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Muhammad Adeel[a, b], Noman Shakoor[b], Muhammad Shafiq[c], Anna Pavlicek[d, e], Florian Part[d, e], Christian Zafiu[d], Ali Raza[f], Muhammad Arslan Ahmad[g], Ghulam Jilani[h], Jason C. White[i], Eva-Kathrin Ehmoser [d], Iseult Lynch[j]， Xu Ming[a] and Yukui Rui[b*]

[a]BNU-HKUST Laboratory of Green Innovation, Advanced Institute of Natural Sciences, Beijing Normal University Zhuhai subcampus, 18 Jinfeng Road, Tangjiawan, Zhuhai, Guangdong

[b]Beijing Key Laboratory of Farmland Soil Pollution Prevention and Remediation and College of Resources and Environmental Sciences, China Agricultural University, Beijing 100193, P.R. China

[c]University of Guadalajara-University Center for Biological and Agricultural Sciences. Camino Ing. Ramón Padilla Sánchez núm. 2100, La Venta del Astillero, Zapopan, Jalisco, México. CP. 45110.

[d]Department of Water-Atmosphere-Environment, Institute of Waste Management, University of Natural Resources and Life Sciences, Muthgasse 107, 1190 Vienna, Austria

[e]Department of Nanobiotechnology, Institute for Synthetic Bioarchitectures, University of Natural Resources and Life Sciences, Muthgasse 11/II, 1190 Vienna, Austria

[f]Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, 38000. Pakistan

[g]Shenzhen Key Laboratory of Marine Bioresource and Eco-Environmental Science, College of Life Sciences and Oceanography, Shenzhen University, Shenzhen 518060, China

[h]Institute of Soil Science, PMAS Arid Agriculture University Rawalpindi, Pakistan

[i]The Connecticut Agricultural Experiment Station, New Haven, CT 06504, United States

[j]School of Geography, Earth and Environmental Sciences, University of Birmingham, Edgbaston, B15 2TT Birmingham, UK

*Corresponding author:

Yukui Rui: ruiyukui@163.com

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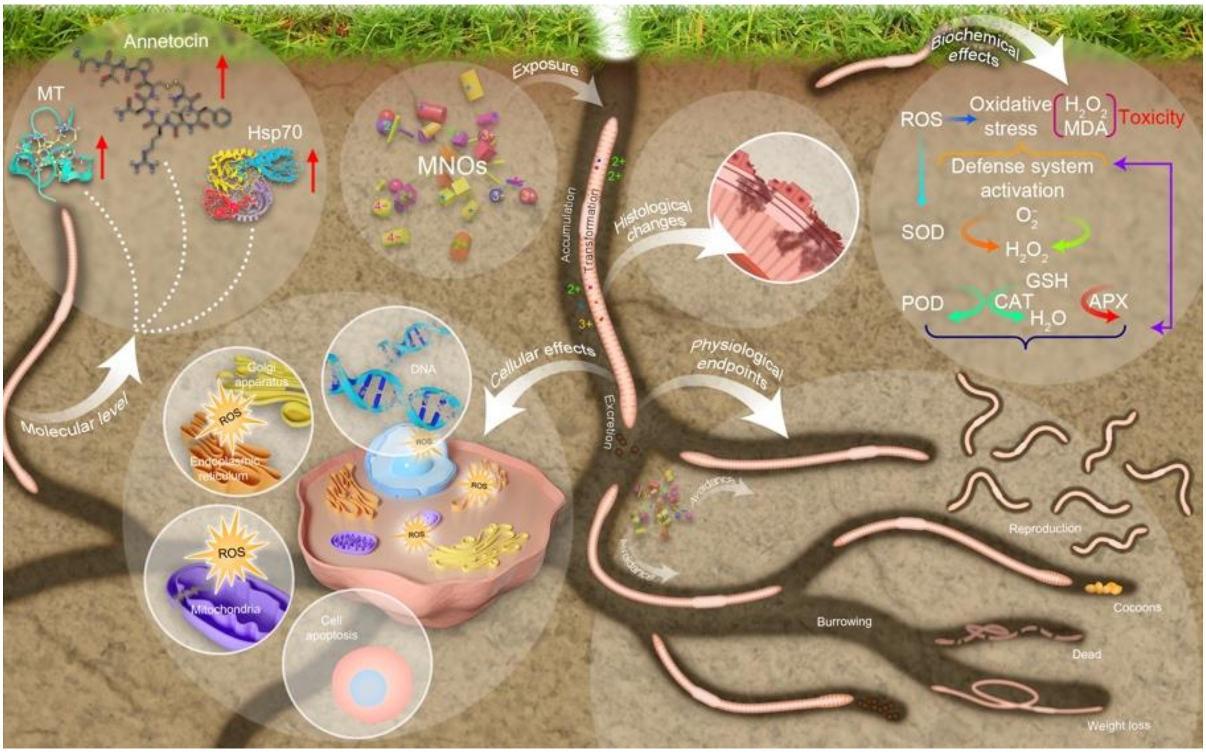
**Abstract**

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.

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

**Graphical Abstract**




## 1. Introduction

Advances in nanotechnology have enabled the development of manufactured nano-sized objects (MNOs), such as nanoparticles (NPs), nanoplates or nanofibers, which have interesting and useful material properties. By definition, NPs have dimensions in the size range between 1–100 nm, and importantly, many materials of relevance for industrial purposes contain toxic elements such as heavy metals. MNOs are widely applied for medical purposes (Shakib et al., 2014; Wu et al., 2011), and present several positively recognized features such as enhancing water security (Alvarez et al., 2018), improving agriculture productivity and food security (Duhan et al., 2017; Kah, 2015a), energy storage (Hussein, 2016), as well as broad applications related to environmental services and remediation (Saratale et al., 2018), electric and electronic (Contreras et al., 2017), fire safety (Olawoyin, 2018), industrial and transportation purposes, (Mathew et al., 2019) among others. With increasing production volumes, the unintentional release of MNOs into the environment increases, which may occur along the entire life cycle of MNO-containing products (Froggett et al., 2014; Keller and Lazareva, 2014; Part et al., 2018a). In addition, MNOs that are used in agriculture as pesticides or fertilizers (nanoagrochemical) are seen as promising solution to safeguard the future food production and may enter the ecosystem in large quantities (Sun et al., 2020). In the last two decades, numerous research projects have assessed the environmental health and human safety implications (Haase and Lynch, 2018). MNOs exhibit nanoscale-specific adverse effects due to their small size, asbestos like needle forms, large surface-to volume area or higher reactivity compared to conventional non-nanoscale (bulk) materials (Du et al., 2018; Glisovic et al., 2017; Hristozov et al., 2016). However, MNOs also have positive nano-specific physiochemical properties useful for relevant purposes, such as in agriculture. Novel nanoformulations combine several surfactants, polymers and inorganic NPs and are referred as nanoagrochemicals, including nanopesticides and nanofertilizers that aim at increasing solubility of poorly water soluble compounds or slow/controlled release (Kah, 2015b; Kah et al., 2013). At the nano-bio-interface, MNOs can be used specifically for plant protection, for example, to dissociate plant viruses, to act as organic based delivery systems of nutrients or to increase antioxidant enzyme activity (Farooq et al., 2021; Zulfiqar et al., 2019). Current research focuses on increasing the efficacy of MNOs but also on risk assessment, if MNOs are to be directly used for large scale agricultural applications. When MNOs and nanoagrochemicals get into the soil, a change physical, chemical or biological transformation processes started that is strongly dependent on soil conditions (e.g., pH, electrolyte and pore water composition, natural organic matter (NOM) content, etc.) (Kah et al., 2013). At the nanobio-interface, biotransformation, heteroaggregation, oxidation-reduction and dissolution are very likely – e.g. proteins, humic / fulvic acids or other NOM can adsorb onto the MNO's particle surface (corona formation) and thus can increase the mobility in the environment, whereas adsorption of electrolytes (e.g. $Ca^{2+}$) rather leads to a decrease in particle mobility (Markiewicz et al., 2018; Quigg et al., 2013). For risk assessment including bioassays it is therefore important to consider transformation processes of MNOs in order to elucidate exposure pathways and possible uptake by the biota. Especially, in agricultural soils invertebrates play a significant role in the formation and maintenance of soil structure and fertility through their direct role in a vast number of biological and biochemical processes. Soil invertebrates are important indicators of soil quality and also play a significant role in the the risk assessment of potential polluting substances as long-used model species in regulatory ecotoxicology. Literature reviews on the potential toxic effects of metal- and carbon-based MNOs on the soil environment have indicated that MNOs affect the entire soil community and may lead to adverse effects on the environment and its biota, as well as human health via trophic transfer within the food chain (Liné et al., 2017; Rocha et al., 2017). In addition, earthworms are common prey to other biota and therefore play a key role in the biomagnification of soil pollutants, often leading to negative consequences for sensitive vertebrate species (Rodríguez-

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

Given the important roles of earthworm species, we focus in this review on the potential negative effects of metal-, metal oxide- and carbon-based MNOs on this group of Oligochaetes. As noted above, these species are model organisms in soil ecotoxicology and they play a significant role in contaminant fate in soils, are recommended test organisms according to international standards such as International Organization for Standardization (ISO) and Organisation for Economic Co-operation and Development (OECD)(ISO 11268-1:2012; OECD, 2016). Mollusks, mites, isopods and collembolans are also used in terrestrial ecotoxicity testing in order to derive conclusions on the hazardous properties (persistence, potential bioaccumulation and toxicity) of MNOs. However, different functional and taxonomic groups have different routes of exposure, intake and response to MNOs. As such, toxicological data on various MNO types within a specific taxonomic group like earthworms, belonging to the subclass of Oligochaetes, must first be generated and then comparedwith other co-habitating taxonomic groups. In such specific environmental compartment one can yield a more generalizable statement on the ecotoxicity potential of MNOs. Herein, we specifically address terrestrial earthworm species of the Oligochaetes group; importantly, these organisms comprise the majority of invertebrate biomass (>80 %) in terrestrial environments and have over 600 million years of evolutionary experience as “environmental engineers” (Fierer, 2019). We evaluated 165 peer-reviewed journal articles that focus on the ecotoxicological effects of MNOs on Oligochaetes. Figure 1 shows that in the last 10 years, the highest number of studies focused on nano-Ag followed by ZnO, CuO, $TiO_2$, Fe-based NPs (FeO, $Fe_2O_3$, zerovalent $Fe^0$), QDs, carbon based materials (nanoplastic, $C_{60}$, graphene oxide (GO), and carbon nanotubes (CNTs), Al, Ni, Ce, Si, as well as others such as Cr, Yb, La, fullerenes or graphene. Importantly, research to assess nanotoxicological effects on terrestrial invertebrates is still needed (Johnson et al., 2018; Mukherjee and Acharya, 2018), particularly given that the chemical transformation of MNOs in soils presents a high level of complexity and consequently, organic/organo-mineral composition under the influence of MNOs renders an unpredictable event. Ecotoxicological studies on earthworms are particularly necessary because these species are relevant indicator organisms with regard to food production and environmental services. More specifically, MNOs released into the soil can lead to bioaccumulation and physiological changes in earthworm species, which in turn can have adverse effects on food production, the environment and finally, MNO release might affect human health.

