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Nanomaterials in the Environment: Perspectives on *in Vivo* Terrestrial Toxicity Testing

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Over the last decade, engineered nanomaterials (NMs) brought a revolutionary development in many sectors of human life including electronics, paints, textiles, food, agriculture, and health care. However, the exponential growth in the number of NMs applications resulted in uncertainties regarding their environmental impacts. Currently, the common approach for assessing the toxicity of NMs such as, carbon—(fullerenes, single- and multi-walled carbon nanotubes), mineral—(gold and silver nanoparticles, cerium and zinc oxide, silicon and titanium dioxide), and organic-based NMs (dendrimers) includes standard guidelines applied to all chemical compounds. Nevertheless, NMs differ from traditional materials as their physicochemical and surface properties influence the toxic rather than their composition alone. Considering such NMs specificities, adaptations in some methods are necessary to ensure that environmental and human health risks are accurately investigated. In this context, the focus of this mini-review is to summarize the current knowledge in nanotoxicology regarding relevant organisms and experimental assays for assessing the terrestrial toxicity of NMs.

Keywords: ***Enchytraeus albidus, Caenorhabditis elegans,*** **nanoparticles, nanotoxicity, standard guidelines**

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INTRODUCTION

Engineered nanomaterials (NMs) are defined by the International Organization for Standardization (ISO) as those intentionally produced to have specific purpose or function, with at least one external dimension, or presenting an internal or surface structure in the nanoscale (∼1 to 100 nm) (ISO/TS 80004-1, 2015). Due to their unique physicochemical properties, the production and use of these NMs are increasing exponentially in all sectors of human life, consequently, their amount released into the environment during all stages of their lifespan (production, use, and disposal).

Ecotoxicity testing of NMs is a challenge considering the lack of information related to fate, potential interactions, and behavior of the NMs in the surrounding environments. Furthermore, abiotic factors can influence specific physicochemical properties of NMs affecting their bioavailability and toxicity (El Badawy et al., 2010; Grillo et al., 2015). Thus, to ensure the reliability and reproducibility of ecotoxicity tests, NMs should be accurately characterized in multiple life cycle endpoints and the experimental parameters should be carefully chosen (Holden et al., 2016).

The Organization for Economic Cooperation and Development (OECD) has published several guidelines for assessing the potential effects of conventional chemicals on human health

and environment[1] Regarding NMs, the consensus in the scientific and regulatory communities is that many current health effects and ecotoxicology test guidelines (TG) for conventional chemicals apply to NMs. However, these TG need revision taking into account the specificities of the NMs and guidance on NMs preparation, delivery, and metrology need development for toxicity testing (OECD, 2009a; ECHA, 2014; Rasmussen et al., 2016).

The *in vivo* studies are still the gold standard to predict human toxicity. Nevertheless, following the 3R principle (Replacement, Reduction, and Refinement), the use of lower organism in the initial screening is recommended to replace and reduce the use of vertebrates test species. The purpose of the present mini-review is therefore to describe some of the more relevant organisms and protocols that have been used for terrestrial toxicity testing of NMs.

CURRENT TERRESTRIAL TOXICITY TESTS

Environmental hazard assessment in the terrestrial media can be performed using a suite of tests as standardized by OECD and ISO, an example update can be found in (Amorim et al., 2016). These tests are performed on animals that may be exposed directly or indirectly to the contaminated soil including microorganisms, soil invertebrates, and vertebrates. Among these models we, highlight the most often used models from lower organism to higher mammal models.

Nematode Toxicity Studies: *Caenorhabditis elegans*

Caenorhabditis elegans is abundant in ecosystems and plays a major role in the decomposition of soil organic matter and nutrient cycling. Many *C. elegans* characteristics are desirable for toxicological studies, such as small size (1 mm in length), optically transparency, readily propagation (generation time approximately 3 days), and simple anatomy (959 identified somatic cells in the adult, in addition to programmed cell death in others 131 cells during development; Brenner, 1974; Sulston et al., 1983). Hence, *C. elegans* can be used in different types of toxicological assays, including high-throughput screening, which is limited in more complex animals. Additionally, the complete sequence of *C. elegans*, finished in 2002, allows a full characterization of new genes and proteins those are relevant in mediating human diseases (*C. elegans* Sequencing Consortium, 1998). Many mutants have been generated, thus permitting the search for mechanistic toxicity. Furthermore, proteins can be tagged with green fluorescent protein (GFP), allowing the visualization of dopaminergic neurons, for example (Chalfie et al., 1994).

