Adeel et al., 2021a).  
739 However, nanosafety concerns still hinder a wide application in agriculture. For CNTs, a PEC value of ca.  
740 35 ng kg<sup>-1</sup> in natural and urban soil and ca. 12 µg kg<sup>-1</sup> in sludge-treated soils was predicted using an exposure  
741 model for the EU (Sun et al., 2016b).

742 There are only 7 published studies investigating the toxicity of CNTs to earthworm species (Table  
743 S1). For example, Scott-Fordsmand et al. (2008) reported that *E. veneta* produced 60% fewer cocoons when  
744 exposed to 495 mg kg<sup>-1</sup> double-walled nanotubes in contaminated food for 28 days. However, physiological

745 endpoints such as hatchability, survival or mortality remained unchanged after 28-d exposure to  
746 concentrations up to 495 mg kg<sup>-1</sup>. Conversely, Calisi et al. (2016) revealed that *E. fetida* exposed to 30 and  
747 300 mg kg<sup>-1</sup> of MWCNTs for 14 days caused changes in cellular and biochemical markers, including  
748 morphometric alteration in immune cells, destabilization of lysosomal membranes, acetylcholinesterase  
749 inhibition and changes in concentration of metallothionein tissues. A number of papers have been published  
750 that focus on how carbon nanotubes alter the toxicity of co-contaminants. Hu et al. (2013) reported that  
751 MWCNTs at 1000 mg kg<sup>-1</sup> increased the bioavailability and toxicity of nonylphenol (NP) to *E. fetida*.  
752 Furthermore, these studies reported that although MWCNTs are not readily absorbed and accumulated in  
753 earthworm tissues, the nanomaterial may cause harmful effects indirectly, including DNA damage, by  
754 releasing contaminants that have sorbed on their surfaces. Hu et al.(2014) examined the combined effect of  
755 MWCNTs and sodium pentachlorophenate (PCP-Na) on *E. fetida* in artificial soil samples and found 100 %  
756 survival of earthworms upon exposure to levels up to 1000 mg kg<sup>-1</sup> MWCNTs for 14 days. In addition, a  
757 laboratory-based study demonstrated that the mixture of MWCNTs and PCP-Na induced different  
758 expression levels of biochemical markers (Zhang et al., 2014). Interestingly, negligible negative effects were  
759 observed upon exposure to MWCNTs or PCP-Na individually, whereas under simultaneous exposure  
760 MWCNTs partially alleviated the toxicity on *E. fetida* due to contaminant adsorption. Notably, the BAF of  
761 MWCNTs in earthworms was low ( $0.015 \pm 0.004$ ) when they were exposed at 60 mg MWNTs kg<sup>-1</sup>  
762 (Kalinowska et al., 2013). Similarly, another study reported the limited accumulation of MWCNTs into body  
763 tissues due to minimum absorption; in addition, an exponential decay model suggested ready elimination of  
764 accumulated MWNTs (Petersen et al., 2011). Importantly, the impact of carbon-coated NPs, either through  
765 intended functionalization or natural processes, on toxicity to earthworms is currently unknown. It is  
766 important to understand the toxicity of these materials under environmentally realistic exposure scenarios.  
767 Importantly, these types of studies will provide useful information for the development of safe and  
768 sustainable novel C-based composites for a range of environmental applications, such as wastewater  
769 treatment and soil remediation.

## 770 **5.2 Buckminsterfullerene**

771 Buckminsterfullerene (C<sub>60</sub>) is carbon-based engineered nanoparticle with high organic carbon  
772 normalized partition coefficients of log K<sub>oc</sub> = 6.2-7.1 (Kausar, 2017) that has been of interest for some time.

773 C<sub>60</sub> can be used, e.g., for solar cells (Nelson, 2011), from which they can be unintentionally released into the  
774 environment. It is noted that we could not find PEC values for soils.