## 2. Review scope and approach

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

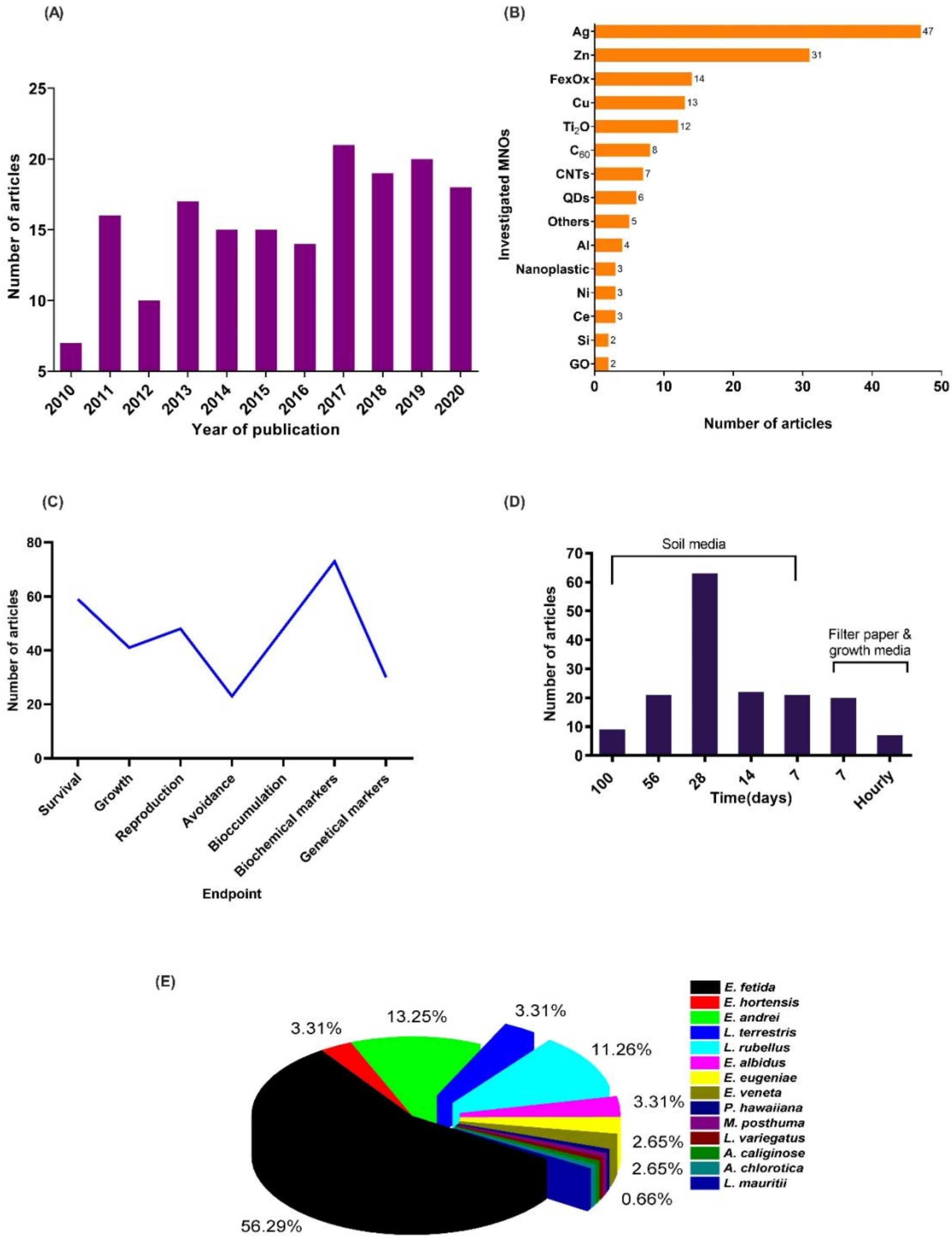



**Figure 1** (A) Number of peer-reviewed publications (165 in total between 2010–2020) investigating the potential toxic effects of manufactured nano-objects (MNOs) on the earthworm species of the group

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

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

Nanotechnology allows for manipulation of material properties through the control of matter, atoms and molecules. This key emerging technology has enabled a wide range of applications, such as sunscreens, water-repellent paints, nanocrystalline semiconductors for thin-film solar cells, nanocarriers or nanotherapeutics for cancer treatment, nanoagrochemicals and many more (Jean, 2020; Kah, 2015a; Lin, 2015; Vance et al., 2015). The total global production of MNOs, such as Ag, $Al_2O_3$, $CeO_2$, CNTs, Cu, Fe, nanoclays, $SiO_2$, $TiO_2$ and ZnO, has been estimated to be approximately 267,000 to 318,300 tons (Future Markets, 2012). According one market survey, 50% of the MNOs were produced in the USA, followed by 19% in the EU, 12% in China, 6% in Korea, 4% in Japan, 3% in Canada, 2% in Taiwan and 4% in other countries. A more recent market study estimated the global production volume of MNOs in 2020 to range from 400,000 to 3,150,000 tons in the case of nano-$SiO_2$ and from 2 to 4 tons in case of nano-Ag (highest and lowest value) (Janković and Plata, 2019). Jankovic et al. (Janković and Plata, 2019) highlighted that MNO production volumes represents only a small share of the total ore production of the mining industry – for example 1% to 0.000002% of the total Ag or Fe ore production, respectively – and therefore had a minor influence on entire life cycle of anthropogenic elements in the mining and manufacturing sectors. In summary, the mass-relevance of MNOs in the global cycle of raw materials or processed materials needs to be further investigated, particularly given that the currently reported quantities can vary widely and are often inconsistent.

In support of exposure assessment, dynamic material flow models have been applied on the data to predict the mass flows associated with release along the product life cycle into soil, water or air (Song et al., 2017a; Sun et al., 2017). Song et al. (2017b) estimated that by 2020, approximately 51% (12,200 tons) of the total global release of $TiO_2$, $SiO_2$ and $FeO_x$ occurred during the use of nano-enabled products (e.g., construction, packaging, medical products etc.), whereas 43% (9,890 tons) were the result of end-of-life releases. The remainder of the releases were during manufacturing. Release models for Europe, developed by Sun et al. (2016a) and Wang and Nowack (2018a), enabled the prediction of environmental concentrations of MNOs that occur in landfills, as well as a natural, urban or sludge-treated soils. Figure 2 summarises the results obtained from these models and shows that the majority of MNOs remain in the in-use stock or are transformed during processes such as wastewater treatment or waste incineration. For example, released nano-Ag either directly attaches to biosolids, dissolves in slightly acidic wastewaters, or transforms to insoluble $Ag_2S$ or $AgCl_2$ species that precipitate or attach to the sewage sludge, which is in turn thermally treated or used directly for agricultural purposes as a soil amendment (Kim et al., 2010b; Schlich et al., 2013a). Chemically more stable MNOs such as $TiO_2$, $Al_2O_3$, $Fe_xO_y$ or $SiO_2$ also attach to biosolids during wastewater treatment and may accumulate in measurable quantities in sludge-treated soils, as shown in Figure 2. Based on the results from the release models for the EU in 2014 (Sun et al., 2016b; Wang and Nowack, 2018a), we summarise (Figure 2) the predicted environmental concentrations (PEC values) of MNOs which are most relevant for soil environments and thus, lead to increased risk of exposure to terrestrial invertebrate species such as earthworms. The lowest MNO concentrations are expected to be ca. $8.4 \times 10^{-9}$ mg $kg^{-1}$ in natural and urban soil (in the case of quantum dots-MNOs), whereas the highest levels are predicted to occur in landfills in the case of nano-$SiO_2$ at a concentration at ca. $4.9 \times 10^{2}$ mg $kg^{-1}$. For comparison, Garner et al. simulated ten years of release of nano-$CeO_2$, -CuO, -$TiO_2$ and -ZnO in the San Francisco Bay area (California, U.S.) and found that the highest concentrations and mass fractions of MNOs are predicted for sewage-treated agricultural soils, as well as freshwater and marine sediments (Garner et al., 2017b). However, taking account of transformation processes such as de-/sorption, leaching or particle dissolution in soil pore water, and species sensitivity distributions (obtained from ecotoxicity data), Garner et al. (2017b) showed that neither nano-$TiO_2$ nor ZnO exceeds the no observed effect concentration (NOEC), even for sewage-treated agricultural soils. Their predictions also indicate no risks in the case of nano-CuO, whereas for nano-$CeO_2$ no quantitative risk assessment was possible due to the lack of toxicity data for soil organisms. Based on the currently known production volume of MNOs and nanoagrochemicals, the predicted environmental concentration of MNOs (apart from quantum dots) in soils is in the range of $10^{2}$ to $10^{-7}$ mg $kg^{-1}$, and in landfills of $10^{3}$ to $10^{-3}$ mg $kg^{-1}$ (Garner et al., 2017a; Sun et al., 2016b).