Several endpoints can be carried out to assess the toxic effects of chemicals on *C. elegans* (**Figure 1**). Worms can be exposed to NMs in different types of media such as, liquid (M9 buffer, saline, K or S medium), solid (nematode growth medium or OP50 medium), or directly in soil samples. The absorption of the chemicals occurs mainly through the mouth, whereas the thick cuticle absorbs very little. Regarding exposure time, worms can be exposed acutely (30 min, for example) or throughout their whole life.

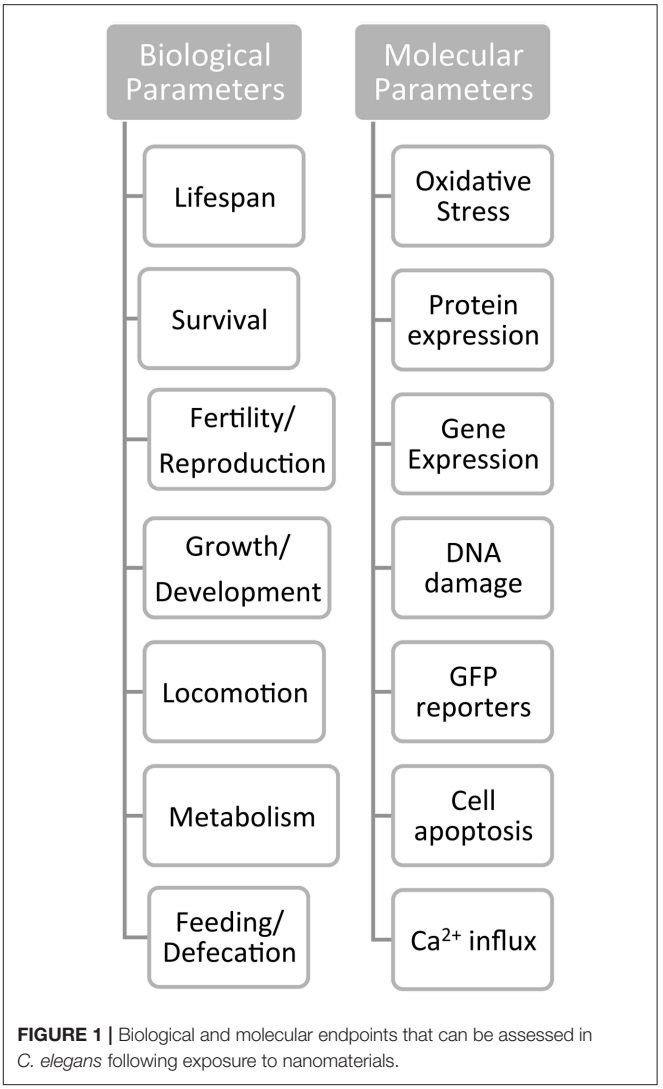

FIGURE 1 | Biological and molecular endpoints that can be assessed in *C. elegans* following exposure to nanomaterials.

Regarding NMs, the evaluation of their potential toxic effects has become necessary, as there is a growing concern on the short and long-terms effects following exposure. Among the available models, *C. elegans* characteristics advise for using this nematode as a living system for the primary screening of NMs toxicity (Gonzalez-Moragas et al., 2015). One of the most tested NMs is silver nanoparticles (AgNPs). In worms, treatment using sublethal concentrations of AgNPs could lead to neurotoxicity. Exposure to 100 mg AgNPs/L reduced the velocity, flex, amplitude, and wavelength of the body bend of exposed worms, which was worsened in the progeny (Contreras et al., 2014). Of note, worms locomotion is regulated by gamma-aminobutyric acid-ergic (GABAergic), cholinergic and dopaminergic neurons, then alteration in these parameters may indicate neuronal damage (Jorgensen, 2005; Rand, 2007). Additionally, AgNP is reported to reduce survival and reproductivity in *C. elegans*, and also to cause severe edema after being in contact with the biological surfaces of the worm (Kim et al., 2012).

[1]http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm.

