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Review

Alternative test methods for (nano)materials hazards assessment: Challenges and recommendations for regulatory preparedness

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ABSTRACT

The outstanding work performed by standardization organizations for guidelines to assess hazards, e.g., OECD (Organisation for Economic Co-operation and Development), is clearly visible by the currently available number and broad coverage, from aquatic to terrestrial organisms dealing with environmental relevant issues. Nevertheless, novel materials challenge the adequateness and fit-for-purpose of such standards, as the standards were developed to assess hazards of "conventional" chemical substances and not advanced materials (e.g. materials that may deliberately change behaviour). While standardization is a well-known process that requires extended time before reaching implementation stage, there is strong support from regulatory bodies for the development of New Approach Methodologies (NAMs) (e.g., updating of current guidelines, development of novel omics-, in vitro-, and in silico- tests including modelling and read-across) that meet regulatory preparedness (i.e. have considered issues important for regulatory testing). There are currently several NAMs available, complying with high quality standards and relevancy, which should be adopted. In the current review, we collected the available literature on NAMs to assess hazards of Nanomaterials (NMs), focusing on the terrestrial environment, and critically discuss the advantages, challenges and gaps. Tests were grouped into 1) Standard tests (OECD/ISO), 2) Standard tests (OECD/ISO) extensions: time course or prolonged exposures and/or multigenerational, and 3) Alternative tests, beyond current OECD/ISO: omics, biomarkers, in vitro, in silico and modelling. The goal is to provide guidance on the best practices and test designs focusing on the specificities of testing NMs, outlining recommendations and way forward.

Contents

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Nomenclature

AOPs	Adverse Outcome Pathways
CEA	Cellular Energy Allocation
DEGs	Differentially Expressed Genes
DEMs	Differentially Expressed Metabolites
DEPs	Differentially Expressed Proteins
EC	Effect Concentration
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EHS	Environmental Health and Safety
ERT	Enchytraeid Reproduction Test
FLC	Full Life Cycle
GEO	Gene Expression Omnibus
GO	Gene Ontology
HTP	High Throughput
ISO	International Organization for Standardization
JRC	Joint Research Centre (from European Commission)
LTP	Low Throughput
MG	Multigenerational
NAMs	New Approach Methodologies
NBM	Nanobiomaterials
NCBI	National Center for Biotechnology Information
NMs	Nanomaterials
NPLs	Nanoplastics
OD	Open Data
OECD	Organisation for Economic Co-operation and Development
ROS	Reactive Oxygen Species
SMS	Soil Multispecies test System
SOPs	Standard Operating Procedures
US EPA	United States Environmental Protection Agency
UV	Ultra Violet
Ag	Silver
Al_2O_3	Aluminium Oxide
Au	Gold
C_{60}	Fullerenes
CeO_2	Cerium Oxide
Co_3O_4	Cobalt Oxide
Cu/CuO	Copper/Copper Oxide
DWCNTs	Doublewalled Carbon Nanotubes
ERC	Environmentally Realistic Concentrations
Fe	Iron
Fe_2O_3	Iron Oxide
GDs	Guidance Documents
GO	Graphene Oxide
LOECs	Lowest Observed Effect Concentrations
MoO_3	Molybdenum Oxide
MWCNTs	Multiwalled Carbon Nanotubes
NCB	Nano Carbon Black
NGOs	Non-Governmental Organizations
Ni	Nickel
NOECs	No Observed Effect Concentrations
nZVI	nano Zerovalent Iron
PECs	Predicted Environmental Concentrations
QDs	Quantum Dots
qPCR	quantitative (or real-time) Polymerase Chain reaction
RA	Risk Assessment
RNAseq	RNA Sequencing
RU	Risk Understanding
SiO_2	Silicon Oxide (or Silica)
TGs	Test Guidelines
TiO_2	Titanium Dioxide
$TiSiO_4$	Titanium Silicon Oxide
WCCo	Tungsten Carbide Cobalt
Zn/ZnO	Zinc/Zinc Oxide
ZrO_2	Zirconium Dioxide

Overview and aims

The organizations responsible for standardization of ecotoxicology guidelines, e.g., OECD (Organisation for Economic Co-operation and Development), ISO (International Organization for Standardization), etc., have for many years done an extensive work on developing guidance and guidelines. The progress is clearly visible by the number of guidelines currently available, covering from aquatic to terrestrial organisms with environmental relevant issues. Nevertheless, novel materials keep being developed, e.g., nanomaterials, advanced smart materials, challenging the adequateness and fit-for-purpose of such standards, standards mostly developed to assess hazards of "conventional" chemical substances and not materials. Nanomaterials (NMs, also used to refer interchangeably to nanoparticles throughout this paper) are among the examples of such materials that challenge the current guidelines. Over the last 20 years it has become clear that the existing test guidelines (e.g., OECD Test Guidelines (TGs) and Guidance

Documents (GDs)) required adaptations for nano-specific issues, and this besides the need of new guidelines for fully novel aspects, to cover regulatory requirements. The OECD Working Party on Manufactured Nanomaterials (OECD WPNM), established in 2006, has been appointed to inform on the environmental and human health safety of manufactured NMs, among which to explore the need for new guidelines or adaptation of the existing ones to address the NM issues. OECD WPNM has already preformed extensive work with initiating globally testing of some of the priority NMs, with adapting some of the current guidelines to make them suitable for NMs, and with developing novel guidelines specifically designed for NMs, e.g., for the characterisation of NMs. Nevertheless, there has in the past decade been an ongoing development which has yet not found its way in the regulatory world.

The scientific (and regulatory) community is well aware of the challenges and has contributed to identify some of these as well as ways forward [1] including adaptations of current OECD guidelines to NMs [2,3]. Some TGs have been updated and other are being developed [4]. The importance of this is also clear for regulatory agencies, e.g., the later EU funds allocated to regulatory aspects after the standardization of methods (e.g., FP7: MARINA, SUN, H2020: BIORIMA). Obviously, not all needs are tackled and there are concurrent initiatives, e.g. the Malta initiative (2017) (https://www.nanosafetycluster.eu/international-cooperation/the-malta-initiative/), where several European countries, including Directorate-General of the European Commission, the European Chemicals Agency (ECHA), authorities, research institutions, NGOs, universities and industry, aim to mitigate and fill the many gaps towards legislation. In relation to this initiative are projects such as NanoHarmony (EU) and NANOMET (OECD), which further promotes this development of guidelines. On a broader level, the overall governance of NMs, as funded by the EU call NMBP13, is being widely discussed and considered under 3 collaborative projects (H2020 NANORIGO, GOV4-NANO, RISKGONE). One of the frameworks' needs [besides data], are fit-for-purpose tools to assess the hazards, and hence the role of standardized tools is a key asset to have consolidated and harmonised between countries. The standardization process is well-known to require extended time before reaching implementation stage. While this is part of a continuous ongoing effort, there is strong support from regulatory bodies for the development of New Approach Methodologies (NAMs) to establish "Alternative Tests" both in EU [5], USA, Canada and Australia (as well as elsewhere e.g., Japan, South Korea) [6]. NAMs usually refer to alternative methods, such as in silico and in vitro, modelling, read-across and omics, etc. There are currently several NAM tests and methods available, complying with high quality standards and relevancy (e.g. [7,8]).

For regulatory purposes there is often a need to meet consensus and define quality criteria, e.g. minimum required descriptors, validation, data analysis outputs. The importance and added-value of alternative methods is well recognized, e.g. the cosmetic industry saw the testing of cosmetic products or ingredients on animals being banned (in force from 2013), under the EU regulation on cosmetic products (1223/2009) hence the urgent need to develop and use alternative testing and meet regulatory preparedness.

Regarding the omics as alternative testing, the mechanistic level of information provides key knowledge, namely the potential predictive and read-across, as well as safer-by-design production of materials, being used for many years in toxicology, e.g. for pharmacology approaches. Naturally, fears associated with novelty like NAMs exist, partly due to the difficulty of adequate communication and the need of maturation. Hence its introduction can be time consuming. Organizations such as EFSA (European Food Safety Authority) have had an important role, promoting the discussion between stakeholders on these matters (e.g. EFSA scientific colloquium, April, 2018 on "Omics in risk assessment: state-of-the-art and next steps" https://www.efsa.europa.eu/en/events/event/180424–0) [9]), focusing on the importance of omics knowledge. There is a need to provide a list of criteria for alternative data (e.g. NAMs) and pilot data sets for verification of tools (e.g. assays/guidelines). There are two major aspects of any read-across application, namely assessing similarity and uncertainty.

There are already good examples of routine implementation of alternative test methods such as subcellular functional assays. For example, for risk assessment of endocrine disrupters, (under the Endocrine Disruptor Screening Program (EDSP)), the US EPA launched a series of guidelines to identify substances that have the potential to interact with the oestrogen, androgen, or thyroid hormone (Tier 1). Among those guidelines, e.g. the H295R Steroidogenesis Assay is intended to identify xenobiotics that affect the steroidogenic pathway beginning with the sequence of reactions occurring after the gonatotropin hormone receptors (FSHR and LHR) through the production of testosterone and estradiol/estrone [10] which was further adopted by OECD [11]. Another example, the Aromatase (Human Recombinant) Assay intends to identify chemicals that may affect the endocrine system (e.g., steroidogenesis) by inhibiting catalytic activity of aromatase, the enzyme responsible for the conversion of androgens to oestrogens [10].