775 Li and Alvarez (2011) reported no significant impact on reproduction and avoidance behavior of *E.*  
776 *fetida* upon exposure to 10,000 mg kg<sup>-1</sup>; suggesting very little hazard associated with that C<sub>60</sub> exposure in  
777 soil (Li and Alvarez, 2011). Similarly, another study found that low concentrations (5 -10 mg kg<sup>-1</sup>) induced  
778 non-significant effects on weight and cuticle fibers of *L. Variegatus* and *E. fetida* (Kelsey and White, 2013;  
779 Pakarinen et al., 2011). Alternatively, Van der Ploeg et al. (2011) found that C<sub>60</sub> at 154.4 mg kg<sup>-1</sup> had  
780 significant effects on cocoon production, juvenile growth rate and mortality after 28 days exposure of the  
781 parental generation. Another study revealed histological tissue injury and that external barriers (cuticle and  
782 gut epithelium) were partly damaged in *L. rubellus* that was exposed to 154 mg kg<sup>-1</sup> C<sub>60</sub> for 28 days.  
783 Interestingly, surviving earthworms showed evidence of tissue repair and recovery (Van Der Ploeg et al.,  
784 2013). Another study reported that *E. fetida* potentially exhibited tolerance to C<sub>60</sub> as evident by adaptive  
785 response at the molecular level. However, reduction in sugars, amino acids (leucine, valine, isoleucine and  
786 phenylalanine) and the nucleoside inosine appeared to be potential biomarkers for C<sub>60</sub> exposure (Lankadurai  
787 et al., 2015). Contradictory results were evident in *L. rubellus* as the down regulation of certain stress  
788 (HSP70) and immune (CCF-1 ) related genes was observed, but no effects on antioxidant enzyme activity  
789 were detected (Van Der Ploeg et al., 2013; van der Ploeg et al., 2014b). As such, we suggest these biomarkers  
790 should be evaluated in a targeted / dose-dependent manner across multiple species. Li et al. (2011) reported  
791 the rapid BSAF of <sup>14</sup>C-labeled C<sub>60</sub> in *E. fetida* at 0.25 mg kg<sup>-1</sup>, which indicates a risk for the food chain  
792 contamination through TTF. This underscores the need for further studies on C<sub>60</sub>, among other ENPs,  
793 regarding TTF, bio magnification potential, and associated sublethal effects under different soil  
794 environments. In addition, the sorption capacity of organic co-contaminants and how those association  
795 processes impact multi-analyte toxicity needs to be evaluated.

### 796 **5.3 Graphene oxide**

797 Graphene oxide (GO) is an advanced two dimensional material that consist of a single sheet of carbon  
798 atoms arranged in a hexagonal network (Bianco, 2013; De Silva et al., 2017; Wang et al., 2010). Although  
799 the production volume of GO increases, no PEC values for soils have been found. Within the past 10 years,  
800 2 studies were published on the effects of GO on earthworm species. Mechanical exfoliation techniques are  
801 used to generate graphene sheets having a highly ordered structure, high surface areas (2630 m<sup>2</sup> g<sup>-1</sup>), high



802 Young's modulus (1 TPa), high thermal conductivity (5000 W mK<sup>-1</sup>), strong chemical durability and high  
803 electron mobility (2.5 10<sup>5</sup> cm<sup>2</sup>V<sup>-1</sup>s<sup>-1</sup>) (Sherlala et al., 2018). It has been reported that multi-layer GO with a  
804 range of morphologies and hydrophobicity have different toxic effects on earthworm species (Zhang et al.,  
805 2020b). Metabolite levels of alanine, phenylalanine, proline, and glutamate in juvenile of *E. fetida* changed  
806 significantly after 7 days exposure to GO-MNOs at 300 mg kg<sup>-1</sup> (Zhang et al., 2020d). Given the increasing  
807 interest in the application of novel 2-dimension materials such as graphene oxide, much additional work is  
808 needed to evaluate the fate and effects of these materials in soil and on key invertebrate species such as  
809 earthworms.

#### 810 **5.4 Nanoplastics**

811 Nanoplastics are an emerging contaminant of concern that are closely related to microplastics. The  
812 largest source of microplastics originate from macro plastic objects that are unintentionally released into the  
813 environment (e.g. by littering) and break down to secondary microplastics (Andrady, 2017; Qi et al., 2020),  
814 with continuous weathering that eventually generates nanoplastics (Gigault et al., 2018). Most research to  
815 date has focused on the microplastic with particles > 10 µm in size, largely due to the resolution limits of  
816 the analytical equipment that is used to identify and quantify these materials (Shim et al., 2017). As such,  
817 there are a limited number of papers investigating nanoplastics and earthworm species; notably the use of  
818 traceable materials such as fluorescently labelled polystyrene (PS) beads enables detection with higher  
819 special resolution through the use of fluorescence microscopy.

820 Jiang et al investigated *E. fetida* exposure to micro- (diameter 1300 nm) and nanoplastic polystyrene  
821 particles (diameter 100 nm) at 1000 µg kg<sup>-1</sup> (Jiang et al., 2020). In general, the results show that the toxicity  
822 of micro- and nanoplastics in *E. fetida* was quite low. However, particle accumulation, which was four times  
823 larger for micro- than for nanoparticles, was observed. In addition, the authors used a comprehensive set of  
824 physiological and biochemical endpoints and concluded that microplastic was more toxic than nanoplastics.