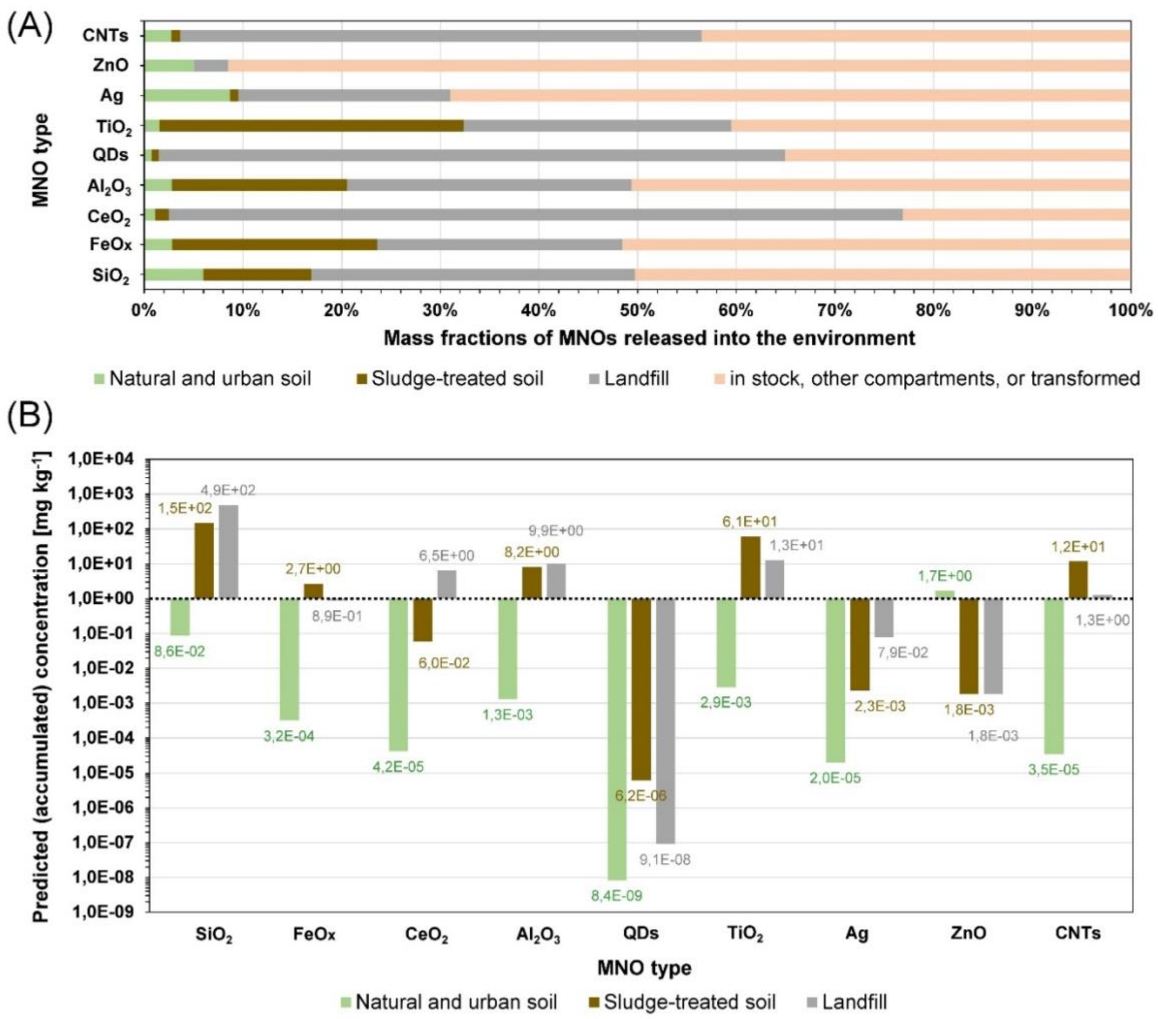



**Figure 2** (A) Mass fractions of the total release of MNOs in the EU in 2014, and (B) the predicted environmental mass concentrations that accumulated since 1990. All figures are based on the modelling results from Sun et al. (2016a) and Wang and Nowack (2018a)

Dynamic flow models for exposure assessment also must consider market dynamics and the lifetime of nano-enabled products. We highlight that MNOs release pathways depend on the type of application, the product's service lifetime, and the waste treatment applied to the product. Natural, urban and sludge-treated soils, as well as landfills for solid wastes with high organic content or with bio-covers (used after landfill closure), are the most relevant sinks for MNOs and represent an important exposure pathway for terrestrial species. With regard to risk assessment and future increases in MNO production volumes, harmful effects on terrestrial species in the near future cannot be excluded and release models must therefore be constantly updated with relevant data as they provide important guidance for ecotoxicity testing under realistic concentration ranges and relevant conditions. For quantitative risk assessment, it is also important to consider possible transformation processes of MNOs in the environment, particularly given that the fate and toxicity of some transformed particles differs greatly from those of pristine MNOs (Adam et al., 2018; Caballero-Guzman and Nowack, 2016; Part et al., 2018b; Svendsen et al., 2020). Predictive toxicological approaches based on high-throughput screening assays or structure activity relationship analysis have revealed that particle dissolution is not the only driver in nanotoxicity, and that processes such as oxidative stress, redox activity and production of oxygen species, cationic stress, photoactivation, embryo hatching interference, and membrane lysis are highly relevant toxicological drivers (Nel et al., 2013). In the last decade, grouping approaches have been developed for regulatory testing in order to find similarities in toxicological effects among different MNO types, where the relationship between toxicity and certain physico-chemical properties will be further investigated (Ha et al., 2018; Hund-Rinke et al., 2018; Lamon et al., 2019; Lynch et al., 2014). A grouping concept for metals and metal oxide MNOs that is based on ecotoxicity tests using algae, daphnids and fish embryos showed that for nano-Ag that, not only the solubility, but also the reactivity of the particle surface, morphology, and shading effects were all relevant and important factors for ecotoxicity (Hund-Rinke et al., 2018). However, such grouping approaches, particularly regarding ecotoxicity to terrestrial species, have many limitations as there is often a lack of data on both physico-chemical properties of a specific MNOs under realistic exposure conditions in the relevant matrices and comparable results from ecotoxicity tests.

Regarding the accumulation of MNOs in soil, Figure 3 shows possible release and exposure pathways which are relevant for earthworm species. MNOs can either be released unintentionally into soil or added intentionally when applied as biocides (e.g., Ag-, ZnO- or CuO-NPs) (Zhang et al., 2020c) in agricultural applications, such as the through the use of nano-pesticides or nano-fertilizers (Kah et al., 2019). Consequently, broad ecosystem exposure may occur after particle uptake and transfer through trophic levels from bioaccumulation or biomagnification. For instance, lab-scale experiments showed that amino acid-conjugated semiconducting quantum dots (QDs) were accumulated by the soil fungi *Penicillium solitum;* in contrast, no MNO uptake was evident without the conjugated amino acid coating. Hou et al. (2013) highlighted in their review that invertebrates such as the earthworm *Eisenia fetida* can be used to quantitatively assess the extent of bioaccumulation by calculating the ratio of MNO tissue concentration to that in water (bioaccumulation factor or BCF), and biomagnification by the ratio of the MNO concentration in the predator to that in its prey (biomagnification factor or BMF). Furthermore, the biota-sediment accumulation factor (BSAF) represents the ratio of the MNO concentration in an organism to that in the sediment (Hou et al., 2013). For example, Hou et al. reported that nanosized $Al_2O_3$ resulted in a lower uptake than their micro-sized equivalents, and that the uptake of metals (e.g., Ag, Au or Cu) in ionic form was higher than their nanoparticulate counterparts. Here, when fullerene ($C_{60}$) and single- or multi-walled carbon nanotubes (SWCNTs or MWCNTs) were exposed for 28 days to soils at concentrations ranging between ca. 0.3–300 mg MNO kg$^{-1}$ dry soils, the BSAF (kg dry soil per kg dry biomass) ranged from 0.0061–0.79 (Hou et al., 2013). As mentioned above, as the concentration of MNOs in soil environment significantly increases, as well as it will be important to understand their impact on soil biota.

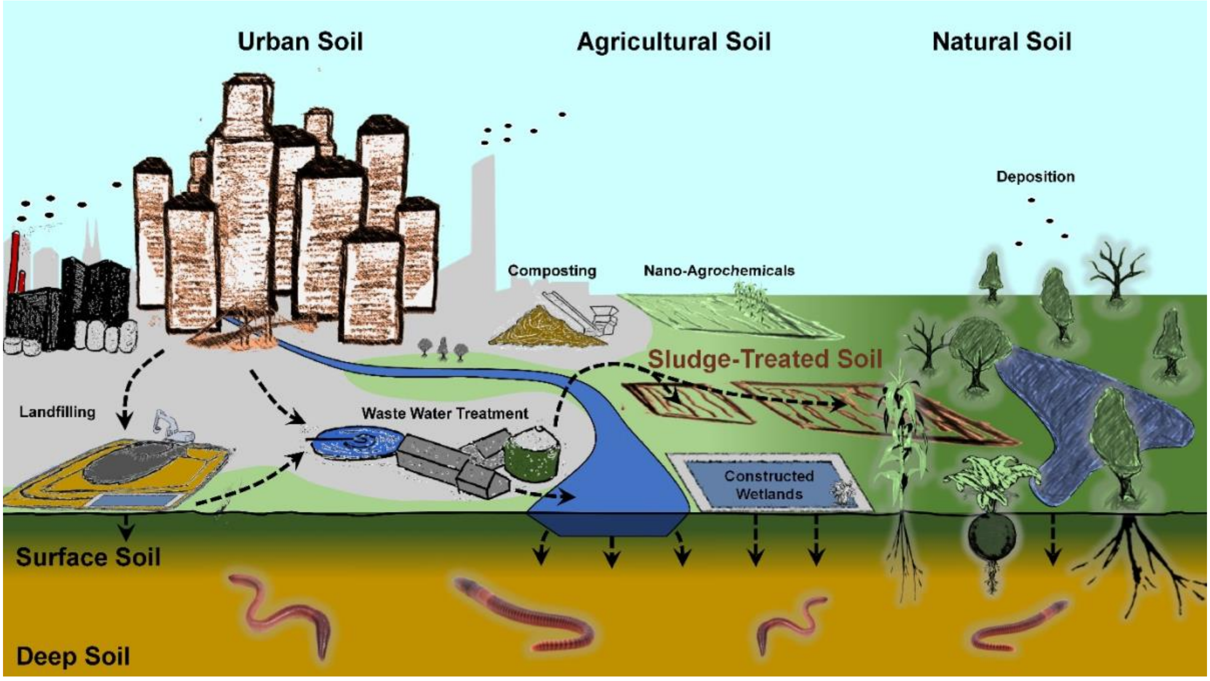



**Figure 3** Summary of possible relevant exposure pathways of MNOs for terrestrial species, such as earthworms.

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

### *4.1 Silver*

Silver nanoparticles (Ag-NPs) are extensively used in industrial and commercial products and intentionally or unintentionally accumulate in the soil environment (e.g. as applied nanoagrochemical and fungicide). PEC values of Ag-NPs for soil differ by global region; the PEC value of native soil in Denmark is 13 - 61 ng $kg^{-1}$ and agricultural soil contains 6 - 21 ng $kg^{-1}$, (Gottschalk et al., 2015) while across European soils the predicted levels range between 17.4 - 58.7 ng $kg^{-1}$, and in the USA 6.6 - 29.8 ng $kg^{-1}$ of Ag-NPs are predicted (Gottschalk et al., 2009a).