Hence, in the current review, we collected the available literature on NAMs to assess hazards of NMs, focusing on the terrestrial environment. We discuss the advantages, challenges and gaps of the use of NAMs for the hazard assessment of NMs, outline recommendations and the way forward. The goal is to provide guidance on the best practices and test designs focusing on the specificities of testing NMs.

Method for literature review

Previous review papers on the (eco)toxicity of NMs, concerning terrestrial ecosystem, focused on specific NMs or groups of NMs (e.g. metal-based [12,13]), or one species/taxonomic group (e.g. earthworms [14]). In this review we have collected available literature on data from standard testing (e.g., OECD/ISO) and alternative testing, i.e. OECD/ISO extensions and beyond standard, where additional endpoints and tests are offered. An overview of the available tests in terrestrial compartment and levels of detail is shown in Amorim et al. [1].

The literature review was performed using the search engine Web of Science (WoS), using the database WoS Core Collection, in the Basic Research mode (at 1st November 2020), using the keywords: nano* , soil, toxicity, ecotox* , gene, and biomarkers in different combinations, yielding a total of 188 papers.

The survey excluded tests made in aquatic media without linkage to soil, e.g. several studies with nematodes (mainly *Caenorhabditis elegans*) where exposure was via aqueous media (e.g. [15,16]), not mimicking soil constituents. The exceptions were 7 studies (among the more than 40 with *C. elegans*), performed in soil and/or soil pore water and thus were included in this review.

From the 188 studies reviewed (Table S1), in terms of species, 50% were performed exclusively with earthworms, 23% with enchytraeids, 9% with collembolans, 5% with isopods, 4% with nematodes, 5% involving 2 test species, and 1% involved more than 2 species of soil invertebrates (i.e., multispecies test); the category other corresponds to 3% of the papers (Fig. 1A).

Regarding the chemical identity of the tested NMs, Ag are in more than 30% of the publications, followed by Zn (almost 16%) and Cu (12%). TiO_2 NMs (6%) and carbon based NMs (8%) are in more than 6% of the publications. Less than 5% of the publications were on Fe based NM (4%), nanoformulations (3%), and nanoplastics (1%). In the

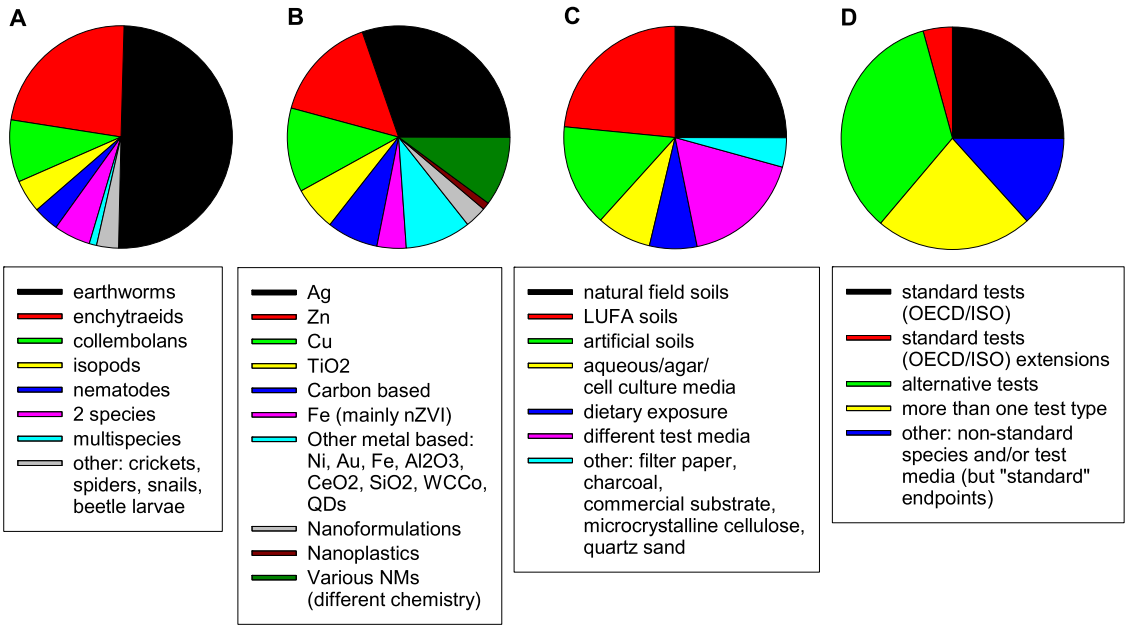


Fig. 1. Distribution of the 188 papers analysed for the revision, by A) group of organism used as test species (single species test, otherwise considered in the sections "2 species" and "multispecies"), B) chemical identity of the tested NMs (single chemical tested, otherwise is considered in the section "various NMs (different chemistry) [different forms of the same NM were considered as one per publication), C) type of test media, and D) type of test/ endpoints used.

category "other metal based" (less than 2% each) are NMs such as Ni, Au, Fe, Al_2O_3, CeO_2, SiO_2, WCCo, Quantum dots (QDs), corresponding to almost 10% of the total publications. Around 10% of the publications include two or more NMs of different chemistries ("other (various NMs, different chemistry)") (Fig. 1B).

Soil is the test medium used in more than 60% of the publications (LUFA natural standard soil in 23%, other natural soils in 25%, and artificial soils in 15% of the studies). Aqueous media (either agar, other cell culture media or reconstituted waters) were used in 8% of the publications. Dietary exposure was performed in 7% of the publications. The category different test media (18%) include studies performed in e.g. more than one type of soil, combination of in vivo (soil) and in vitro exposure, or combination of soil and filter paper tests. In the category "other" (4%) are the studies using exclusively filter paper contact test, activated charcoal, inert quartz sand, microcrystalline cellulose (Fig. 1C).

The data concerning other than standard (OECD/ISO) tests (1) were grouped in two major classes: 2) Standard tests (OECD/ISO) extensions, prolonged exposures and/or multigenerational, and 3) Alternative tests, beyond OECD/ISO, where sub-organismal endpoints like omics, biomarkers, in vitro tests, in silico and modelling are included.

Around 25% of the publications included data from standard tests (OECD/ISO) alone; 4% from standard (OECD/ISO) extensions (extra sampling times); 35% from alternative tests, here including a selection of gene/cell level endpoints, in vitro testing; 23% of the studies included more than one test type data source (e.g. standard tests and alternative tests). In the category "other" (13%) are included studies that were done with non-standard test species and/or test media, but include traditional standard endpoints (e.g. survival, reproduction, bioaccumulation, etc.) (Fig. 1D). Currently there are 31 studies from transcriptomics (alternative tests), of which 19 focus on a small number of target gene transcripts (using qPCR), and 12 are high-throughput, covering thousands of transcripts (based on microarrays or RNAseq technologies).

The high-throughput studies were done on NMs of Ag [17–20], Cu [21,22], Ni [23], Ti [24,25], Fe [26], and Zn [27,28] [the study from Starnes et al. [27] was performed in synthetic soil pore water].

The referred ecotoxicological studies are summarised in Table S1.

Standard tests (OECD/ISO)

Many of the standard tests for the terrestrial environment have been developed for invertebrates (just surpassed by the tests on soil microbial communities (e.g. [29]), and most of these model species have been used for many years, providing the basis for risk assessment and soil protection. They represent robust tools and the provided knowledge has consolidated on the traditional endpoints. The most common standard endpoints in soil ecotoxicology studies are survival and reproduction, as assessed in earthworms [30–33], enchytraeids [34,35], collembolans [36,37], mites [38,39], nematodes [40], snails [41], and beetle larvae [42] [this latter has not been used].

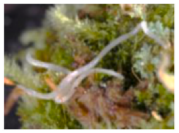
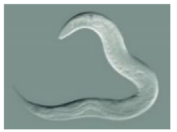
Avoidance tests are standardized for earthworms [43] and collembolans [44]. Bioaccumulation test is available for oligochaetes [45]. There are also several plant tests available, covering mono- and di-cotyledonous species [46–49] not included in this literature review, although also key to the ecosystems functioning. An overview of key invertebrate test soil living species is provided in Table 1.

These tests are very important within the current regulatory framework yet they are insufficient in number (coverage) and quality (understanding). A simple example can be illustrated by the common endpoints survival and reproduction assessed via standard tests, where we obtain key information on the impact after a certain time period, but nothing about in-between. This is a typical black box concept (Fig. 2), we don't know when, how or why it happens.

Standard tests (OECD/ISO) extensions

Based on the standard OECD/ISO guidelines, i.e. all procedures follow the standard, there has been considerable development for: a) additional endpoints, e.g. full life cycle (FLC) tests [51–55], where besides survival and reproduction, the hatching success, growth and maturity status can be assessed providing longer exposure period and sampling points; b) additional time points, where an endpoint is followed over time (e.g. avoidance tests monitored at 24, 48, 72, 96 h, instead of 48 h alone [56]); and c) extended exposure period,

Table 1
Overview of all standardized key soil living invertebrate species and characteristics, including adult length size, biomass, reproductive output, function in the system, assumed species interaction type and predicted living layer in the soil. Please note the pictures are a case-by-case scale size for improved visualization. * in a 28 days LUFA 2.2 soil test; $ per posture; # in a reproductive cycle; † in a 28 days artificial soil test; +depends on the presence of microbe-rich decomposing plant material.
Source:Adapted from [50].