825 Overall, the available literature on the hazardous effects of nanoplastics in earthworms is limited  
826 and indicates that hazards are mainly driven by mechanical damage to the intestine. Importantly, larger  
827 microscale particles appear to be more hazardous than nanoscale plastics as determined by this endpoint.  
828 However, a range of other potential toxicity mechanisms are possible for nanoplastics in earthworm species,  
829 including toxicity caused by leaching of plastic additives that could exert additional negative impacts after  
830 exposure.

831

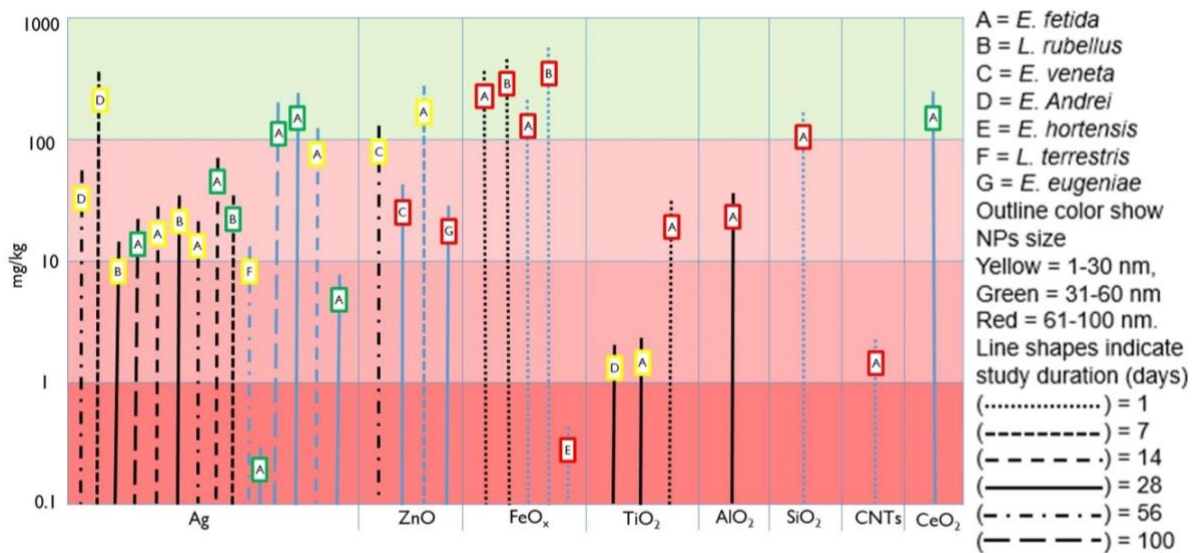
## 832 **6. Summary of current knowledge regarding MNOs toxicity to earthworms**

833 In soil, the mobility and bioavailability of metallic MNOs are controlled by physio-chemical  
834 properties of the material (e.g., size, surface charge and surface functionality) as well as by a series of  
835 different soil characteristics, of which pH and organic matter generally have the greatest impact on toxicity.  
836 It should be noted that further soil parameters (e.g., pore size, soil surface properties, hydraulic parameters  
837 or texture) and pore water characteristics (e.g., pH, content and composition of electrolytes and dissolved  
838 organic matter) do also play a role (Kah et al., 2013). However, metal and metal oxide based MNOs are  
839 prone to degrade in soils and liberate potentially toxic ions. Thus, this is a significant transformation process  
840 and often confounds attempts to understand nanoscale specific species response. As a function of dose,  
841 metallic MNOs can reduce adult and juvenile growth rates, reproduction, and respiration, as well as increase  
842 avoidance response, lethality, subcellular damage and oxidative stress. Oxidized metal oxides may have  
843 different effects than pure metal particles of the same element; for example, zero-valent Fe NPs are generally  
844 less toxic to earthworms than FeOx NPs. Harmful effects of zero-valent Fe NPs, including avoidance,  
845 mortality, and weight loss, are typically reported only at high concentrations. The toxicity of materials such  
846 as TiO<sub>2</sub>-NPs depends significantly on the size, purity, surface coating, crystallinity, shape, and solubility.  
847 These studies show that both material properties and environmental conditions control the behaviour and  
848 fate of TiO<sub>2</sub>-NPs in soil environments. In the case of Al<sub>2</sub>O<sub>3</sub>-NPs, the reviewed studies indicated that these  
849 NPs are only toxic at very high concentrations unlikely to be found in the environment. NiO-NPs reduced  
850 reproduction and were shown to affect embryo development and induce oxidative stress. NiO-NPs also had  
851 a negative effect on the soil microbial community and soil enzymes and therefore could significantly affect  
852 other terrestrial invertebrates. Exposure to CeO<sub>2</sub>-NPs showed no effect on the survival or reproduction, while  
853 Ce salt (ammonium cerium nitrate) affected both reproduction and survival at high doses. In the case of  
854 silver, the studies showed that Ag-NPs are often more toxic than Ag ions (typically AgNO<sub>3</sub> salts). In addition,  
855 soil samples containing aged Ag-NPs were more toxic to the earthworms than non-aged Ag-NPs, which led  
856 to the conclusion that the fate of the NPs was determined largely by their interaction with organic matter  
857 which increased particle toxicity through yet to be characterized transformation processes. Median values of  
858 EC<sub>50</sub> and LC<sub>50</sub> are depicted in Figure 6 as determined from 35 published papers. EC<sub>50</sub> and LC<sub>50</sub>  
859 concentrations for each MNOs significantly varied, largely due to differences in experimental design such