From 2010, 47 studies on the effect of Ag-NPs on earthworm species were published; this is the highest number for any of the MNO types (Table S1). In general, the studies showed that Ag-NPs were more toxic than Ag ions, which were typically presented as $AgNO_3$ salt. Toxicity in general is dependent on several factors, including particle size, exposure concentration and duration, experimental conditions and Ag-NPs properties, such as surface stabilization or coating, surface speciation, shape etc. (Bourdineaud et al., 2019; Mukherjee et al., 2017). A consistent finding was that the toxicity of AgNPs increased with decreasing size of the NPs; this is likely due to the higher surface to volume ratio of smaller NPs that results in both faster dissolution and likely greater uptake of discrete particles (Hu et al., 2012). Specifically, Hu et al. reported that 20 nm NPs were more toxic than 80 nm (500 mg Ag-NPs $kg^{-1}$); the authors speculated that greater passage of the 20 nm NPs through the cell membranes via membrane proteins (porins) resulted in more significant interference of metabolic pathways and damage to cellular constituents of *E. fetida*. At the cellular level, the coelomocytes of earthworms (*E. fetida*) and human cells (THP-1 cells, differentiated THP-1 cells and peripheral blood mononuclear cells) showed a similar response to Ag-NPs (5.91 μg Ag-NPs $mL^{-1}$) cytotoxicity after 24 hours. The early molecular responses to Ag-NPs involved an apparent transition from stress-related to immune-related activation of genes in both cellular models, with a distinct induction of the metallothionein encoding genes in THP-1 cells (Hayashi et al., 2012). Regarding the lifecycle of earthworms, several studies reported that the toxicity of Ag-NPs/$Ag^+$ depended on the developmental stage of the earthworms at exposure. The strongest effects on avoidance behavior were observed at 12.5 mg $kg^{-1}$ (Ag-NPs/$Ag^+$); adverse effects on survival were most significant at 100 mg $kg^{-1}$. Brami et al., (Brami et al., 2017) indicated that these effects were induced by oxidative stress originating from released $Ag^+$ ions. In addition, it was observed that the Ag concentration in the tissue of earthworms increased strongly with dose, which indicates the bioaccumulation of either Ag ions or nanoparticles or both (Antisari et al., 2016). Exposure studies conducted for the evaluation of the acute toxicity (1-14 days) have reported accumulation of the particles or ions in the gut and tissue (Hu et al., 2012; Li et al., 2015; Shoults-Wilson et al., 2011c). However, Makama et al.(2016) could not find any significant changes in Ag concentration (NPs and ions) in the soil after exposure and also, no changes on the growth levels and mortality of *E. andrei* during 28 days of $AgNO_3$

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 Ag-NPs identified interference with different enzymes and metabolic activity in earthworms, as well as glutathione reductase, glutathione S-transferase, acid phosphate and ATPase (Das et al., 2018; Gao et al., 2012; Hu et al., 2012).

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

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

We found contradictory reports about impacted physiological endpoints and behavior of Ag-NPs and ions on earthworms. However, the majority of studies showed that Ag-NPs lead to greater Ag accumulation in earthworm tissue than Ag ions (Coutris et al., 2012a; Shoults-Wilson et al., 2011a) and that earthworms showed avoidance behavior from both Ag-NPs/$Ag^+$ (Shoults-Wilson et al., 2011b). Importantly, the effects of Ag-NPs on earthworms can be complex and can manifest as growth reduction (van der Ploeg et al., 2014a), decrease in cocoon production (Schlich et al., 2013b; Shoults-Wilson et al., 2011b), juvenile mortality and reduced reproduction (Heckmann et al., 2011), oxidative stress (Hayashi et al., 2012; Hayashi et al., 2013) damage to proteins (Tsyusko et al., 2012) and DNA resulting in reduced enzymatic activities (Hu et al., 2012) and gene expression changes. Despite a relatively large number of studies addressing Ag-NPs toxicity to earthworm species, there are still significant knowledge gaps. For example, long-term multi-generational experiments are needed to analyze the impact of Ag-NPs under realistic exposure scenarios. An understanding of the threshold doses of Ag-NPs in soils that are not expected to affect earthworm growth and development need to be characterized. In addition, given the complexity of interactions and transformation processes of Ag-NPs with soil components, an understanding of the role of soil properties in particle fate and effects relative to earthworm species is needed.

### *4.2 Zinc oxide*

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

Within the past 10 years, 31 studies were published on the effects of ZnO-NPs on earthworm species, describing bioaccumulation, toxicity, and oxidative stress (Table S1). Most incubation studies were conducted for 28 days. A possible mechanism of toxicity involves the release of $Zn^{2+}$ from ZnO-NPs by dissolution, which depends strongly on the surrounding medium. Dispersions of 1000 mg $kg^{-1}$ ZnO-NPs in agar medium at different ratios resulted in 100% mortality for *E. fetida* within 4 days. The authors indicated that the high mortality was caused by a successive loss of antioxidant enzyme protections at this very high dose, such as superoxide dismutase (SOD). An increased activity of SOD was observed at the lower dose of 50 mg $kg^{-1}$, and levels decreased with increasing ZnO NP dose, suggesting that the protective enzyme potential was lost in a dose-dependent fashion and that the worms suffered from excessive oxidative stress (Li et al., 2011)(figure 4). Laycock et al.,(2015) reported no significant difference between the uptake of two forms of $^{86}Zn$ isotopes ($^{86}ZnO$-NPs and $^{68}ZnCl_2$) at 5 mg $kg^{-1}$ in *L. rubellus*, with the dietary and dermal pathways accounting for 95% and 5 % uptake, respectively .

Cañas et al. found that ZnO-NPs exposure induced a range of negative impacts on earthworms: for example, at 10,000 mg $kg^{-1}$ ZnO-NPs (unrealistic dose) caused mortality, reduced the cocoon and juvenile production (*E. fetida*). Toxicity was attributed to dissolution of the NPs to $Zn^{2+}$ (Cañas et al., 2011). Conversely, another study in soil showed little effect on earthworm (*E. andrei*) survival at concentrations up to 4000 mg $kg^{-1}$, ($EC_{50}$ estimated as 1020 mg $kg^{-1}$). However, reproduction and the number of juvenile offspring were significantly affected (Alves et al., 2019). Another study was published that reports long-term exposure (140 days) of ZnO-NPs to earthworms in different soil types (entisol and tropical artificial soil). The reproductive endpoints for *E. andrei* were in accordance with the short-term exposure studies (28 days); the reproductive rate was reduced to 45% at 500 and 1000 mg $kg^{-1}$, but no significant effect was found on growth and survival (Romero‐Freire et al., 2017). In an interesting co-contaminant study, combined exposure of ZnO-NPs with chlorpyrifos (CPF) (125/40 mg $kg^{-1}$) for 28 days showed no effect on survival but decreases (21-43%) in growth and fertility were reported. Furthermore, the enzymatic activities of CAT, GST and MDA were not affected. However, acetylcholinesterase (AChE) activity was diminished as compared to control and the extent of inhibition increased with increasing concentration of chlorpyrifos (García-Gómez et al., 2019; Uwizeyimana et al., 2017). A similar study conducted for 56 days evaluated the combined effects of ZnO-NPs with CPF on $2^{nd}$ generation earthworms after exposure. The results showed that the growth of the $2^{nd}$ generation was affected by the parental exposure and that this progeny had lower body weights, produced fewer cocoons and consequently, fewer (33.2 %) juvenile earthworms. GST and CAT were upregulated in organisms simultaneously exposed to a mixture of ZnO and CPF as compared to controls. Other biomarkers such as AChE activity were inhibited more strongly in the $1^{st}$ generation compared to the $2^{nd}$ generation (Lončarić et al., 2020). Stress responsive gene expression analysis following dietary exposure to ZnO-NPs in *E. fetida* showed that Zn induced expression of SOD (increase 3.35 fold), CAT (3.03 fold) and MT (7,68 fold) and the heat shock protein 70 (5.05 fold) following exposure to 500 mg $kg^{-1}$ ZnO for 15 days (Xiong et al., 2012). However, a detailed molecular mechanism of ZnO-NPs response was not described. Li et al showed that exposure of earthworms to ZnO-NPs (10, 50 and 250 mg $kg^{-1}$) caused a dose-dependent upregulation of antioxidant biomarkers, including ROS, SOD, and MDA (Li et al., 2019). Across the 31 studies, contradictory lethal doses of ZnO-NPs were reported, and the reason for this variability remains unknown. Given this and the potential for bioaccumulation and long-term effects of ZnO-NPs, additional study is needed. In addition, many of the knowledge gaps noted above for Ag-NPs also exist for ZnO-NPs. In addition, the literature on ZnO-NPs highlights the importance of additional mechanistic work on co-contaminant exposure and interaction studies, as well as on the development of important biomarkers for exposure and response.

## 4.3 *Copper*

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

A comparative study of CuO-NPs and $CuCl_2$ reported that CuO-NPs were 2-8 fold more toxic to *Enchytraeus albidus* than was $CuCl_2$ as measured by reproductive output ($EC_{50}$ 95 mg $kg^{-1}$) and induced avoidance behavior ($EC_{50}$ 241 mg $kg^{-1}$) (Amorim and Scott-Fordsmand, 2012). Similarly, a comparative study exploring gene expression through microarray analysis of *E. albidus* grown in soil amended with 400-1000 mg $kg^{-1}$ Cu-NPs or CuO-$Cl_2$ for 48 h reported significantly different gene responses (increase/decrease) and that CuO-NPs effects on soil ecotoxicology and ecotoxicogenomic parameters were likely caused by the NPs themselves and not by released ions (Gomes et al., 2012b). Furthermore, both nanoscale and ionic Cu induced oxidative stress, with overt differences evident between the two copper forms (Gomes et al., 2012a). CuO-NPs showed no effects on mortality or the growth rate of *Capitella teleta* under sediment exposure at 250 mg CuO $g^{-1}$ , whereas the ionic form caused a 26% increase in mortality, demonstrating that Cu in the ionic form is more toxic than the nanoscale form (Dai et al., 2015). Gomes et al. concluded that both nanoscale and ionic Cu induced oxidative stress, with overt differences evident between the two copper forms (Gomes et al., 2012a). At 1000 mg $kg^{-1}$ of CuO-NPs, a significant reduction was reported in *Metaphire posthuma* SOD levels, total coelomocyte count (with maximum depletion as 15.45 and 12.5 cells $mL^{-1}$), and population density as compared to copper sulphate (Gautam et al., 2018). Mudunkotuwa et al. demonstrated that organic acids in soils and natural environments highly affect the mobility and aggregation behaviour of Cu-NPs and CuO-NPs (1.0 g $L^{-1}$) through ligand promoted dissolution (Mudunkotuwa et al., 2012). Furthermore, organic acid adsorption to NP surface drives dissolution and the release of metal ions (Schrand et al., 2010). Cu bioavailability determines toxicity in the environment as demonstrated by the fact that: 1) small CuO-NPs are more toxic than larger particles; 2) NPs toxicity is enhanced due to positive charges facilitating interactions between cells and NPs; and 3) CuO-NPs dissolution and toxicity depends heavily on pH and temperature of the solution (Chang et al., 2012).

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

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

The most common forms of FeO-NPs are magnetite ($Fe_3O_4$), maghemite ($\gamma$-$Fe_2O_3$), and hematite ($\alpha$-$Fe_2O_3$) (Ali et al., 2016). Nano-zerovalent iron (FeO, nZVI) is known as a cost-efficient agent used for the degradation of several environmental pollutants (Yang et al., 2010). As such, there has been interest in the toxicity of nZVI to earthworm species. Between 2010-2020, 12 studies were published that report on ecotoxicity of Fe-based NPs to earthworms (Table S1). Due to this modest number of publications and the complexity of Fe chemistry in soils, the influence of nanoscale Fe based NPs on bioavailability and toxicity is not well understood.