The toxicity of titanium dioxide (TiO_2), zinc oxide, and silicon dioxide (SiO_2) nanoparticles (NPs) has been compared using different approaches such as, lethality, locomotion, growth, reproduction, and the production of reactive oxygen species (Wu et al., 2013). Particularly, TiO_2 NPs led to a substantial decrease in both head thrash or body bend in superoxide dismutase (SOD)-2, SOD-3, metallothionein-2, and heat shock protein-16.48 mutants compared to the wild-type (Wu et al., 2014). These findings suggest that the lack of SOD isoforms, metallothioneins, and heat shock proteins, which are proteins involved in oxidative stress protection and metal elimination, render worms more susceptible to these NPs. In an attempt to reduce NPs toxicity, polymeric coatings have been used to increase water solubility, to reduce toxicity and to direct site-specific metal delivery (Subbiah et al., 2010; Thanh and Green, 2010).

Long-term early onset exposure to Cadmium Telluride quantum dots (CdTe QD-0.1 and 1 μg/L) caused abnormal foraging behavior, which is related to the altered function of the motor neurons (Zhao et al., 2015). In accordance, there was a decreased fluorescence of motor neurons cell bodies, indicating an alteration in their development. Furthermore, authors demonstrated that these CdTeQDs crossed the intestinal barrier and reached RME neurons, which are GABAergic motor neurons.

Regarding organic based NMs, hydroxylated fullerene NPs were reported being able to induce apoptosis in *C. elegans* (Cha et al., 2012). In fact, organic polymeric NPs toxicity assessment is still scarce in the literature. It has been demonstrated that melatonin-loaded-lipid core nanocapsules showed better antioxidant activity than free melatonin (Charão et al., 2015). Besides, a study with acute exposure to polysorbate coated polymeric nanocapsules loaded with clozapine showed that the nanoencapsulation reduced the toxicity of the antipsychotic (Sanches Moraes et al., 2016). However, this study demonstrated an acute toxicity of the formulation without the drug, which was not observed in a long-term manner. Notably, a recent study with nanopesticides showed that unloaded formulations have a strong toxic effect in worms, reducing survival, brood size, and delaying worms development (Jacques et al., 2017). By using fluorescent formulations, it was observed that they remained in the intestine of the worms, which may be the cause of the toxic effects.

Enchytraeid Toxicity Studies: *Enchytraeus crypticus*/*Enchytraeus albidus*

Enchytraeids are abundant soil organisms with worldwide distribution. They often live in the actual soil layer and provide important functions to the soil ecosystem, mostly indirectly, facilitating the organic matter turnover. They are sensitive to chemicals and stressors; hence the enchytraeid reproduction test (ERT), a chronic laboratory test for the testing of chemicals and soil quality assessment, was developed for *E. albidus* (Römbke and Moser, 2002). It has standard guidelines by ISO (ISO 16387, 2014) and OECD (OECD, 2016) where several test species can be used. *E. crypticus* has a shorter life cycle and is among the test species of choice in recent years. It has been given considerable focus and has currently an extensive suite of tools available for hazard assessment, far beyond the standard ecotoxicity test battery of many other species. The tools cover bioaccumulation (Amorim et al., 2011), avoidance (Bicho et al., 2015a), full life cycle test (Bicho et al., 2015b, 2016, 2017a; Santos et al., 2017), embryotoxicity (Gonçalves et al., 2015), full life span (Gonçalves et al., 2017), multigenerational (Bicho et al., 2017b), multispecies (mesocosm) system (SMS) (Menezes-Oliveira et al., 2013), full transcriptome and microarray tool (Castro-Ferreira et al., 2014; Gomes et al., 2017), oxidative stress biomarkers (Ribeiro et al., 2015), energy metabolism and cellular energy allocation (Gomes et al., 2015a), genotoxicity via the comet assay (Maria et al., 2017), and metabolomics and proteomics. This species has further potential as it can be exposed via water for a short period and hence allows the assessment of other routes of exposure (Gomes et al., 2015b).