Class/Sub-class	Oligochaeta	Oligochaeta	Gastropoda (Filo Mollusca)	Chromadoria (Filo Nematoda)	Arachnida (Acari)	Arachnida (Acari)	Collembola	Insecta
Species	*Eisenia fetida*	*Enchytraeus crypticus*	*Cornu aspersum* (prev. *Helix aspersa*)	*Caenorhabditis elegans*	*Hypoaspis aculeifer*	*Oppia nitens*	*Folsomia candida*	*Oxythyrea funesta* (larvae)
								
Standard guideline	OECD 207/ ISO 11268–1; OECD 222 /ISO 11268–2; ISO 17512–1; OECD 317	OECD 220/ISO 16387; OECD 317	ISO 15952	ISO 10872	OECD 226	ISO 23266	OECD 232/ ISO 11267; ISO 17512–2	ISO 20963
Endpoints	Survival Growth Reprod. Avoidance behaviour Bioaccum.	Survival Reprod. Bioaccum.	Growth Survival	Growth Fertility Reprod.	Survival Reprod.	Survival Reprod.	Survival Reprod. Avoidance behaviour	Survival (of larvae)
Adult lenght (mm)	100	7	25–40 (shell)	1	1	0.51	2.5	8–12
Biomass (mg)	300–600	0.25	6000–15000	0.001	0.07 (♀)		0.16	
Reproduction (juv/ad)	10 *	60 *	80$	300–1000#	10 *	2†	120 *	
Living layer	Top	Middle	Surface	+	Upper-Middle	Upper	Upper-Middle	Surface
Function	Decomposer	Decomposer; Fungivore; Grazer; Prey for mites	Saprophagous; Phytophagous	Decomposer; Fungivore; Grazer; Prey for mites	Predator	Polyphagous fungivores	Decomposer; Fungivore; Grazer; Prey for mites	Phytophagous

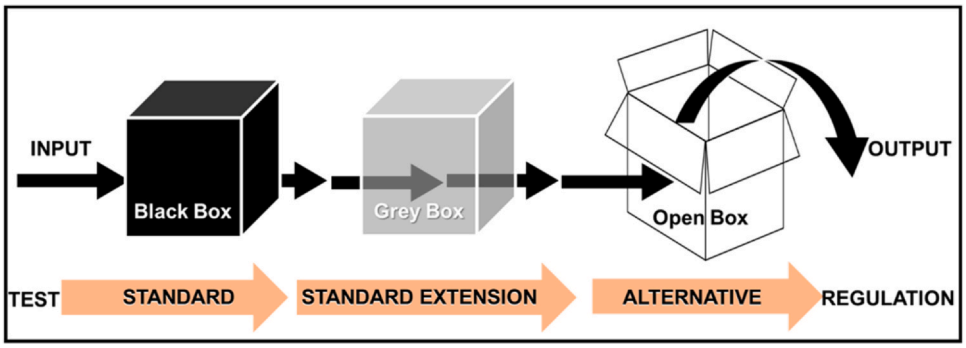


Fig. 2. Schematic representation of the concept analogy for testing and boxes: standard test – black box, standard extension test – grey (see through) box and alternative test – open box.

for which the time frame exceeds by far the standard test, assessing the endpoints over prolonged exposures (e.g. to survival and reproduction/population in 56 or 84 days instead of the 21–28 of the standard reproduction test [57,58]), in several generations (e.g. multigenerational tests [59]), or in a full life span [60]. For each species-/test-system there has to be a careful consideration as to what is covered both with a life cycle and beyond.

Such developments represent examples for obvious integration onto the standard OECD/ISO tests, e.g. as annex to the standard recommended [3]. Still "grey box" test type concept, grey being a see-through type (Fig. 2), but the refinement allows to understand substantially more than with the standard alone, with (often) minor additional effort. For instance, in a FLC we can elucidate whether the reduction in reproduction was due to reduced hatching success, adult mortality or an actual reduction of the reproductive output.

Alternative tests

Many developments are offered in other key fields, e.g. omics, where there is no similar standardization history and hence these require background establishment before they can be lifted to a routine use for future acceptance in a standardized mode. There are many advances provided by -omics techniques, mostly these provide data to unravel the mechanisms of response of the organisms to stressors often referred to as Adverse Outcome Pathways (AOPs) (Fig. 3).

This is an "open box" approach because it provides the understanding of what, how and why an observed response happens.

The potential of NAMs, in particular omics techniques, has been acknowledged long ago, by its specificity and fast response (e.g. [63]), and the mechanistic information provided [64]. More recently the integration of NAMs into regulatory risk assessment has emphasized the discussion on the aspects of minimum required descriptors, data analysis and outputs, i.e. for standardization purposes [5,9,65]. The importance and added-value of alternative methods is particularly recognized for NMs, that are entering the market at a speed never seen before for any class of chemicals, which would benefit from the predictive and read-across potential of NAMs, also for a safer-by-design production of materials.

Even though omics are not yet applied to meet standard information requirements during regulatory hazard and risk assessment [66], their integration within a system toxicology approach has potential to improve several layers of the process, as detailed in Van Ravenzwaay [67]. Finally, future alternative approaches may also include in silico techniques, where modelled data are used to predict risk, both by single materials or based on read across many materials (e.g., [68–70]). These in silico data may either be used to support novel testing, or indeed in the future be used for regulatory decisions, e.g., for grouping of hazards of NMs.

Advantages of integrating: lessons learned from nanotoxicology

Despite the large literature data volume on the (eco)toxicology of NMs, it is widely diverse in terms of NMs, test methods (standard, alternative), exposure media, duration, etc. This creates difficulty when trying to interpret and integrate or extrapolate results. Hence, in the following sections, we will present the main findings and advantages of the integration of standard with standard extension and alternative test results, this done per each NM (based on

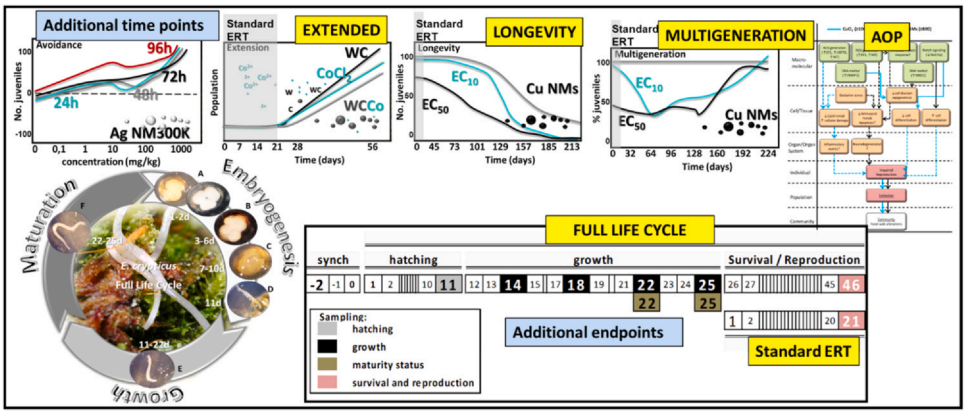


Fig. 3. Developments based on standard tests. Schematic for comparison of outputs (additional time points [61] and endpoints) between Standard ERT (Enchytraeid Reproduction Test- 21 days), Full Life Cycle (46 days) [52], extension (56 days) [57], longevity (213 days) [60], multigeneration (224 days) [59]. AOP: Adverse Outcome Pathways - linking effects at different levels, integrating knowledge from various levels to provide mechanistic explanations for toxicity (the AOP presented can be found in [62]).

chemical identity), covering NMs of Ag, ZnO, Cu/CuO, TiO_2, carbon based, and other NMs with fewer studies available (e.g., Fe, Ni, Au, metal oxides, nanformulations and nanoplastics).

Case studies

Silver (Ag) nanoparticles/nanomaterials

Ag NMs are the most studied ENMs in soil living invertebrates, this based on standard OECD/ISO tests.

In terms of survival, overall, the standard test in soils showed very low to no effect of Ag NMs, e.g. earthworms survive up to 1000 mg Ag/kg soil (e.g. [71–74]), enchytraeids, slightly more sensitive, showed an LC50 ranging from 300 to 700 mg Ag/kg soil [53,75,76]). Reproduction is a more sensitive endpoint for earthworms, enchytraeids, and collembolans. The reference Ag NM300K (JRC material, [77]) was overall more toxic when compared to other (non-dispersed) Ag NMs (e.g. on *F. candida* [78,79]). When the exposure time was prolonged, in earthworms, from the standard 4 weeks to 10 months, significant reproductive effects were observed at 10 times lower concentrations [80]. Similarly, in enchytraeids, the performance of the longer FLC test (which starts from cocoons (2 days after laying) and lasts 46 days, with many sampling time points to assess various endpoints ((11 days: hatching, 22–25 days: maturity status, 46 days: survival and reproduction) in addition to growth and survival throughout the test) showed higher toxicity compared to the standard ERT (21 days), particularly in terms of survival, and showed that hatching success (day 11) was the most sensitive endpoint [53]. This is particularly interesting because, as the exposure starts in the early developmental stages (cocoons), the endpoint hatching success specifically targets embryo developmental effects and/or death of the just hatched juveniles, which cannot be directly derived from the ERT (since the exposure starts with adult organisms, and the reproductive ECx can result from cumulative effects on the parental generation). Both examples show that the current standard guidelines failed to predict the long-term effects of Ag NMs. Further, with little extra effort in comparison to the standard ERT, the FCL test showed that embryos/juveniles were more sensitive towards Ag NM exposure than adults.