Liang et al (2017) reported that exposure to nZVI (~50 to 100 nm) coated with 1-nm iron oxide shells at 500 and 1000 mg $kg^{-1}$ for 7, 14, 21, or 28 days increased avoidance response and inhibited the growth and respiration in *E. fetida* (Liang et al., 2017). Interestingly, the inhibitory effects of nZVI at 1000 mg $kg^{-1}$ appeared to be both time and dose dependent, with growth reduction and avoidance behaviour at 15 and 40%, respectively, at the highest nZVI concentration (Liang et al., 2017). This kind of inhibition behaviour was attributed to the depletion of glycogen and lipid content, as well as decreased in protein content. After incubation of nZVI with earthworms for 28 days, the SOD activity was significantly inhibited in worms exposed to low (100 mg $kg^{-1}$ nZVI) and high (1000 mg $kg^{-1}$ nZVI) concentrations (Liang et al., 2017). Liang et al. (2017) proposed that nZVI induced oxidative stress by upregulating antioxidant enzyme activities of SOD and CAT in response to the accumulation of reactive oxygen species (ROS) in the tissues. However, despite the high nZVI concentration (1000 mg $kg^{-1}$), no changes in the body weight or reproduction of *E. fetida* were observed (Yoon et al., 2018). Similarly, the survival of the *E. fetida* was not affected by nZVI even at concentrations as high as 3000 mg $kg^{-1}$. However, DNA damage and lipid oxidation were reported (Yirsaw et al., 2016). Another study showed negative effects of nZVI at 500 and 1000 mg $kg^{-1}$ on earthworm dermal tissues, such as disorganized texture, symptoms of dehydration, and lacerations in the intersegmental furrows or setae (Liang et al., 2017). Liang et al. suggested that this damage originated from reactive oxygen species (ROS) induced after nZVI exposure (Liang et al., 2017). Valerio-Rodríguez et al. (2018) reported that $Fe_2O_3$-NPs above 150 mg $kg^{-1}$ were highly inhibitory to *E. fetida* reproduction but that no effects were observed on reproduction at doses lower than 150 mg $kg^{-1}$ (Ali et al., 2016). Samrot et al. (2017) reported that synthesized magnetite Fe-NPs (17 and 28 nm) in aqueous study easily penetrated *E. eugeniae* epithelium, causing the deposition of lipofuscin in the circular muscle (fibrosis), erosion of the epithelium, and gut disintegration at 0.2 and 0.4 mg in 0.01 $L^{-1}$.

El-Temsah and Joner (2012) exposed *E. fetida* and *L. rubellus* to nZVI that had been aged in non-saturated soil for 30 d prior to earthworm addition. Earthworm reproduction was negatively affected at 100 mg $kg^{-1}$, but nZVI toxicity was significantly reduced when compared to non-aged soils. High nZVI concentrations (≥500 mg nZVI $kg^{-1}$ soil) induced avoidance, weight loss, and mortality (79% and 89% mortality was observed for *E. fetida* and *L. rubellus* in sandy loam soil, while no mortality was observed at the same concentration with LUFA 2.2 soil). More specifically, the $LC_{50}$ values for acute toxicity (14 days exposure) were 399 and 447 mg $kg^{-1}$ of soil-aged nZVI for *E. fetida* and *L. rubellus*, respectively. In addition, experiments conducted under the OECD test guideline yielded acute $LC_{50}$ value of 866 mg $kg^{-1}$ nZVI for *L. rubellus* after 14 days in soil. This study showed that avoidance behaviour was quite similar at 511-582 mg $kg^{-1}$ for both types of soil (sandy loam and LUFA 2.2 soil) and earthworm species (El-Temsah and Joner, 2012).

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

### *4.5 Titanium oxide*

$TiO_2$-NPs have a wide range of uses, mostly in commercial products (Hu et al., 2010). Nano-$TiO_2$ can also be used as a nanoagrochemical (Kah et al., 2013). The PEC value of $TiO_2$-NPs in Europe has been estimated at 1.01-4.45 μg $kg^{-1}$, 70.6-310 μg $kg^{-1}$, 100-433 mg $kg^{-1}$ in soil, sludge-treated soil and sewage sludge, respectively (Gottschalk et al., 2015; Gottschalk et al., 2009a); this suggests that sewage sludge poses the greatest source of $TiO_2$-NPs hazard for earthworms. Importantly, even soils that are treated with such $TiO_2$-NP contaminated sewage sludge exhibit concentrations that are orders of magnitude lower than the concentrations used in many publications (≥1000 mg $kg^{-1}$) (Hu et al., 2010). Between 2010-2020, there were 14 published reports on titanium dioxide nanoparticle ($TiO_2$-NPs) toxicity to earthworms (Table S1), consisting of 9 full life cycle (28-58 days) studies and 5 shorter term studies (7-14 days).

As with other particles, the toxicity of $TiO_2$-NPs can depend on particle size, purity, surface coating, crystallinity, shape, and solubility (Di Virgilio et al., 2010; Shah et al., 2017). McShane et al. (2012) investigated the reproduction, juvenile growth and avoidance behaviour of earthworms (*E. fetida* and *E. Andrei*) in soils spiked with $TiO_2$-NPs by two methods: 1) liquid dispersion and 2) dry powder mixing. Both amendment protocols yielded similar findings; low (200 mg $kg^{-1}$) and high (10,000 mg $kg^{-1}$) concentrations of pure $TiO_2$-NPs (5, 10 and 21 nm) showed no adverse effects on *E. andrei* and *E. fetida* on any parameters, including avoidance behaviour, juvenile survival and growth, adult earthworm survival, cocoon production, cocoon viability and total number of juveniles hatched from cocoons. Heckmann et al. (2011) used uncoated

$TiO_2$-NPs that were characterized for particle size, surface charge, agglomeration, purity and chemical composition; the authors reported a 49% reduction in the number of juveniles upon exposure to 1,000 mg $kg^{-1}$ crystallite sized spherical, multi-faced and elongated $TiO_2$-NPs (nominal particle size: 21 nm) under similar incubation conditions. Similar to other NPs, aggregation, agglomeration and surface area of $TiO_2$-NPs are factors governing the relationship between earthworm reproduction and avoidance behaviour. Conversely, no negative effects were reported on the number of adults, reproduction, juvenile growth, number of cocoons, and number of shells of *E. fetida* cocoons in a long term study (over 90 days) at lower doses of 150 or 300 mg $kg^{-1}$ $TiO_2$-NMs (Sánchez-López et al., 2019).

In soils amended with 200 and 10,000 mg $kg^{-1}$ $TiO_2$-NPs, the reproductive rate of *E. andrei* was up to twice as high as that of *E. fetida* but did vary with experimental conditions, such as artificial and natural soil exposure (McShane et al., 2012). This highlights the importance of species-specific responses to NPs exposure in general (Domínguez et al., 2005). Lapied et al. (2011) reported that no lethal effects for *L. terrestris* at 100 mg $kg^{-1}$ $TiO_2$-NPs. In the same study even at high concentrations (1000 mg $kg^{-1}$), an aqueous suspension $TiSiO_4$-NPs of < 50 nm caused no toxicity to *E. andrei*. However, $TiO_2$-NPs induced mitochondrial injury at 5000 mg $kg^{-1}$ in *E. fetida* (Hu et al., 2010); interestingly, the authors indicate that $TiO_2$-NPs toxicity was probably due to changes the crystal structure. $TiO_2$-NPs are known to cause oxidative stress originating from reactive oxygen species (ROS) generation (Khalil, 2015); histopathology has shown this leads to cuticle loss from the body wall and muscle damage by breakage and shrinkage of cells (Priyanka et al., 2018). Moreover, Ti bioaccumulation in *E. fetida* was significantly increased when soil was amended with 150 mg $TiO_2$-NPs $kg^{-1}$ compared to the control treatment (0 mg $kg^{-1}$ NPs) (Valerio-Rodríguez et al., 2020), although no toxic effects on growth parameters were found (Zhu et al., 2020).

At the biochemical level, environmentally relevant concentrations (1-100 mg $kg^{-1}$) of $TiO_2$-NPs caused significant impairment of key processes such as reducing *Pheretima hawaiiana* acetylcholinesterase (AChE) by 70%, SOD activity by 130%, CAT activity by 161%, glycogen content by 6%, total lipid by 92% and total soluble proteins by 82% (Khalil, 2015). Similarly, Lapied et al. (2011) reported that exposure of *L. terrestris* to 15 mg $kg^{-1}$ $TiO_2$-NPs in the soil for four weeks significantly increased apoptotic frequency in the cuticle and intestinal epithelium.

Importantly, the findings of the long-term lab-based studies with $TiO_2$-NPs do not align well with the shorter-term studies. An explanation for this difference may be found in the fate of the $TiO_2$-NPs. For example, when nanoscale $TiO_2$ is exposed to clay particles, the materials can heteroaggregate over time, effectively minimizing accumulation by and exposure to earthworm species (Sánchez-López et al., 2019; Shi et al., 2017). This highlights that both particle properties and environmental conditions control fate of $TiO_2$-NPs, and can explain unexpected variation in toxicological data (Peijnenburg et al., 2015; Wagner et al., 2014). Additional studies are needed that address the effects of $TiO_2$-NPs on earthworms at the molecular level, including the use of critical biomarkers to enable understanding of the mechanisms of response.

### *4.6 Aluminum oxide*

Aluminium oxide NPs are produced in the form of nanocomposites with other metals and have demonstrated efficiency in the removal of fluoride pollutants for environmental applications (Liu et al., 2015). There are 5 studies investigating $Al_2O_3$-NPs toxicity to earthworm species from 2010-2020 (Table S1).

When nanoscale and microparticles of $Al_2O_3$ were introduced into soil containing adult *Dendrobaena veneta*, a strong correlation was found between particle size and the levels of water-soluble and EDTA-extractable aluminium. Importantly, earthworm soil activities such as digestion, excretion and repeated ingestion substantially affect the speciation of Al and can result in reduced bioavailability of the metal. Bystrzejewska-Piotrowska et al. (2012) reported the bioaccumulation of Al in earthworm tissues in both nanoscale and microparticles (MPs) form, although uptake was 95% higher for the smaller particles. $Al_2O_3$ NPs at 3.69 mg $kg^{-1}$ increased the Al content in *D. veneta* as compared to $Al_2O_3$ MPs (1.89 mg $kg^{-1}$) after depuration. However, $Al_2O_3$-NPs in soil eluates and *D. veneta* tissues were determined after 1 and 10 days before and after gut cleansing (depuration) but no accumulation of Al was found. Furthermore, the presence of $Al_2O_3$-NPs in soils can significantly influence the bioavailability and toxicity of metals *D. veneta* (Bystrzejewska-Piotrowska et al., 2012). Interestingly, disk-shaped $Al_2O_3$ NPs were not toxic to *D. veneta* after a 10 day exposure even at the unrealistically high concentration of 10 g NPs $kg^{-1}$ of soil (Bystrzejewska-Piotrowska et al., 2012). However, high NPs concentrations in soil greatly affect soil-metal equilibrium and metal extractability. Reproduction of *E. fetida* was negatively affected at 3,000 mg $kg^{-1}$ of large sized $Al_2O_3$ (50-200 µm), but agglomerated nanometric $Al_2O_3$ (11 nm) was found to be non-toxic. However, exposure to high concentrations (3,000-5,000 mg $kg^{-1}$) of both micro- and nanometric $Al_2O_3$ induced avoidance behaviour of *E. fetida* (Coleman et al., 2010). Importantly, these high concentrations are highly unrealistic in the soil environment. Similarly, no mortality or negative influence on the reproductive behaviour of *E. fetida* were observed upon exposure to 1000 mg $kg^{-1}$ $Al_2O_3$-NPs (Heckmann et al., 2011). At 3,000 mg $kg^{-1}$, a significant reduction in earthworm enzyme activities, reproduction, and survival was observed. These two studies also highlight contradictory findings as a function of dose on reproduction. The SOD and CAT activities in *E. fetida* decreased following exposure to $Al_2O_3$-NPs at 3,000 mg $kg^{-1}$ (Yausheva et al., 2017). Drawing further conclusions on $Al_2O_3$-NPs is difficult given the limited existing literature and as such, there is clearly much work to be done involving multiple dosing and exposure regimes and involving a broad range of biochemical, physiological and molecular endpoitns.