An essential element of a systems toxicology approach is to have a broad combination of tools and endpoints being able to cover many levels of biological organization; therefore *E. crypticus* is an exquisite model at the moment. With this species, effects can be assessed by the transcriptome and metabolome, which can underpin mechanisms but also generate hypothesis for further testing. This is very important in an intelligent testing strategy context where efforts must be dedicated and prioritized. At the cellular level, various tools are optimized covering oxidative stress biomarkers, histology, embryo development, or cellular energy allocation. Organism level effects are broadly populated with results for avoidance behavior, survival, reproduction, and kinetics (bioaccumulation). More recently, a full life cycle test is also available, including hatching success, growth, and maturity besides survival and reproduction endpoints. This species has also an established procedure for a full life span test and multigenerational. The latter allows the assessment of epigenetic potential.

E. crypticus has been successfully used for assessing the effects of several NMs including silver, copper, nickel, TiO_2, silica, iron oxide, tungsten carbide cobalt, multi-wall carbon nanotubes (MWCNTs), organic pigments, and nanopesticides. Adaptations to existing guidelines or developments of novel tools have been made. For example, the full life cycle test (46 days), an extension of the standard reproduction test (21 days), where effects could be discriminated between nano and non-nano form materials such as silver. Bicho and co-workers observed that embryo development, hatching, and survival of juveniles were less affected by silver nitrate than AgNPs (Ag NM300K) (Bicho et al., 2016). In this case study, it was clear that the standard guideline was too limited compared to the full life cycle test.

Concerning novel tools, the full life span test was developed considering the issues of long term testing and NMs persistency. As shown, exposure to copper oxide nanomaterials at the reproduction EC_{50} caused shorter longevity than exposure to copper-salt, bringing a novel concept to ecotoxicology (Gonçalves et al., 2017).

In sum, the tools are available and the potential for in-depth studies should be explored. Although, *E. crypticus* will not cover the effects of all terrestrial ecosystem and the use of different species is always recommended, preferably including differing life traits and taxonomic groups.

Mammalian Toxicity (Rodents)

The ecological soil screening levels for mammals considered two potential exposure pathways to chemicals: incidental ingestion of contaminated soil, and ingestion of contaminated food items (EPA, 2007). Regarding NMs occupational safety, the importance of gastrointestinal tract is nil in comparison to respiratory airways. However, if the NMs are applied on food items the evaluation of oral toxicity become essential (Pachapur et al., 2015).

Oral and inhalation toxicity studies should be performed in at least one mammalian species. Following OECD TG, rats are the preferred rodent species for the studies unless a species more representative of human toxicity is known. Rodents have been preferred due to their extensive use in pharmacological and toxicological studies, relatively short life cycle, susceptibility to tumor induction, and availability of characterized strains. Consequently, their physiology and pathology characteristics have been well-documented in several studies.

To test acute oral and inhalation toxicity, there are four OECD TG (420, 423, 425, and 403) and two US Environmental Protection Agency (EPA) TG (OPPTS 870.1100, 870.1300). In these guidelines, female rats are normally used as their Lethal dose (LD50)-value is slightly lower than in males indicating a higher sensitivity. However, if males could be more susceptible to the test compound, then males or both sexes should be used. Following the single treatment using the test compound, animals should be observed daily for mortality, body weight changes, and clinical signs of toxicity including alterations in behavior patterns and physiological functions that indicated an abnormal function of circulatory, respiratory, autonomic, and central nervous systems. After 14 days, the animals should be sacrificed, and pathological changes evaluated (EPA, 1998a, 2002; OECD, 2002a,b; OECD, 2008b, 2009b).

Additionally, sub-chronic and chronic toxicity studies should be performed to determine the toxicological profile of the new compound after repeated oral (OECD TG 407, 408; OPPTS 870.3050, 870.3100) or inhalation exposure (OECD TG 412, 413; OPPTS 870.3465). These studies provide detailed information about the potential target organs of toxicity, the exposure-response relationships, as well as the potential reversibility of the toxic effects (EPA, 1998b,c, 2000; OECD, 1998, 2008a, 2009c,d). Furthermore, significant data on nervous, immune, endocrine, and reproductive systems can also be obtained even specific guidelines are available to investigate these endpoints. **Table 1** summarizes the OECD guidelines for acute and repeated dose toxicity studies.