Important output: Standard OECD/ISO tests extension, e.g., extension of a survival/reproduction test - via Full Life Cycle, extended exposure period, additional sampling points - provide key interpretation data and deliver effects not foreseen via the standard alone - currently such improved level exists but is not mandatory/done. Avoidance behaviour has been among the most sensitive endpoints, for e.g. earthworms (effects were observed at concentrations 7–100 times lower than for reproduction reduction [71,72,81]), and for other species' groups (e.g. enchytraeids [61] and isopods [82,83]).

Standard avoidance tests have a 48 h exposure duration. Extensions to the standard, increasing the number of time points from one (48 h) to four (24, 48, 72, 96 h) allows further understanding of the avoidance process. This has been shown to vary, e.g., it was observed that for *E. fetida* the response varied little within 96 h [56], while for *E. crypticus* the avoidance rate was reduced for longer exposure periods, i.e. from 24 to 96 h [61]. This reduced capacity to avoid Ag, when extending exposure, can be partly explained by the measured neurotoxic effects (via gamma-aminobutyric acid (GABA) up-regulation) which causes animals paralysis. Ag NMs are known to slowly dissolve over time in aqueous media [84], and even slower in solid media [56] (often without reaching an equilibrium state [85]). Hence, avoidance behaviour as assessed in the 24–48 h likely reflects the detection of NMs and not only released Ag ions. The ability of an animal to avoid represents a highly relevant ecological trait, as it can strongly impact its survival. Based on the observed variation [61], and bearing in mind that avoidance should be detected in a short time interval, a period between 24 and 96 h is recommended, but obviously also depending on the material properties.

Important output: Avoidance behaviour to NMs is a sensitive endpoint and can vary with time - a 24–96 h period is recommended instead of 48 h standard alone.

Studies where different sizes and shapes, etc. are compared are scarce, e.g. when comparing 10, 30 and 50 nm, indications were that size had limited influence on Ag NMs toxicity via soil exposure [71,72]. On the other hand, shape [86], coating and surface charge [87] seem to play a more important role: 1) for example plates-Ag NMs were more toxic to *C. elegans* than spheres [86], 2) particles with BSA coating [bovine serum albumin (BSA, negatively charged] were more toxic than particles coated with chitosan [Chitosan, positive charge], and polyvinylpyrrolidone [PVP, neutral charge] for *Lumbricus rubellus* (another species of earthworm) [87]. Similar evidence on the importance of coating was drawn from a study using a simplified exposure media (aqueous solutions added to inert quartz sand) [88], where PVP-Ag NMs were more toxic than citrate (Cit)-Ag NMs. The pH was also shown relevant for Ag NMs toxicity either via soil exposure [81] or soil pore:water extracts [89].

The use of simplified (aqueous) media as soil surrogate, that aims to reduce complexity soil factors and the associated characterization problems, has been a good stepwise approach to integrate effects of individual factors, hence improving the understanding of the whole, being also very important to support the development of robust models that can be used to describe the fate and toxicity of NMs in natural systems.

Important output: the use of simplified media as surrogates of complex media - e.g. sand, soil:water extracts, reconstituted water, as soil surrogate - provide important stepwise data to deliver effects of individual factors.

Aging and the associated biotransformation processes significantly affect Ag NMs toxicity, with evidences for increased toxicity with increasing aging time [90]. In the presence of biosolids (sewage sludge from wastewater treatment plants) the same pattern of increased toxicity with aging time was reported for *F. candida* [91]. Further, higher toxicity of Ag NMs, in comparison to $AgNO_3$, was reported to *E. andrei* (but not to *F. candida*) when both Ag forms were added to soil via spiked sludge [74], even though a reduction in toxicity was expected with the sulfidation of the Ag NMs in the presence of biosolids. The authors [74] suggested that the sulfidation of the Ag NMs was not total, and that some of the Ag NMs remained as dispersed particles, thus resulting in higher pools of Ag^+ available (compared to $AgNO_3$). In more realistic scenarios (prolonged aging of the NMs with the biosolids), the degree of sulfidation would be higher.

Bioaccumulation results showed low bioaccumulation factor for pristine Ag NMs (ranging from 0.01 to 0.89 [71,72,92,93]), but with low elimination rates (up to 50% within 21–28 days of depuration [93,94]), indicating potential for biomagnification. In the presence of less soluble Ag NMs (sulfidized Ag NMs), bioaccumulation was reduced, and elimination was almost total [93,94] indicating that the Ag uptake was mostly based on its soluble fraction. An alternative, less conventional study, conducted with L. *rubellus* with sealed and unsealed mouthparts, showed that up to 75% of Ag uptake, both Ag NMs and ions, occurred through oral/dietary route [95]. This, questions the application of current metal bioavailability models, where soil solution is the dominant route of exposure, for bioavailability assessment and modelling of metal-based NMs.

Overall, if merely based on standard tests, Ag NMs toxicity was lower compared to Ag ions. When testing beyond, e.g. via standard extension, prolonged exposures showed increased toxicity of Ag NMs [80], or that toxicity of Ag NMs and Ag ions merge to a common value [90]. This highlights the importance of standard tests extension, as better predictors for toxicity in real-case scenarios (longer-term exposures).

Much evidence supports that toxicity of Ag NMs is mostly explained by the soluble fraction of Ag^+. In soils, the aggregation of Ag NMs is likely higher during the early phases of exposure, with the dissolution of the aggregated NMs taking place later in time [96], and working as a continuous source of Ag ions over time [90]. So, although there is a dissolution effect, the fact is that Ag NMs provide a long-term source and delivery of Ag ions and toxicity can increase with exposure time and be equivalent to persistent substances.

Important output: standard extension tests, i.e. prolonged exposure, showed that toxicity of Ag NMs was similar to that of soluble salts like $AgNO_3$.

Non-monotonic dose-response (e.g., higher toxicity at intermediate concentration than at higher) was reported for Ag NMs (and also for other NMs, such as Ni [54]), in *E. crypticus* when based on a FLC test [53]. This is probably related with the agglomeration/aggregation behaviour of the NMs in soils, that will be smaller for lower concentrations, with consequent presence of higher amount of single particulates, that in turn can undergo faster dissolution [12]. It may however, also be related to concentration related uptake pattern, since endocytosis is rate and size limited. The non-monotonic dose-response pattern is not always captured, simply because it is not tested or it depends on other aspects, like the measured endpoint exposure media or time lapse [54]. The testing done in *E. crypticus* with Ag NM300K seems to indicate that this intermediate higher toxicity occurs at a rather narrow concentration interval, e.g., Rodrigues et al. (2020) studied the closest neighbour concentrations to 20 mg/kg, the 10, 20, 30 and 40 mg/L range in an aqueous exposure, showing 40 > 20 > 30 > 10 mg/L toxicity for time to hatch.

Important output: NMs can induce non-monotonic dose-response effects, with highest toxicity tending to occur at a low dose where dispersion is maximal in a certain media.

Alternative tests data, e.g. high-throughput (HTP) omics, clearly further elucidate the mechanisms for Ag materials. The pathways by which Ag (ions) exert toxicity are relatively well studied, and include disruption of energy metabolism, cell redox homeostasis, ribosome function and DNA processes. Those processes were identified, based on RNA-sequencing (RNAseq) and microarray data, in response to exposure to $AgNO_3$ and Ag NMs in earthworms and enchytraeids [17–20], which in this case seems to corroborate the role of Ag ions from Ag NMs toxicity. Additionally, the above mentioned HTP omics techniques showed different patterns of internalization between Ag forms, with involvement of endocytosis [17] and transcytosis [18] for the nanoforms. In *E. crypticus* cocoons, it was shown that the uptake of Ag (both Ag NM300K and $AgNO_3$) by the embryos was not by transcytosis via the transferrin receptor (TfR); Ag NM300K specifically affected calcium metabolism via complete inhibition of L type Ca channels (LTCC), which could explain the disruption of embryonic development [97]. These results support the Trojan-horse like mechanism [in which the Ag NMs serves as a delivery system of Ag ions inside the cells] being particularly relevant because they were obtained from in vivo exposure of multicellular organisms (earthworms and enchytraeids) in an environmentally relevant exposure media (soil, as opposed to in vitro). Differentiation between different Ag NMs (with varying characteristics such as size or coating) was also evident based on HTP omics [19,20], such a differentiation was not possible based on standard test endpoints (providing similar EC values among materials). Overall, the HTP genomic studies identified mechanism of response towards Ag NMs, while also provide information on key events in the development of Adverse Outcome Pathways (AOPs) (e.g. [66,98]). HTP omics are *premium* techniques to generate novel hypothesis, besides identifying the molecular initiating events (as initiated for Ag NMs [19]), and supporting evidences of key events at different levels of biological organization and across species. For instance, omics from short-term Ag exposure showed evidences of activation of processes related to epigenetics [19], indicating that effects could be transgenerational, i.e. be transferred to the next generation. Transgenerational effects, by parental transfer, were reported in *C. elegans* exposed to Ag NMs via simulated soil pore water (SSPW) [99], and confirmed in a follow up study (global levels of histone 3 lysine 4 dimethylation (H3K4me2) and histone 3 lysine 9 trimethylation (H3K9me3)) as probably related to the transgenerational toxicity induced by Ag NMs [100].