### *4.7 Nickel oxide*

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

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

Another study found that exposure of 250 mg $kg^{-1}$ NiO-NPs in vegetable residues resulted in Ni retained in earthworm gut for over four weeks (Antisari et al., 2015). Furthermore, exposure over 7 and 28 days to Ni-NPs at higher concentrations (>500 mg $kg^{-1}$) caused oxidative stress which led to DNA damage and increased of proteolysis, apoptosis and inflammatory response, as well as interference with the nervous system (Gomes et al., 2019). Importantly, nothing is known about the TTF of NiO-NPs, as longer-term studies has thus far only focussed on understanding the impacts on behavior, as well as biochemical and molecular responses of the exposed species. There are several significant knowledge gaps related to NiO-NPs which need to be addressed for thorough and accurate risk assessment: 1) Fate and transport mechanism of NiO-NPs in the presence of earthworm species; 2) longer term exposure experiments at environmentally relevant doses, and 3) NiO-NPs effects on gene expression and key biomarkers (SOD, CAT, etc.).

### *4.8 Cerium oxide*

Ce is widely used as $CeO_2$-NPs as catalyst, a fuel additive (Auffan et al., 2014a) and also in optics, which has raised production levels of the nanoscale form in global market up to 1000 metric tons/year. Cerium (Ce) covers approximately 0.0046% of the earth's crust by weight (Johnson and Park, 2012), and exists in threepossible oxidation states; Ce are $Ce^{2+}$, $Ce^{3+,}$ and $Ce^{4+}$. The most common and stable valence for Ce is Ce (IV) oxide ($CeO_2$), followed by cerium (III) oxide ($Ce_2O_3$) (Adeel et al., 2019a).

Limited data is available concerning the PEC of $CeO_2$-NPs; Denmark's uncultivated and agricultural soil PECs are estimated at 24-1500 ng $kg^{-1}$ and 10-530 ng $kg^{-1}$, respectively (Gottschalk et al., 2015). Data on $CeO_2$-NPs toxicity to terrestrial invertebrates is generally scarce; there are 3 published studies addressing toxicity, and as such, drawing conclusions is difficult as there are still large knowledge gaps. The $EC_{50}$ and $LC_{50}$ values for *E. fetida* after 28-days of exposure were 294.6 and 317.8 mg Ce $kg^{-1}$, respectively (Lahive et al., 2014). Some researchers have focused on direct effects of Ce-NPs to earthworms as described in Table S1. For example, Lahive et al reported that *E. fetida* exposure to $CeO_2$-NPs at 41-10,000 mg $kg^{-1}$ for 28 days had no effect on survival or reproduction, whereas Ce in a salt form (ammonium cerium nitrate) negatively affected both reproduction and survival at 10,000 mg Ce $kg^{-1}$ (Lahive et al., 2014). However, histological analysis revealed potential toxicity by cuticle loss from body wall and some loss of integrity of the gut epithelium exposed to $CeO_2$-NPs. This suggests that the earthworms are negatively affected by exposure to lower doses, although proper endpoint selection will be an important factor in accurately assessing toxicity and risk.

A complex link between NP accumulation and toxicity has been reported (Auffan et al., 2014b). Ce accumulation in *L. rubellus* tissues (5.3 mg $kg^{-1}$) and feces (49 mg $kg^{-1}$) was evident after 7 days of exposure to $CeO_2$-NPs amended soil (at 5000 mg $kg^{-1}$) (Antisari et al., 2012). No accumulation of Ce was evident *E. fetida* as the NPs were rapidly removed (excreted) when worms were moved to clean soil (Carbone et al., 2016). In addition, no studies have been done to assess the TTF of $CeO_2$-NPs from earthworm species to their predators. Clearly additional work is needed to understand the risk of TTF to the food web and potentially to human health.

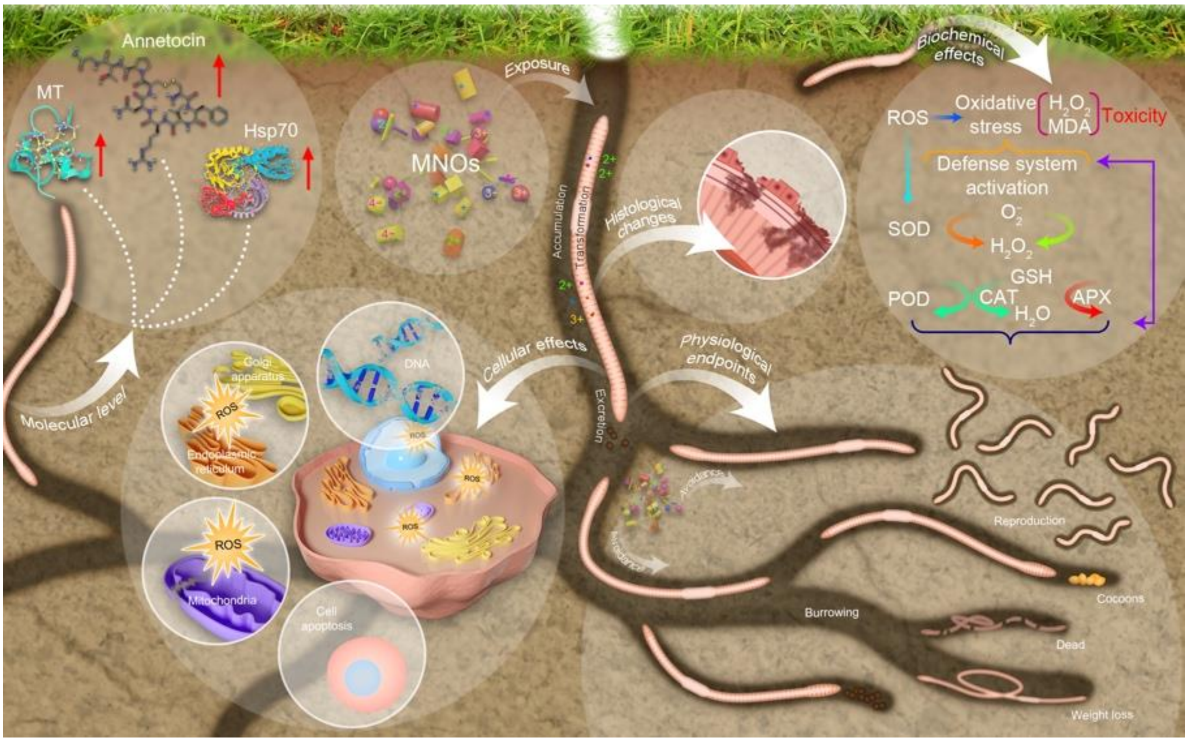



**Figure 4** Potential MNOs toxic effects and mechanisms reported for earthworms at the organism, organ, cellular, biochemical and genetic level. At the organism level, MNOs exposure can cause changes in endpoints such as the avoidance, survival, growth and locomotion. At the organ level, dermal and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

### *4.9 Lanthanum*

Lanthanum (La) is often applied as manufactured NPs mainly exist in lanthanum hydroxide and lanthanum oxide. Lanthanum nanoparticles (La-NPs) are white spherical metal particles typically 40 nm in size with surface area range 130-150 $m^2g^{-1}$ (Brabu et al., 2015). China is one of the leading countries in production of La and total production of La exceeds 30,000 metric tons worldwide (Reilly, 2019). Recently, Adeel et al exposed *E. fetida* to various concentration level (25, 50, 100, 200, 500 and 1000 mg $kg^{-1}$) of both forms (nanoscale and bulk) of $La_2O_3$, concluded that at 100 mg $kg^{-1}$, nanoscale and bulk $La_2O_3$ induced earthworm mortality by 33-35% and reduced reproduction by 10-32% respectively. Ultrastructural observations revealed that nanoscale and bulk REOs at higher doses (500 and 1000 mg $kg^{-1}$) induced abnormalities in the internal organelles, including mitochondria, Golgi apparatus and chloragosomes. Nanoscale $La_2O_3$ significantly reduced the earthworm digestive (glutathione S-transferase, total glutathione, glutathione reductase, glutathione peroxidase) and cast enzymes (SOD, POD, CAT and MDA) by 20-80% at medium and higher concentrations as compared to bulk $La_2O_3$. Results suggest that beta-glucosidase and alkaline phosphatase were the most sensitive and lipase was the least sensitive digestive enzyme to $La_2O_3$-NPs toxicity. Interestingly, study reported that earthworms provided a protective role to minimize the toxic effects of nanoscale and bulk $La_2O_3$ on the microbial biomass carbon and soil enzymes at 100-200 mg $kg^{-1}$ (Adeel et al., 2021c). Overall, long-term field studies are needed to enhance the understanding of processes and bioaccumulation of rare earth oxides (REOs) in *E. fetida*, and to elucidate the risk and recovery potential of earthworms in the presence of agricultural plants.

### *4.10 Ytterbium*

Ytterbium nanoparticles (Yb-NPs) is a unique material, has been growing rapidly and applied in diverse fields, such as nano electronic devices including light emitting diodes, solid-state lasers, fertilizer industry and environmental remediation (Venkata Krishnaiah et al., 2013; Zhu et al., 2014). Nowadays, China is one of the leading country containing REOs reservoirs of about 44 million metric tons (Adeel et al., 2019a). Global demand of $Yb_2O_3$ was estimated in 2017 to be 5,000 to 7,000 tons according to Mineral commodity summaries 2018, Reston, Virginia, USA (Ober, 2018). Recently, Adeel et al., (2021) reported that nanoscale and bulk $Yb_2O_3$ induced earthworm mortality by 13-15%, and reduced reproduction by 10-12%, at 100 mg $kg^{-1}$. In another study Adeel et al. determined the biochemical, genetic, and histopathological effects on *E. fetida* exposed to $Yb_2O_3$ at 50, 100, 200, 500 and 1000 mg $kg^{-1}$. This study reported that $Yb_2O_3$ treatment induced neurotoxicity in earthworm by inhibiting acetylcholinesterase by 22-36% at 500 and 1000 mg $kg^{-1}$. Additionally, $Yb_2O_3$ at 100 mg $kg^{-1}$ significantly down-regulated the expression of annetocin mRNA in the parental and progeny earthworms by 20%, which is crucial for earthworm reproduction. Similarly, expression level of heat shock protein 70 (HSP70) and metallothionein was significantly upregulated in both generations at medium exposure level (Adeel et al., 2021b; Adeel et al., 2021c). In future, long term field experiments should be conducted to understand the underlying processes and potential risks associated with Yb containing fertilizers. Additional long-term research is needed to understand the underlying processes and potential risks that nanoscale fertilizers could pose to soil ecology.