860 as exposure duration and MNOs size. In terms of species, *E. fetida* and *L. rubellus* were the most commonly  
 861 investigated organisms.

862 Exposure to nanoscale CdTe QDs, regardless of the respective coating material, caused greater  
 863 toxicity at the microscale, and the toxicity increased after soil aging, indicating that heavy metal ions are  
 864 leached out with time. However, colloiddally stable MNOs, including QDs, can also move through the food  
 865 chain by TTF. Due to their persistence, they can therefore also accumulate in higher organisms and/or have  
 866 toxic effects. This poses a risk not only to the organisms themselves but also to other species within the food  
 867 chain. Surprisingly, we found very few reports investigating the toxicity of rare-earth-based MNOs on  
 868 earthworms; this is a point of concern given that their application is dramatically increasing in a number of  
 869 industries. We identify knowledge gaps in the fate and effects of several elements, including La, Yb, Cr, and  
 870 Se MNOs.

871 The toxicity of carbon-based MNOs that have been intentionally functionalized or that have been  
 872 transformed/coated through natural processes to earthworms is currently poorly understood, with both  
 873 limited and contradictory results in the literature. More studies are needed to examine the impact of carbon-  
 874 based MNOs on bioaccumulation (bioconcentration and biomagnification) and TTF from soil biota to higher  
 875 levels of the food chain. Thus, targeted/dose-dependent long-term exposure experiments at environmentally  
 876 relevant concentrations are proposed .



877 **Figure 5** EC<sub>50</sub> and LC<sub>50</sub> of selected MNOs to different earthworm species recovered from the published  
 878 literature. Capital letters correspond to earthworm species, the colour outline of the capital letters signifies

879 the size of MNOs, line colour representing the EC<sub>50</sub> and LC<sub>50</sub> (Black = EC<sub>50</sub> and blue=EC<sub>50</sub>) and line shapes  
880 indicated the duration of experiment.

881

## 882 **7. Conclusions and future perspective**

883 This literature review summarized and evaluated the findings from 165 studies that focused on the  
884 toxicity of 16 types of MNOs to earthworm species. Earthworms represent organisms that interact strongly  
885 with natural organic and inorganic matter in soils and can be used as reporter organisms for assessing eco-  
886 toxicity (OECD, 1984). We found a large range of EC<sub>50</sub> values from 3.25 to 532 mg kg<sup>-1</sup> and LC<sub>50</sub> 0.2 to 866  
887 mg kg<sup>-1</sup> for different types of MNOs (Figure 5). The extreme range of data produced from the extensive  
888 variation in experimental design associated with geometric parameters of the MNOs that were used and often  
889 not mentioned. For example, particle sizes, dispersion and organic ligands used for the particle coating,  
890 rarely considered. Furthermore, little information was generally provided about the MNO behavior in the  
891 matrix, such as the dispersion status and colloidal stability, which is utterly important to assess the true  
892 environmental toxicity of the MNOs (Zhang et al., 2020a). Finally, most of the experimental designs aimed  
893 for a particular environmental scenario and were not used to model environmental events, such as  
894 precipitation or drought which can lead to dispersion or aggregation of the MNOs. Therefore, the results  
895 have to be regarded skeptically, when it comes to an assessment of ecological risks. In regard to the future  
896 application of nanoagrochemicals to safeguard the food production under challenging conditions of climate  
897 change, we can expect a large increase in the quantity of MNOs in soils that will inevitably disperse in the  
898 environment and will almost certainly affect soil organisms in a positive or negative way. This future use of  
899 nanoagrochemical in large-scale has to undergo a thorough risk assessment, that includes also data of long-  
900 term toxicity studies, which are rarely performed. Such a precautionary approach is necessary, since the  
901 depollution of soil is time-intensive and also represents a loss of arable land.