Important output: HTP omics alternative tests provide a source for broad understanding of the mechanisms underlying adverse outcomes, thus being an important tool to the development of AOPs. Further, it provides discrimination between materials, not possible via standard tests alone.

Low-throughput (LTP) studies, e.g. gene qPCR or Reactive Oxygen Species (ROS) markers, although providing a lower screen, carry added knowledge, e.g. as shown by the measurement of metallothionein (MT) and catalase (CAT) genes (by qPCR) in earthworms' immune cells – coelomocytes: 5 nm Ag NMs induced MT and CAT up-regulation in coelomocytes collected from in vivo exposed worms [101] which was not observed in worms (whole organism) exposed to similar Ag NMs [102,103].

In vitro experiments with earthworms' coelomocytes showed that Ag NMs impair the immune system (e.g. [80,104,105]). Further, a study by Hayashi et al. [106] showed that coelomocytes and THP-1 cells (phagocytic human acute monocytic leukemia cell line) exhibited similar molecular response to Ag NMs (early regulation of oxidative stress genes and subsequent alteration of immune signalling processes), adding to evidences that the immune response is an evolutionarily conserved mechanism across the animal kingdom [106].

At sub-cellular level, the investigation of oxidative stress biomarkers (e.g., determination of lipid peroxidation (LPO), catalase (CAT), superoxide dismutase (SOD), etc.) is often available although usually found from different experimental designs and materials. Oxidative stress is a recognized response to Ag NMs exposure, and has been reported in several studies (e.g. [78,107–110]) and often associated with DNA damage (e.g. [103,111]).

Important output: LTP alternative tests have an important role in providing mechanistic confirmation, being quantitative, and easier than HTP to implement in laboratory.

The vast majority of studies focus on direct effects on the model organisms (e.g., earthworms/enchytraeids/collembolans and effects of NMs on their reproduction, gene expression, etc.). There has been an increasing number of toxicological studies focussing on the importance of the gut microbiome, a symbiotic community. Ag NMs have been shown to disturb the gut bacterial community of earthworms' (with effects on nitrate cycling [112]), and collembolans' [113]. The abundance and diversity of Antibiotic Resistance Genes, which can be a serious threat to global health, decreased in collembolans' gut when exposed to Ag, but the further implications of this to the organisms' health (e.g., via nutrient absorption and the immune system) are unknown and might occur later. Effects on gut microbiome community were found at concentrations that did not affect survival, reproduction or growth, providing indications that this could be a sensitive indicator to Ag NMs exposure [113].

Although Ag are among the most studied NMs, their numerous applications lead to the production of Ag NMs with all sort of variations in size, shape and coating, that are certainly not covered in terms of effect assessment (Fig. 4 depicts the main physicochemical properties of NMs known to condition toxicity, with indication of those that are currently investigated in the soil ecotoxicological studies reviewed). Further, the test designs used are also extremely variable, which limits the possibility of read-across. There is no easy solution but this could be supported through larger harmonization,

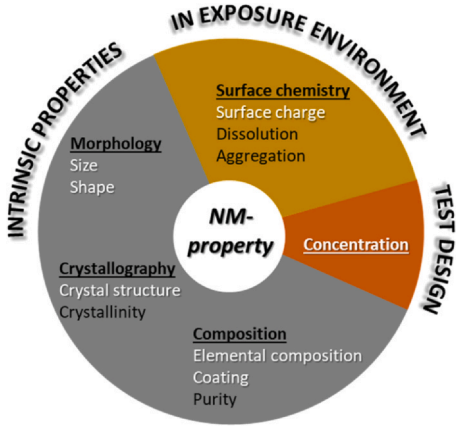


Fig. 4. Schematic illustration of the main physicochemical properties of nanomaterials that condition its (eco)toxicity, with indication if the properties are intrinsic to the NMs (as synthesized), governed by the exposure environment, or determined by the test design. The white text indicates the properties that have been investigated in the soil ecotoxicological studies reviewed.

e.g. for descriptors, endpoints, test duration, etc., as often recommended [2,3,4,114–116] and harmonization of alternative methods [5,9,65].

One of the alternatives lies on the use of NMs libraries (e.g. [117–120]). A NM library consists of a certain NM, e.g. TiO_2, that are synthesized to vary one property (e.g. size, shape, composition, etc.) at a time, while keeping all other constant. Their use make possible to study the impact of specific material descriptors individually, as much as possible. See further details in way forward section.

Important output: The variety in properties of tested NMs is too wide to make adequate comparisons between studies of the same element NM; one partial alternative is the use of NMs libraries, where one descriptor varies, e.g., size, while keeping others constant.

Zinc oxide (ZnO) nanoparticles

Similar to reports on Ag, in terms of survival, overall the standard OECD/ISO tests in soils showed very low to no effect of ZnO NMs, e.g. in earthworms (up to more than 1600 mg Zn/kg [28,121–125]), enchytraeids (up to more than 1000 mg Zn/kg [126]), or collembolans (up to more than 6400 mg Zn/kg [127,128]) via soil exposure. Reproduction was a more sensitive endpoint (EC_{50} ranging from around 600 mg Zn/kg in enchytraeids [126] to 900 mg Zn/kg in earthworms [121]). The lower sensitivity of survival, compared to reproduction is well known in almost all areas of ecotoxicology.

Important output: Survival is an endpoint with very low sensitivity. Chronic effects, like reproduction, and preferably with longer exposure, are recommended.

The importance of size for the toxicity is not clear or comparable between studies, with reports of no influence on ZnO toxicity between nano and bulk or also when comparing nanosizes of 20 versus 200 nm ZnO NMs [127], but other showed that 140 nm ZnO NMs were more toxic to *F. candida* than 15 nm NMs (EC_{50} of around 400 and 1500 mg Zn/kg for 140 and 15 nm ZnO NMs, respectively [124]).

On the other hand, additional factors like soil pH have been shown to play an important role in ZnO toxicity [121,128–130], showing an increase in ZnO toxicity with decreasing pH, being associated to increased solubility of ZnO [121]. Another important factor was found related to organic material, when adding ZnO NMs to soil via sludge, the toxicity increased in *E. fetida* (reproduction), probably due to the effects of the natural organic carbon on the stability of the ZnO NMs in the soil pore water [123]. The same pattern was reported in agar media, with increased toxicity in the presence of humic acids, which increased the dissolved fraction of ZnO [131]. On the other hand, the artificial sulfidization and phosphatization of ZnO NMs, which limits the release of Zn ions from the particles, reduced its toxicity to *C. elegans* [27]. These differences must be also linked to the time required for the organic materials to interact with particles and prevent its dissolution (i.e., in the artificially sulfidized/phosphatized ZnO NMs, the NMs were added to the test media previously being chemically stabilized [using Na_2HPO_4 for the phosphatization, and Na_2S for the sulfidization]).

Notably aging has an impact, but not always independent of other variables across studies, e.g. reproductive toxicity increased in *E. fetida* with aging of ZnO NMs up to 6 months' time in LUFA 2.2 soil, but not in other natural soils [122]. For *F. candida* there was a decrease in toxicity with up to 12 months of aging [129]. Soil properties, such as pH, and the related fraction of available Zn (ZnO NMs dissolution peaks from 3 to 6 months but continues, although slower, up to 12 months [129]) likely explain the differences in toxicity. This may in part be due to interaction with soil constituents, as seen in other areas of ecotoxicology, but also due to changes in the NMs.

Important output: Aging of NMs plays an important role on toxicity, e.g., for ZnO NMs, the impact of aging on toxicity is associated with other variables, e.g., organic matter content.

Toxicokinetics studies showed ZnO NMs and $ZnCl_2$ were assimilated at different rates (with overall smaller accumulation and elimination rates for the NMs) [132,133] and Zn accumulation depended on Zn form, with the nanoparticulate form dominating accumulation (75%) and toxicity (100%) patterns (based on Zn ions release measurements) [133]. Further ZnO NMs were identified in worms tissues by scanning electron microscopy [134].

The effect of NMs concentration and its optimal dispersibility and bioavailability point, seems to also impact the bioaccumulation patterns, e.g. at low concentrations of 5 mg Zn/kg, ZnO NMs undergo fast dissolution, with high uptake rate of Zn by L. *rubellus* earthworms [135]. By sealing the earthworms' mouthparts, the uptake of Zn decreased in 95%, showing that oral/dietary is the main uptake route [135]. In isopods, there were no distinctive patterns of uptake or toxicity between ZnO NMs, non-nano ZnO, and $ZnCl_2$, either via soil [136] or dietary exposure [137].