### *4.11 Silica*

Silica (Si) NPs are highly crystalline materials (Tchalala et al., 2018) primarily used in agricultural and commercial products (Kah et al., 2013) (Buchman et al., 2019). For example, porous hollow $SiO_2$-NPs can be used as carriers to control the release and shield from UV of neurotoxins or antibiotics (Li et al., 2007; Li et al., 2006). In 2016, annual production of Si-NPs was approximately 93,300 tons worldwide which is 3rd largest production followed by $nTiO_2$ and nFeOx and increased gradually year by year (Song et al., 2017c). The PEC value of $SiO_2$-NPs can range from 86-150000 µg $kg^{-1}$ in natural, urban or sludge-treated soils (Wang and Nowack, 2018b). However, their deteremental effects on soil biota is not well understood. A recent study evaluated the avoidance behavior of five soil species, including *E. fetida*, in $SiO_2$-NPs-contaminated soil. At 100 mg $kg^{-1}$, $SiO_2$-NPs induced 50% avoidance behavior (Santos et al., 2020), clearly highlighting the need for further studies to understand toxicity mechanisms in soil environment. Similarly, Di Marzio et al (2018) investigated the effect of surface charge of Si -NPs on coelomic cells from *E. fetida;* the data suggested a strong genotoxic effect at 1 µg m $L^{-1}$ with $LC_{50}$ values of 73.9 µg m $L^{-1}$ (negative charge) and 116.9 µg $mL^{-1}$ (positive charge). The observation that negatively charged NPs were twice as toxic as positively charged particles highlights the need to understand how material properties control nanoscale fate and effects (Di Marzio et al., 2018). With only two published studies, clearly more research is needed to understand not only the mechanisms of toxicity to key species, but also the role of material properties and environmental factors controlling toxicity, TTF, and risk.

### *4.12 Quantum Dots*

Semiconducting quantum dots (QDs) have attracted significant attention for use in energy, electronics, solar cells and for biomedical applications. These fluorescent nanocrystals have a size that varies depending on the material composition and synthesis method, with most types being approximately 1 to 10 nm (Liu et al., 2020). In 2012, global production of QDs was approximately 135 tons and increased gradually year by year (Future Markets, 2012). QDs are used at laboratory scale as fluorescent markers in nanosafety research (Murray et al., 2018), but they are currently not used as nanoagrochemical. Although QDs are already used for micro-LEDs, TV screens and solar cells or as fluorescent markers in medicine and environmental research, only limited data are available on possible negative environmental and health risks of QDs. The PEC value for QDs was predicted to be very low at ca. 8 pg $kg^{-1}$ in natural and urban soils in the EU (Wang and Nowack, 2018b), but it must be stressed that available exposure model results are currently very inaccurate, as there is a lack of data on production volume of QDs in particular.

Over the last 10 years, 6 studies have been published on the effects of QDs on earthworm species, describing both bioaccumulation and toxicity (Table S1). From an ecotoxicological perspective, QD uptake can clearly lead to the liberation of dissolved metal components (e.g., $Cd^{2+}$ , $Zn^{2+}$ etc.) to lead to toxic effects (Rocha et al., 2017). For example, QDs, passing the biological barriers, can be taken up into the intracellularly via endocytosis, leading to the production of reactive oxygen species (ROS) that can induce oxidative stress and bimolecular damage. Likewise, QDs can penetrate cell membranes and might have a growth-inhibiting effect on the cell or on the organism (Barroso, 2011). Interestingly, earthworms have the ability to biosynthesize QDs *L. rubellus* were exposed to soil amended with $CdCl_2$ and $Na_2TeO_3$ for 11 days and metal detoxification processes in the earthworm gut (chloragogen cells) resulted in the production of biocompatible CdTe QDs (Tilley and Cheong, 2013). The earthworms did not seem to be physiologically affected by the presence of CdTe QDs in their systems, possibly because the generated QDs are coated with a passivating layer that stabilizes the QDs in biological media. This highlights an interesting metal detoxication strategy; however, the presence of Cd-containing QDs could still pose a risk to organisms within the food chain that feed on these earthworm species (Tilley and Cheong, 2013).

Kominkova et al., (Kominkova et al., 2014) also studied the biosynthesis of QDs in *E. fetida*. Earthworms exposed to CdTe QDs showed oxidative stress and antioxidant activity, but earthworms exposed to CdTe were less negatively affected than with dissolved $Cd^{2+}$. The expression of metallothionein was partly responsible for the ability of organisms to cope with heavy metals exposure. Stürzenbaum et al., studied the biosynthesis of QDs in *L. rubellus*. Earthworms were exposed to soil amended with $CdCl_2$ and $Na_2TeO_3$ for 11 days, resulting in the biosynthesis of CdTe QDs that showed optical characteristics typical of quantum-confined semiconductor materials useful for cell imaging applications (Stürzenbaum et al., 2013). The bioaccumulation potential and toxic effects of CdTe QDs to *E. fetida* was studied by Tatsi et al (2020) according to procedures outlined in OECD TG 222. The earthworms were exposed to 50, 500 and 2000 mg CdTe QD $kg^{-1}$ (dry weight) for 28 days, as well as in aged soil (Tatsi et al., 2020). The aged soil was the same soil used in the initial fresh soil experiments but the earthworms were added only after 6 months of incubation. No effects on survival were noted, but some reductions of growth were observed at the higher doses, with juvenile production being the most sensitive endpoint. The nominal $EC_{50}$ of values were > 2000, 108, 65, 96 mg CdTe $kg^{-1}$ for bulk, PEG-, $COOH^{-}$ and $NH^{4+}$-coated CdTe QDs in fresh soil (Tatsi et al., 2020), again highlighting the significance of particle (surface) chemistry to overall toxicity. The accumulation of QDs from six-month aged soil was higher, leading to reduced growth and survival of the adult worms relative to unaged soil. The nominal $EC_{50}$ values for juvenile production in the aged soil were 165, 88, 78 and 63 mg CdTe $kg^{-1}$ for microscale material, PEG-, COOH- and $NH_4^+$-coated CdTe QDs, respectively (Tatsi et al., 2020). Thus, exposure to nanoscale CdTe QDs, regardless of coating, caused greater toxicity than the microscale CdTe materials, and toxicity increased after aging of the materials in soil (Tatsi et al., 2020). Apart from toxicity to individual cells or organisms, QDs could also enter higher levels of the food chain by TTF. This poses a risk not only to the organisms themselves, but also to the food chain and potentially human health; as such, additional work in this area is needed.

# 5. Carbon-based nano-objects

## *5.1 Carbon nanotubes*

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

There are only 7 published studies investigating the toxicity of CNTs to earthworm species (Table S1). For example, Scott-Fordsmand et al. (2008) reported that *E. veneta* produced 60% fewer cocoons when exposed to 495 mg $kg^{-1}$ double-walled nanotubes in contaminated food for 28 days. However, physiological endpoints such as hatchability, survival or mortality remained unchanged after 28-d exposure to concentrations up to 495 mg $kg^{-1}$. Conversely, Calisi et al. (2016) revealed that *E. fetida* exposed to 30 and 300 mg $kg^{-1}$ of MWCNTs for 14 days caused changes in cellular and biochemical markers, including morphometric alteration in immune cells, destabilization of lysosomal membranes, acetylcholinesterase inhibition and changes in concentration of metallothionein tissues. A number of papers have been published that focus on how carbon nanotubes alter the toxicity of co-contaminants. Hu et al. (2013) reported that MWCNTs at 1000 mg $kg^{-1}$ increased the bioavailability and toxicity of nonylphenol (NP) to *E. fetida*. Furthermore, these studies reported that although MWCNTs are not readily absorbed and accumulated in earthworm tissues, the nanomaterial may cause harmful effects indirectly, including DNA damage, by releasing contaminants that have sorbed on their surfaces. Hu et al.(2014) examined the combined effect of MWCNTs and sodium pentachlorophenate (PCP-Na) on *E. fetida* in artificial soil samples and found 100 % survival of earthworms upon exposure to levels up to 1000 mg $kg^{-1}$ MWCNTs for 14 days. In addition, a laboratory-based study demonstrated that the mixture of MWCNTs and PCP-Na induced different expression levels of biochemical markers (Zhang et al., 2014). Interestingly, negligible negative effects were observed upon exposure to MWCNTs or PCP-Na individually, whereas under simultaneous exposure MWCNTs partially alleviated the toxicity on *E. fetida* due to contaminant adsorption. Notably, the BAF of MWCNTs in earthworms was low (0.015 ± 0.004) when they were exposed at 60 mg MWNTs $kg^{-1}$ (Kalinowska et al., 2013). Similarly, another study reported the limited accumulation of MWCNTs into body tissues due to minimum absorption; in addition, an exponential decay model suggested ready elimination of accumulated MWNTs (Petersen et al., 2011). Importantly, the impact of carbon-coated NPs, either through intended functionalization or natural processes, on toxicity to earthworms is currently unknown. It is important to understand the toxicity of these materials under environmentally realistic exposure scenarios. Importantly, these types of studies will provide useful information for the development of safe and sustainable novel C-based composites for a range of environmental applications, such as wastewater treatment and soil remediation.

### *5.2 Buckminsterfullerene*

Buckminsterfullerene ($C_{60}$) is carbon-based engineered nanoparticle with high organic carbon normalized partition coefficients of log Koc = 6.2-7.1 (Kausar, 2017) that has been of interest for some time.

C60 can be used, e.g., for solar cells (Nelson, 2011), from which they can be unintentionally released into the environment. It is noted that we could not find PEC values for soils.