902 The study results currently do not allow a clear statement on safety of MNOs in agricultural soils,  
903 as data on the physicochemical properties and applied nanoformulations are lacking – in particular,  
904 information on particle size and specific surface area as well as on the used surfactants or organic ligands to  
905 modify the nanoparticle's surface in order to increase colloidal stability (dispersibility), provide functionality  
906 or control solubility of MNOs. Quantitative risk assessment is currently not possible because the actual

907 amounts of intentionally applied nanoagrochemicals or unintentionally released MNOs from nano-enabled  
908 products is unknown, while comparable toxicity studies and bioassays are lacking. However, the reviewed  
909 studies showed that both metal- and carbon-based MNOs can be toxic to earthworms, depending on the  
910 recipient species, duration of exposure, concentration/dose, method of exposure and especially the  
911 physicochemical properties of a specific MNO (type, size, shape, surface charge, surface functionalization  
912 etc.). The literature survey revealed that the deleterious effects of MNOs are clearly related to their size and  
913 therefore the relative surface area. Release of MNOs into soils is often followed by an ageing process, which  
914 can include transformation process such as dissolution, adsorption, hetero-aggregation and  
915 biotransformation. Earthworm biotransformation and stress modulation mechanisms of MNOs are also  
916 poorly characterized; an understanding of these processes is necessary to fully characterize risk in terrestrial  
917 ecosystems. Additionally, standardization of analytical methods as well as toxicity assays, that demand the  
918 reporting of parameters on the investigated MNOs and the matrix, must be further developed and applied  
919 worldwide (e.g. at OECD level), which would increase the comparability of study results. A clear  
920 understanding of the importance of, in particular long-term, dosage at relevant low environmental  
921 concentrations (in the ppb range) has to also be a part of future work, both as this relates to cross-material  
922 and cross-species comparisons. Importantly, reports on adverse effects of MNOs (e.g., Se, GO, Yb, La) on  
923 earthworms are missing for some material classes.

924           We note that using “fresh” artificially amended soil should be considered with care because particle  
925 bioavailability may change significantly during aging. Several studies have documented MNOs behave  
926 differently over time, and the associated toxicity to earthworms is not well understood. As with many  
927 contaminants, additional work is needed investigating the impacts of MNOs to earthworm species under  
928 environmentally realistic exposure scenarios, including chronic exposures over multiple generations.  
929 Importantly, very little work has been done with earthworms under multi-species or microcosm type of  
930 systems that simulate a “real-world” exposure and may include additional stressors such as low to moderate  
931 doses of additional contaminants. An understanding of species tolerance mechanisms to toxic NPs exposure  
932 is also lacking. In addition, earthworms are common prey of many vertebrates and given the documented  
933 uptake and accumulation of MNOs in earthworms, TTF of MNOs to higher levels of the food chain needs  
934 additional investigation. For risk assessment of nanoagrochemicals, it must be stressed to consider possible

935 transformation processes, as the fate and toxicity of transformed particles can vary greatly from pristine  
936 MNOs. In order to comply with this in a laboratory, analytical techniques, such as electron microscopy or  
937 mass spectrometry, must be combined, which allow the MNO quantification (to determine, if possible,  
938 specific particle surface area and both particle number and mass concentration) as well as the differentiation  
939 of MNOs from the background noise and possibly released MNO components (e.g., surfactants or dissolved  
940 metal ions). In the case of metallic MNOs, differences between the nanoparticulate and ionic forms and the  
941 correlation with biological responses of soil organisms such as earthworms should be investigated to identify  
942 nano-specific toxic effects. A clear understanding of the dynamic nature of important transformation  
943 processes and MNO uptake mechanisms in earthworms needs to be a part of these investigations. Future  
944 work should also include a focus on identifying the molecular initiating events that result in adverse  
945 outcomes such as reduced reproductive capacity or genotoxicity and epigenetic effects in subsequent  
946 generations. With regard to long-term exposure studies, gene expression and biomarker assessment (SOD,  
947 CAT, etc.) should be addressed to understand the toxicological mechanisms of MNOs. Additionally,  
948 oxidative stress, redox activity and production of oxygen species, cationic stress, photoactivation, embryo  
949 hatching interference and membrane lysis are further important toxicological drivers to be considered.  
950 Application of MNOs in soil should be reviewed critically for nano-enabled agriculture.

951

952

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