Important output: NMs can have non-monotonic dissolution rates, with highest dissolution tending to occur at lower concentrations in media, hence there is a direct implication for toxicokinetics.

Alternative tests data, like HTP omics, also showed that the toxicity of ZnO NMs was related to both its dissolution (Zn ions) and the NMs itself e.g. via HTP genomics (either via soil [28], or via SSPW exposure [27]), and via metabolomics (via simulated soil solution-quartz sand system [138]) studies. The mechanisms activated, which were common to both Zn NMs and ions, were related to oxidative stress and antioxidant defence, membrane disturbance and energy metabolism [138]. Responses unique to nano were identified [27,28,138], e.g. while the expression of genes coding for Zn transporters was significantly affected by both Zn salt ($Zn(NO_3)_2$) and ZnO NMs, ZnO NMs specifically affected a Zn transporter present in the lysosome and endocytic related processes, confirming differentiated mechanisms [28]. Based on these results, it was suggested that while the toxicodynamic [Zn ions are the trigger of toxicity] for both Zn forms may occur through a similar process, the pathways [in this case, concerning the cellular uptake and distribution] may differ, with ions being taken up into cells by (membrane) ion transporters and ZnO NMs by endocytosis [28], highlighting the fundamental role of molecular/mechanistic analysis to explain the observed toxicity.

Alternative test data based on sub-cellular endpoints (e.g. enzyme quantification, etc.) confirm oxidative stress impairment as

one of the causes of ZnO toxicity, both in vivo [139–141] and in vitro [139]. Yet, as described for Ag NMs, the information obtained is often from varying test designs and variable.

In vitro testing showed coelomocytes are a susceptible target to ZnO NMs [139,142], as also shown for Ag NMs, in the latter observed for both in vitro and in vivo.

Copper (Cu) and copper oxide (CuO) nanoparticles/nanomaterials

Standard OECD/ISO tests (chronic) results showed very low to no effect of Cu NMs in terms of survival. Among the most common standard soil invertebrates (earthworms, enchytraeids and collembolans) there were larger differences between species sensitivity e.g. while for collembolans, CuO NMs did not cause effects (on survival and reproduction) up to 6400 mg [143], for earthworms, 1000 mg/kg of Cu NMs reduced reproduction in about 90% [73]), and for enchytraeids, reproduction EC_{50} ranged from 95 mg/kg in *E. albidus* [144] to around 1500 mg/kg in *E. crypticus* (for different Cu/CuO NMs varying in size and oxidation state [52,145]).

Additional data from standard OECD/ISO test extension, based on a FLCt in *E. crypticus*, further clarified the impact showing that CuO NMs affects juveniles, causing an overall delay in development (growth and maturation) [52]. This increased level of detail compared to the standard alone, allowed not only to identify a specifically affected developmental stage (CuO NMs affected juvenile stage) but also to differentiate between CuO NMs and the Cu salt form ($CuCl_2$, which affects embryo development, further confirmed by histological analysis [62], an alternative test).

That chronic and longer-term exposure to NMs poses higher risk to soil invertebrates is clear, but much less so is the information from higher tier, like mesocosms or multispecies tests type, where higher complexity/ecological relevance is added by including several species and its interaction, besides the prolonged exposure. The standard OECD/ISO test extension soil multispecies test system (SMS) (6 Species: *E. crypticus*, *F. candida*, *Proisotoma minuta*, *Hypogastrura assimilis*, *Mesophorura macrochaeta*, *Hypoaspis aculeifer*, and 3 sampling times: 28, 56 and 84 days), confirmed clearly higher toxicity at prolonged exposure, and also that species interactions greatly affect toxicity (EC_{50} (SMS) < EC_{50} standard individual species) [50].

One of the longest test performed in a single organism, which covered its lifespan, was performed on *E. crypticus* [60] and showed that longevity was more affected by CuO NMs compared to $CuCl_2$ exposure (when exposed at a similar reproduction effect concentration, EC_{50}), i.e. *E. crypticus* have a shorter life when exposed to CuO NMs than when exposed to $CuCl_2$, not predicted based on the standard test. Further, the performance of multigenerational (MG) tests with CuO NMs in soil media, showed e.g. that at low effect concentrations (reproduction EC_{10}), CuO NMs toxicity to *E. crypticus* increased over generations (tested up to F5) [59], although reset when transferred to clean media, whereas MG exposure to $CuCl_2$ decreased toxicity for EC10 and EC50, but the transfer to clean media "revived" the initial effects. On the other hand, for *F. candida*, another soil invertebrate, no increased toxicity was observed due to MG exposure to CuO NMs [146]. Currently there is an increasing amount of evidences that NMs can induce epigenetic changes [147], which play a key role in regulating gene expression and phenotypic impact. Follow up studies in *E. crypticus,* using a MG exposure to CuO NMs showed epigenetic effects in terms of both global DNA methylation and regulation of individual gene transcripts related to epigenetic mechanisms [148], being also confirmed via immunostaining of epigenetics related antibodies [62], these changes persisted after transfer to clean soil which is indicative of transgenerational effects. This highlights the importance of combining tools to assess longer-term effects of NMs, where the phenotypic and underlying molecular mechanism can be understood.

Important output: Longer term tests like multigenerational (MG) exposure to NMs reveal that effects can be transferred between generations and with epigenetics mechanisms activated.

Investigation on the mechanisms of toxicity of Cu/CuO NMs was done using alternative tests in enchytraeids, based on HTP transcriptomics [21,22], proteomics [149], and metabolomics [150]. All omics supported evidences for different mechanisms of response between NMs and the salt forms, despite commonalities as well [aligned with findings for other metal NMs that undergo oxidation, such as Ag and ZnO, with concomitant release of ions]. For Cu, X-ray diffraction analysis studies [145,151] showed the partial oxidation of Cu NMs in soils, with Cu ions release, while core Cu (0) particles still remain [145].

Important output: Evidences are that metallic (M) NMs (e.g., Ag, Zn, Ni, Cu) oxidise and release M^+, although to uneven extent, e.g. Cu NMs to a less extent than Ag and Zn.

Results from energy metabolism showed clear differences between Cu/CuO NMs and Cu-salt [21,149,150], even though Cu (independently of its form) induces an increase in energy consumption [152]. Specific effects of CuO NMs on neurotransmission were also suggested [149,150]. Transcriptomic information, anchored to organism level standard endpoints was used to provide a first AOP for Cu materials, including NMs [22], already complemented for CuO NM with additional endpoints/key events (e.g. at intermediate levels such as cell/tissue) [62]. Although in its infancy, the development of AOPs can greatly improve the risk assessment of NMs, providing mechanistic understanding, the basis for read across materials and species.

Among the characteristics expected to have influence on NMs physic-chemical/biological interactions, the effects of coating/surface charge on CuO NMs have been investigated. In terms of acute toxicity (14 days) to *E. fetida*, results were reported highly dependent of the coating agent [higher effect for the neutral carboxylate –COOH coating, followed by the positively charged ammonium ionized -NH_4^+, the uncoated CuO NMs and the negatively charged polyethylene glycol – PEG coated CuO NMs were not toxic; after 1 year of aging, the patterns of toxicity were altered, and only -NH_4^+ and uncoated CuO NMs caused toxicity to *E. fetida* [153]], even though the differences between coatings could not be explained by the Cu bioavailable fraction. A study using earthworms' coelomocytes (in vitro) also showed differences in cytotoxicity for different CuO-coated NMs [from the most to the least toxic: citrate -CIT, polyethylenimine -PEI, ascorbate -ASC, polyvinylpyrrolidone -PVP, and uncoated CuO] [154], reporting a high correlation between toxicity and NMs size (hydrodynamic diameter), and a less strong correlation with NMs dissolution (higher toxicity for the NMs that solubilize more). The effects of the same CIT, PEI, ASC, PVP and non-coated CuO NMs were investigated in a soil multispecies test system (SMS) [155]. In soil, based on the SMS, CIT- and ASC-CuO NMs were the most toxic, and there was a strong correlation between the community effects and the NMs' zeta potential (higher toxicity for the negatively charged NMs), and again, species interactions played a key role and impacted the toxicity of the CuO NMs (SMS EC_{50} < standard single species EC_{50}) [155]. These studies show that surface coating significantly affect CuO NMs hazards. Further, adding complexity to the system, i.e. species interaction among many species, revealed higher risks than predicted based on each of the individual species standard tests.

Important output: Higher tier tests mesocosms, where both long-term exposure and multispecies are combined, reveal that interactions between species play a key role in the system, being a highly sensitive endpoint system and thus are recommended.

Alternative test data can be even less conventional although of interest, as long as useful in eliminating hypothesis. For instance, as reported above, the experiments conducted with earthworms with sealed and unsealed mouthparts, showed that Ag and ZnO NMs were

mainly taken up via dietary uptake (up to 95% of total uptake) [95,135], and there is no major difference anticipated for Cu. Once in worms' (and in collembolans') gut, NMs will be in contact with the gut microbiome, which was shown to be affected by Cu/CuO NMs [156–158]. Although the alterations of gut microbiome induced by Cu NMs did not lead to altered susceptibility to a bacterial infection [135], other negative effects (e.g. prolonged ones) cannot be ruled out.