Li and Alvarez (2011) reported no significant impact on reproduction and avoidance behavior of *E. fetida* upon exposure to 10,000 mg $kg^{-1}$; suggesting very little hazard associated with that $C_{60}$ exposure in soil (Li and Alvarez, 2011). Similarly, another study found that low concentrations (5 -10 mg $kg^{-1}$) induced non-significant effects on weight and cuticle fibers of *L. Variegatus* and *E. fetida* (Kelsey and White, 2013; Pakarinen et al., 2011). Alternatively, Van der Ploeg et al. (2011) found that $C_{60}$ at 154.4 mg $kg^{-1}$ had significant effects on cocoon production, juvenile growth rate and mortality after 28 days exposure of the parental generation. Another study revealed histological tissue injury and that external barriers (cuticle and gut epithelium) were partly damaged in *L. rubellus* that was exposed to 154 mg $kg^{-1}$ $C_{60}$ for 28 days. Interestingly, surviving earthworms showed evidence of tissue repair and recovery (Van Der Ploeg et al., 2013). Another study reported that *E. fetida* potentially exhibited tolerance to $C_{60}$ as evident by adaptive response at the molecular level. However, reduction in sugars, amino acids (leucine, valine, isoleucine and phenylalanine) and the nucleoside inosine appeared to be potential biomarkers for $C_{60}$ exposure (Lankadurai et al., 2015). Contradictory results were evident in *L. rubellus* as the down regulation of certain stress (HSP*70*) and immune (CCF-1 ) related genes was observed, but no effects on antioxidant enzyme activity were detected (Van Der Ploeg et al., 2013; van der Ploeg et al., 2014b). As such, we suggest these biomarkers should be evaluated in a targeted / dose-dependent manner across multiple species. Li et al. (2011) reported the rapid BSAF of $^{14}C$-labeled $C_{60}$ in *E. fetida* at 0.25 mg $kg^{-1}$, which indicates a risk for the food chain contamination through TTF. This underscores the need for further studies on $C_{60}$, among other ENPs, regarding TTF, bio magnification potential, and associated sublethal effects under different soil environments. In addition, the sorption capacity of organic co-contaminants and how those association processes impact multi-analyte toxicity needs to be evaluated.

### *5.3 Graphene oxide*

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

Young's modulus (1 TPa), high thermal conductivity (5000 W $mK^{-1}$), strong chemical durability and high electron mobility (2.5 105 $cm^2V^1s^1$) (Sherlala et al., 2018). It has been reported that multi-layer GO with a range of morphologies and hydrophobicity have different toxic effects on earthworm species (Zhang et al., 2020b). Metabolite levels of alanine, phenylalanine, proline, and glutamate in juvenile of *E. fetida* changed significantly after 7 days exposure to GO-MNOs at 300 mg $kg^{-1}$ (Zhang et al., 2020d). Given the increasing interest in the application of novel 2-dimension materials such as graphene oxide, much additional work is needed to evaluate the fate and effects of these materials in soil and on key invertebrate species such as earthworms.

### *5.4 Nanoplastics*

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

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

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

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

In soil, the mobility and bioavailability of metallic MNOs are controlled by physio-chemical properties of the material (e.g., size, surface charge and surface functionality) as well as by a series of different soil characteristics, of which pH and organic matter generally have the greatest impact on toxicity. It should be noted that further soil parameters (e.g., pore size, soil surface properties, hydraulic parameters or texture) and pore water characteristics (e.g., pH, content and composition of electrolytes and dissolved organic matter) do also play a role (Kah et al., 2013). However, metal and metal oxide based MNOs are prone to degrade in soils and liberate potentially toxic ions. Thus, this is a significant transformation process and often confounds attempts to understand nanoscale specific species response. As a function of dose, metallic MNOs can reduce adult and juvenile growth rates, reproduction, and respiration, as well as increase avoidance response, lethality, subcellular damage and oxidative stress. Oxidized metal oxides may have different effects than pure metal particles of the same element; for example, zero-valent Fe NPs are generally less toxic to earthworms than FeOx NPs. Harmful effects of zero-valent Fe NPs, including avoidance, mortality, and weight loss, are typically reported only at high concentrations. The toxicity of materials such as $TiO_2$-NPs depends significantly on the size, purity, surface coating, crystallinity, shape, and solubility. These studies show that both material properties and environmental conditions control the behaviour and fate of $TiO_2$-NPs in soil environments. In the case of $Al_2O_3$-NPs, the reviewed studies indicated that these NPs are only toxic at very high concentrations unlikely to be found in the environment. NiO-NPs reduced reproduction and were shown to affect embryo development and induce oxidative stress. NiO-NPs also had a negative effect on the soil microbial community and soil enzymes and therefore could significantly affect other terrestrial invertebrates. Exposure to $CeO_2$-NPs showed no effect on the survival or reproduction, while Ce salt (ammonium cerium nitrate) affected both reproduction and survival at high doses. In the case of silver, the studies showed that Ag-NPs are often more toxic than Ag ions (typically $AgNO_3$ salts). In addition, soil samples containing aged Ag-NPs were more toxic to the earthworms than non-aged Ag-NPs, which led to the conclusion that the fate of the NPs was determined largely by their interaction with organic matter which increased particle toxicity through yet to be characterized transformation processes. Median values of $EC_{50}$ and $LC_{50}$ are depicted in Figure 6 as determined from 35 published papers. $EC_{50}$ and $LC_{50}$ concentrations for each MNOs significantly varied, largely due to differences in experimental design such as exposure duration and MNOs size. In terms of species, *E. fetida* and *L. rubellus* were the most commonly investigated organisms.

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

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

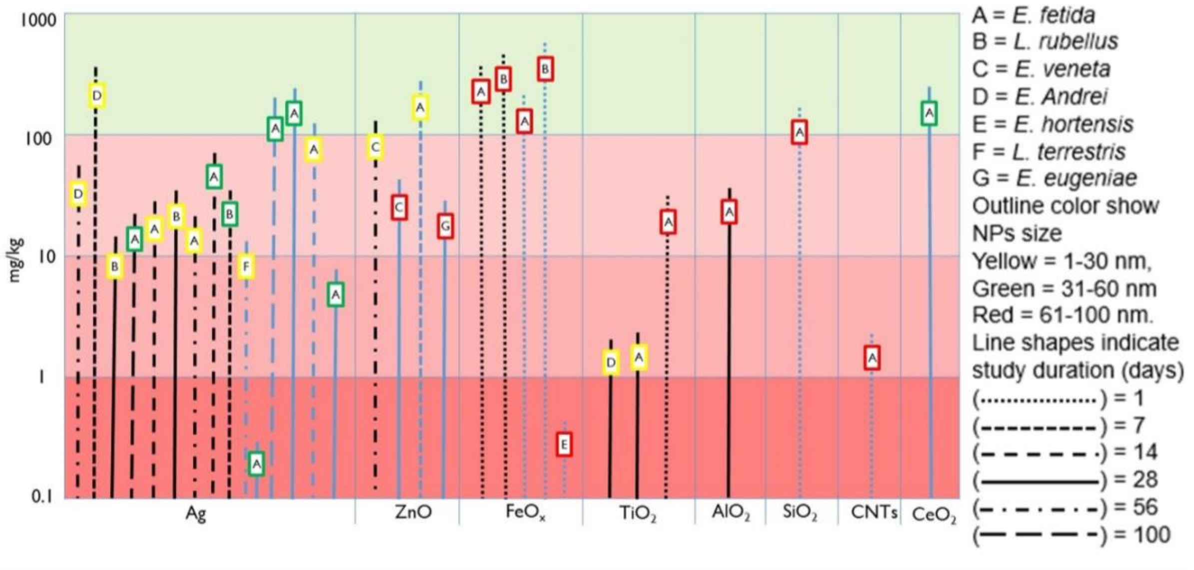



**Figure 5** $EC_{50}$ and $LC_{50}$ of selected MNOs to different earthworm species recovered from the published literature. Capital letters correspond to earthworm species, the colour outline of the capital letters signifies

the size of MNOs, line colour representing the $EC_{50}$ and $LC_{50}$ (Black = $EC_{50}$ and blue=$EC_{50}$) and line shapes indicated the duration of experiment.

## 7. Conclusions and future perspective

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

The study results currently do not allow a clear statement on safety of MNOs in agricultural soils, as data on the physicochemical properties and applied nanoformulations are lacking – in particular, information on particle size and specific surface area as well as on the used surfactants or organic ligands to modify the nanoparticle's surface in order to increase colloidal stability (dispersibility), provide functionality or control solubility of MNOs. Quantitative risk assessment is currently not possible because the actual amounts of intentionally applied nanoagrochemicals or unintentionally released MNOs from nano-enabled products is unknown, while comparable toxicity studies and bioassays are lacking. However, the reviewed studies showed that both metal- and carbon-based MNOs can be toxic to earthworms, depending on the recipient species, duration of exposure, concentration/dose, method of exposure and especially the physicochemical properties of a specific MNO (type, size, shape, surface charge, surface functionalization etc.). The literature survey revealed that the deleterious effects of MNOs are clearly related to their size and therefore the relative surface area. Release of MNOs into soils is often followed by an ageing process, which can include transformation process such as dissolution, adsorption, hetero-aggregation and biotransformation. Earthworm biotransformation and stress modulation mechanisms of MNOs are also poorly characterized; an understanding of these processes is necessary to fully characterize risk in terrestrial ecosystems. Additionally, standardization of analytical methods as well as toxicity assays, that demand the reporting of parameters on the investigated MNOs and the matrix, must be further developed and applied worldwide (e.g. at OECD level), which would increase the comparability of study results. A clear understanding of the importance of, in particular long-term, dosage at relevant low environmental concentrations (in the ppb range) has to also be a part of future work, both as this relates to cross-material and cross-species comparisons. Importantly, reports on adverse effects of MNOs (e.g., Se, GO, Yb, La) on earthworms are missing for some material classes.

We note that using “fresh” artificially amended soil should be considered with care because particle bioavailability may change significantly during aging. Several studies have documented MNOs behave differently over time, and the associated toxicity to earthworms is not well understood. As with many contaminants, additional work is needed investigating the impacts of MNOs to earthworm species under environmentally realistic exposure scenarios, including chronic exposures over multiple generations. Importantly, very little work has been done with earthworms under multi-species or microcosm type of systems that simulate a “real-world” exposure and may include additional stressors such as low to moderate doses of additional contaminants. An understanding of species tolerance mechanisms to toxic NPs exposure is also lacking. In addition, earthworms are common prey of many vertebrates and given the documented uptake and accumulation of MNOs in earthworms, TTF of MNOs to higher levels of the food chain needs additional investigation. For risk assessment of nanoagrochemicals, it must be stressed to consider possible transformation processes, as the fate and toxicity of transformed particles can vary greatly from pristine MNOs. In order to comply with this in a laboratory, analytical techniques, such as electron microscopy or mass spectrometry, must be combined, which allow the MNO quantification (to determine, if possible, specific particle surface area and both particle number and mass concentration) as well as the differentiation of MNOs from the background noise and possibly released MNO components (e.g., surfactants or dissolved metal ions). In the case of metallic MNOs, differences between the nanoparticulate and ionic forms and the correlation with biological responses of soil organisms such as earthworms should be investigated to identify nano-specific toxic effects. A clear understanding of the dynamic nature of important transformation processes and MNO uptake mechanisms in earthworms needs to be a part of these investigations. Future work should also include a focus on identifying the molecular initiating events that result in adverse outcomes such as reduced reproductive capacity or genotoxicity and epigenetic effects in subsequent generations. With regard to long-term exposure studies, gene expression and biomarker assessment (SOD, CAT, etc.) should be addressed to understand the toxicological mechanisms of MNOs. Additionally, oxidative stress, redox activity and production of oxygen species, cationic stress, photoactivation, embryo hatching interference and membrane lysis are further important toxicological drivers to be considered. Application of MNOs in soil should be reviewed critically for nano-enabled agriculture.

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