In a 28-day rat oral toxicity study, the effects of 1,000 mg/kg bw/day of barium sulfate, two surface-functionalized zirconium dioxide, and four amorphous SiO_2 NPs with or without surface functionalization were evaluated according to OECD TG 407. None of the tested nanomaterials cause local or systemic effects (Buesen et al., 2014). Similarly, studies performed by Matsumoto and co-workers did not reveal toxic effects after acute and

TABLE 1 | OECD guidelines for acute and repeated toxicity studies.

Guideline n°	Guideline title	Duration	Species/Sex	Principal endpoints
OECD 220	Enchytraeid reproduction test	42 days	*Enchytraeus albidus*	Mortality; Reproduction; Median Lethal Concentration (LC50); Median Effective Concentration (EC50); No Observed Effect Concentration (NOEC) and/or Effective Concentration (ECx)
OECD 420 OECD 423 OECD 425	Acute oral toxicity—fixed dose procedure Acute oral toxicity—acute toxic class method Acute oral toxicity: up-and-down procedure	14 days (single dose)	Rats/Normally females	Clinical signs of toxicity; body weight changes; gross necropsy; histopathological examination*; LD50
OECD 407 OECD 408	Repeated dose 28-day oral toxicity study in rodents Repeated dose 90-day oral toxicity study in rodents	28 days (daily dose) 90 days (daily dose)	Rodents (rat preferably)/Males and females	Clinical signs of toxicity; body weight and food/water consumption; hematology and clinical biochemistry; gross necropsy/organs weight; full histopathological examination.
OECD 403	Acute inhalation toxicity	14 days (One exposure session) Traditional: fixed period of time (up to 6 h); Nose- or whole body-only C x t: multiple time durations; Nose-only	Rat preferably/Susceptible sex.	Clinical signs of toxicity; body weight changes; gross necropsy; lung weight; microscopic examination of the entire respiratory tract; histopathological examination*
OECD 412	Subacute inhalation toxicity: 28-day study	28 days (6 h/daily exposure session)	Rodents/Males and females	Clinical signs of toxicity; body weight changes; hematology and clinical biochemistry; gross necropsy/organs weight; full histopathological examination.
OECD 413	Subchronic inhalation toxicity: 90-day Study	90 days (6 h/daily exposure session)	Preferred species are rats/Males and females	Clinical signs of toxicity; body weight changes; hematology and clinical biochemistry; gross necropsy/organs weight; full histopathological examination.

**Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 or more hours, and organs known or expected to be affected.C × t, Concentration × Time protocol.*

repeated oral doses of single- or multi-wall carbon nanotubes (Matsumoto et al., 2012).

The toxicity of MWCNTs is more adverse in the case of inhalation as compared to oral exposure. Rats exposed to 2.5 mg/m^3 MWCNTs for 6 h/day during 5 consecutive days using a head-nose system demonstrated signs of pulmonary toxicity such as, microgranulomas and diffuse alveolar histiocytosis (Ma-Hock et al., 2013). After a 13-week exposure period, the inhalation of 2.5 mg/m^3 MWCNTs induced systemic inflammation and histopathological abnormalities in the lungs, lymph nodes, nasal cavity, larynx, and trachea (Ma-Hock et al., 2009).

The inhalation models performed in rats have some limitations, and it is important to consider the interspecies differences in physiology. Animal models exhibited differences in the breathing pattern when compared to humans however they can provide evidence on the fate of inhaled nanomaterials and their biological interactions (Fröhlich and Salar-behzadi, 2014).

CONCLUSIONS

The exponential growth of nanomaterials has broad ecotoxicological implications because of their potential impacts on the environment. The use of internationally accepted test guidelines helps to improve the reproducibility, reliability, and data quality of the studies and contribute to support a common regulatory framework. Herein, we summarize some of the current test guidelines and the understanding of NMs toxicity to terrestrial representative species of various trophic levels, including *E. crypticus*/*E. albidus*, *C. elegans*, and rodents. Combining observations assessed on organisms from different phyla and using different methodologies allows for more accurate environmental monitoring and is recommended.

AUTHOR CONTRIBUTIONS

DÁ wrote the section "Nematode toxicity studies: Caenorhabditis elegans". MA wrote the section "Enchytraeid Toxicity Studies: Enchytraeus crypticus/Enchytraeus albidus". MM and CR wrote the section "Mammalian Toxicity (Rodents)". MdJ conceived the idea and reviewed the manuscript text. All authors read and approved the final manuscript.

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