A proof of concept – Cu salt vs Cu NMs case study in E. crypticus

Toxicity of Cu materials has been studied in detail in *E. crypticus*, including the 1) standard (OECD/ISO) test, including whole organism endpoints like survival and reproduction [52,145], 2) standard (OECD/ISO) test extension: full life cycle test with hatching, growth, maturity status, besides survival and reproduction [52], multigeneration with survival and reproduction [59], and full life span with longevity [60] and 3) Alternative tests, where sub-organism endpoints like transcriptomics [22], proteomics [149], metabolomics [150], epigenetics [62,148], cellular energy allocation (CEA) [152] and immunohistochemistry [62]. Finally, studies have also been performed with multiple species [50,155].

Whereas the standard test showed that $CuCl_2$ was more toxic than CuO NMs (reproduction EC50 = 179 mg $CuCl_2$/kg and EC50 = 1377 mg CuO NM/kg), results of exposure of the cocoons via a full life cycle test [52] showed similar impact to reproduction (EC50 = 218 mg $CuCl_2$/kg and EC50 = 1075 mg CuO NM/kg), although further clarifying that while $CuCl_2$ affected embryo hatching with high correlation with reproduction (EC50 (hatching) = 210 mg $CuCl_2$/kg), CuO NM caused a delay and decrease in the juvenile development, i.e. growth and maturation, without significant impact on hatching.

Important output: based on the standard reproduction test alone, $CuCl_2$ was more toxic than CuO NMs, based on the FLC the toxicity was similar. Further, FLC revealed that $CuCl_2$ affected embryo hatching with high correlation with reproduction and CuO NM caused a delay and decrease in the juvenile development, i.e. growth and maturation, without impact on hatching.

When *E. crypticus* were exposed via multigenerational (MG) test [59], results showed that:

CuO NMs MG caused increased toxicity for EC10 exposed organisms (EC50 did not change), and transfer to clean media reset effects, whereas $CuCl_2$ MG reduced toxicity for EC10 and EC50, but the transfer to clean media "revived" the initial effects, i.e. close to EC50 levels in F7. A further inspection of results (Alternative tests) in terms of epigenetic markers [148] showed MG exposure to Cu increased global DNA methylation which corresponded with phenotypic effects (reproduction). Gene expression analyses (qPCR) showed changes in the epigenetic, stress and detoxification gene targets, depending on the generation and Cu form, also occurring in post-exposure generations, hence indicative of transgenerational effects.

Important output: MG exposure to $CuCl_2$ caused increased tolerance that could be explained by the activation of general stress response mechanisms, i.e. metallothioneins (MT), heat shock protein (HSP) and elongation factor (EF); the transgenerational increased toxicity seems to be the result of a higher Cu homeostasis level, and hence a deficiency when transferred to Cu absence.

Important output: MG exposure to CuO NMs, caused similar (maintained at the EC50) or increased (EC10 > EC50) toxicity, but eliminated (or recovered) when transferred to clean media, while organisms still have activated compensatory mechanisms of protein synthesis and detoxification, and impaired developmental mechanisms.

Hence, the apparent good fitness of the population hides active stress mechanisms at gene level. The immunohistochemistry study [62] confirmed at cell/tissue level that MG exposure to Cu materials affected the measured epigenetic markers at gene level [148]: non-coding RNA (Ago1); histone modification (H3- dimethyl) and DNA methylation (5 mC). CuO NMs affected the Notch signalling pathway, whereas $CuCl_2$ caused both oxidative stress and affected the Notch signalling pathway.

Omics studies, e.g. proteomics [149], showed differences between $CuCl_2$ and CuO NM, both in terms of numbers of differentially expressed proteins (DEPs) and diversity of biological processes (GO: Gene Ontology). $CuCl_2$ was more intrinsically regulated (controlled) than CuO NM, as seen from the increase in DEP for CuO NM with exposure time. This could be related with a different uptake mechanism of the nanoform or slower oxidation of CuO NM (i.e., release of Cu ions), hence effects of Cu ions would occur comparatively later. Interestingly, the parallel results of metabolomics, same species and same exposure design, [150] showed the similar pattern in terms of time and Cu material form. The corresponding transcriptomic study testing similar Cu forms in *E. crypticus* [22] showed that the Cu salt did not induce differential expression of genes (DEG) after 3–7-day exposure, whereas Cu NM activated DEG after 3 days, with a turn down at day 7. Since gene regulation precedes most of protein expression, this is in good agreement with the observations at protein level, i.e., virtually no change in protein expression for $CuCl_2$ exposure.

Important output: Because Cu is an essential element, organisms have mechanisms in cells which continuously regulate its uptake, intracellular traffic, storage, and efflux. The lack of Cu exposure signals can be explained by the regulation of Cu via constitutive genes being transcribed continually, up to a certain threshold. In contrast, CuO NM induced gene regulation, hence transcription of facultative genes seems required for equivalent effect concentrations. This is a clear mechanistic difference.

If the absence of differential gene and protein expression for $CuCl_2$ exposure is due to constitutive gene response, then there were still extra energy costs and hence the apical effects (reproduction decrease). A study by Gomes et al. [159] on energy budgets showed a high energy consumption (Ec) when exposed to $CuCl_2$ EC10 with consequent CEA decrease, which means that the effects of Cu on the total energy budget of *E. crypticus* are due to an enhanced metabolic rate and not due to the reduction in energy assimilation. This could be one of the reasons for the observed reduction in reproduction. Another study for Cu NMs with a similar design [152] also showed an increased Ec, although the CEA increased with time, being associated with an increase in proteins and lipids that probably compensated the increased Ec.

Upregulated DEPs affected the gluconeogenesis (e.g., pyruvate carboxylase protein) and the tricarboxylic acid cycle (e.g., succinate dehydrogenase) processes [149]. The "extra" cellular glucose demand may imply their production by non-carbohydrate substrates (e.g., lactate, glycerol, and glycogenic amino acids) suggesting a disruption in energetic metabolism. Although proteins are constitutive (as opposed to storage/energetic carbohydrates and lipids), their mobilization occurs under stress (contaminants and/or long exposure periods) as observed in *E. crypticus* exposed to Cu NMs.

Effects of CuO NM occur via a different gene and protein regulation as a response to stress.

Important output: Evidences are that CuO NM is not "seen" nor handled by the cell in the same way as $CuCl_2$.

So far, studies on mechanisms of Cu cell uptake support evidences that CuO NM enter the cell via endocytosis and they are internalized in lysosomes [160] where oxidation to Cu ions ($Cu^+/^{2+}$) takes place due to a low pH, allowing Cu ions to be liberated and exported into the cytosol [161]. Excess of cytosolic Cu ions can then be stored in metallothioneins (MT) or as complex with glutathione; handled similarly to the Cu^+ that enters the cell from Cu salts. Copper chaperones rapidly bind cytosolic Cu ions to shuttle them to their designated cellular targets. The mRNAs for the copper

chaperones Cox17, copper chaperone for superoxide dismutase, and antioxidant protein 1 are believed to be involved.

The cellular mechanisms for Cu salt and Cu nano have been reported as the same [162], except that Cu is taken up by the cell via a different route (membrane transporters versus endocytosis) [160]. Indeed, different protein profiles were observed in the $CuCl_2$ and CuO NM exposed organisms, among other, induction of proteins (e.g., neurofibromin (NF1)) influencing the phagocytosis activity, whereas exposure to $CuCl_2$ had higher impact in a shorter time period; organisms have conserved mechanisms that allow Cu handling and detoxification; CuO NM caused higher impact after a longer exposure period and although the same conserved mechanisms can be used for Cu ions (from both CuO NM and $CuCl_2$), the phenotypical effects were different. This could be due to different mechanisms: 1) the cell uptake route is different for Cu NM and Cu ions, 2) internalized Cu NM can result in a Trojan horse boom effect, 3) the cascade of events occurs in a different time order, and 4) the organisms' uptake changes with different life stages, i.e., cocoons have a thickened surface that protects from the entry of NM, whereas juveniles have facilitated uptake via tegument and for adults the oral uptake gains increased importance.

Important output: CuO NM activates more stress response mechanisms in longer exposure period, compared to $CuCl_2$, with good agreement between DEPs, DEGs, and DEMs. $CuCl_2$ causes higher impact in shorter time-periods, but organisms have conserved mechanisms (constitutive genes) that allow Cu handling and detoxification. CuO NM causes higher impact after a longer exposure period, inducing regulation of facultative genes with a whole differentiated paradigm and cascade.

Important output: Multispecies testing, including *E. crypticus* [50], showed that in longer term exposure, toxicity of CuO NM became similar to $CuCl_2$ levels.

The added value of combining many endpoints and tools, beyond the standard test, shows the potentially large error gap to assess risks. The ways to integrate all knowledge onto RA may not always be so straight forward but improvements can be expected in terms of e.g. reducing the uncertainty in safety factors. In this particular Cu case-study we suggest the following approach to RA:

Important output for RA: Toxicity of Cu NMs is similar to Cu salts when tested in longer term exposure period via e.g. FLC or also in a soil multispecies system (SMS), as opposed to the results obtained via the standard test alone.

Important output for RA: Evidences of potential epigenetics of CuO NMs should increase the RA safety factor.

Titanium dioxide (TiO_2) nanoparticles /nanomaterials

Standard OECD/ISO tests (chronic) results showed that very low to no effect of TiO_2 NMs occurred in terms of survival and reproduction, although results vary within test designs and species. Exposure of *E. fetida* to TiO_2 NMs via filter paper contact test (14 days) did not affect survival up to 10.000 mg/L [163], nor via soil exposure up to 10.000 mg/kg in both *E. fetida* and *E. andrei* in terms of survival and reproduction [for several TiO_2 NMs and soil types [164]]. Exposure of *E. crypticus* resulted in no toxicity up to 1000 mg/kg [several TiO_2 NMs, in artificial soil, and in the presence of UV radiation [165]]. However, a (much) higher toxicity was reported for the non-standard earthworm *Pheretima hawayan* with a 24 h LC_{50} of 145 mg/kg for TiO_2 NMs [166], and significant reproductive effects were also reported in *E. fetida* earthworms at 1000 mg/kg [73,163].

Still within standard OECD/ISO tests, avoidance seems a more sensitive endpoint than survival or reproduction, e.g. *E. andrei* avoided TiO_2 NMs (and not the micro TiO_2) in soil at concentrations of 1000 mg/kg at which no effect occurred for survival or reproduction [164].

Alternative test data, with exposure via reconstituted water (5 days), where bioavailability is maximized, followed by transfer to clean soil (21 days), toxicity was observed (for non-coated TiO_2, nano and bulk) when combined with UV radiation. [165]. The same experiment in exposure via soil:water extracts, where dissolved organic matter is added, no toxicity occurred [165]. Because TiO_2 is photoactive [it absorbs photons of energy above 3.20 eV for anatase and 3.02 eV for rutile (corresponding to 384 nm and 410 nm wavelengths, respectively)], a property that has been explored, for instance, for remediation purposes, the toxicity is expected to differ when under UV radiation. Hence, for photocatalytic NMs a standard exposure under standard light, where the excitation is not induced, does not meet the needed adequacy of a test to assess its hazards nor its relevancy to predict risks. Photoactivation mechanism leads to Reactive Oxygen Species (ROS) formation, which is known to damage living organisms, as shown at both organism level [165], and transcriptomics level, as mechanisms were interpreted using HTP (microarray), after short-term exposure [prior the occurrence of effects on the organisms] [24].

Important output: NMs specificities must be considered for the testing of hazards of NMs. e.g., the photocatalytic properties of nano TiO_2 that rely on the ability of TiO_2 NMs to form reactive oxygen species (ROS) on its surface when excited with UV light, should require TiO_2 NMs hazard assessment exposure to be done in the presence of (at least realistic) UV radiation.

Alternative tests via in vitro studies, using coelomocytes, showed no cytotoxicity of TiO_2 NMs up to 25 mg/L (for one of the reference TiO_2 NMs, P25, Evonik Degussa, or NM105) [167,168]. Although not cytotoxic (also no oxidative stress, via Superoxide Dismutase (SOD), Catalase (CAT) or Glutathione S-transferases (GST)), TiO_2 NMs were internalized by coelomocytes (probably by endocytosis) and affected the cells immune response (alterations in the expression of fetidin and coelomic cytolytic factor protein coding genes) [168]. Further, in vivo studies, in exposure from 7 up to 28 days, showed that TiO_2 NMs can induce both oxidative stress and damage, [140,166] [oxidative stress was also observed in isopods exposed via TiO_2 NMs spiked food [169]]. After 120 days of exposure, TiO_2 NMs reduced Superoxide Dismutase (SOD) and glutathione (reduced/oxidized) GSH/GSSG ratio (suggesting oxidative stress), but did not induce oxidative damage or effects at organism level (growth or reproduction) [25]. Another study, a HTP transcriptomics (microarray), showed that short-term (5 days) exposure to several TiO_2 NMs (the JRC standard NM103, NM104 and NM105 [170] and bulk) also induced oxidative stress and apoptosis, but only when combined with UV radiation [24], indicating that oxidative stress response would probably take longer (at least longer than 5 days) to occur. Both transcriptomics studies [24,25] indicate that energy and nucleotide metabolism are targets for TiO_2 NMs, however there might be a shift from down- to up-regulation with time. Prolonged exposure to TiO_2 NMs affect the antioxidant system, metabolomic [25,171] and transcriptomic profiles of earthworms, even though no effects at organism level were reported [25]. However, it is not possible to anticipate for how long the triggered response can protect the organisms, and thus there is a risk for longer term population effects.

It may be noted that TiO_2 NMs are among the most produced NMs worldwide, often applied in food and cosmetic industry (e.g. [172]). For instance, the use of TiO_2 NMs in cosmetic sunscreens increased the debate about ethical aspects of the Environmental Health and Safety (EHS) of nanotechnology in already marketed products [173]. Since the beginning of 2020, France banned the TiO_2 NM food additive E171 (used as colorant in foodstuffs such as sweets, chewing-gums, and cakes frequently consumed by children and other vulnerable sections of the population), as uncertainties remained to whether TiO_2 is safe for consumers (https://www.europarl.europa.eu/doceo/document/E-9–2019–003009_EN.html). Based on the same principle "that prevention is better than cure",

the European Parliament called on the European Commission to adopt the same ban (https://www.europarl.europa.eu/news/en/press-room/20201002IPR88447/parliament-objects-to-legislation-on-food-products-that-might-be-harmful-to-kids).

Carbon based nanomaterials (multiwalled carbon nanotubes (MWCNT), double walled carbon nanotubes (DWCNT), fullerenes (C_{60}), graphene)

As for metal based NMs, carbon based NMs are very diverse not only in the type of NM (e.g., carbon nanotubes_CNTS, being multi-walled_MWCNTs, or double-walled_DWNTs, fullerenes: C_{60}, graphene and its modifications) but also in its characteristics (e.g., size, length, diameter, etc.). This sort of diversity, associated with the fact that there are few studies for each type of carbon based NM causes obvious limitations to interpret their risks. Due to the limited studies, they are evaluated together here.

Based on standard OECD/ISO tests, indications are that MWCNTs induce low to no toxicity to standard soil invertebrates, for both survival [174,175] and reproduction, up to 3200 mg/kg in *E. crypticus* [176] or up to 6400 mg/kg in *F. candida* [143]). One study reports significant effects on *E. fetida* reproduction at 0.3 mg/kg [177]. DWCNTs were toxic to *Eisenia veneta* when exposed via spiked food (reproduction EC50 of 176 mg/kg [178]).

Alternative test methods, showed that MWCNTs induce coelomocytes alterations at the same concentration, increasing metallothionein (MT) levels and decreasing acetylcholinesterase (AChE) activity [177]. Despite being markers of general stress response, these results show that cellular and biochemical parameters were sensitive to short-term (up to 14 days) exposure to MWCNTs.

Standard OECD/ISO test extension test showed that exposure of *E. crypticus* to MWCNT in an extended period of 60 days (instead of 28) caused a significant increase in toxicity [58].

CNTs are known to adsorb other chemicals, hence some research has focused on the toxicity of CNTs combined with organic chemicals, but currently available data indicate a case-by-case evaluation is needed. For instance, while the absorption of nonylphenol to MWCNTs seem to increase nonylphenol bioavailability and its toxicity [179], the adsorption of Sodium Pentachlorophenol to MWCNTs seem to alleviate its [174,180]. It is worth noticing that for soil, the availble surface binding sites in many cases are higher on soil particles than on the available NMs.

Results from standard OECD/ISO (or similar) tests for exposure to C_{60} showed toxicity ranging from no effect on survival or reproduction of *E. fetida* up to 10.000 mg/kg [181] to significant reduction in cocoon production of L.*rubellus* at 154 mg/kg [182], or at 1000 mg/kg, for *Eisenia veneta* exposed via spiked food exposure [178].

A standard OECD/ISO test extension, using L. *rubellus*, in a prolonged exposure to C_{60} over 326 days, showed C_{60} affected population growth rate in a dose-dependent way, and that juvenile stage is probably more sensitive [182]. A follow up study showed that external barriers (cuticle and gut tissues) were affected by C_{60} exposure, and that while cuticle damage was progressive (more pronounced for prolonged exposure), gut tissue seemed to adapt (lower effects for prolonged exposure indicate tissue repair, also supported by the down-regulation of heat shock protein 70 (HSP70) coding gene) [183]. Further, there was no indication of tissue inflammation, that together with the down-regulation of the cytokine-like protein CCF-1 coding gene, indicate immunosuppression [183]. This hypothesis was corroborated by in vitro studies, using coelomocytes, in which viability did not decrease with C_{60} exposure [rather CCF-1 coding gene was down-regulated] [184]. This is again a good proof of concept where alternative tests complemented and explained the effects observed at organism level. Metabolomics data indicated higher energy demands to cope with C_{60} toxicity (increase in enzymes production) that cause a shift in energy allocation from functions such as growth and reproduction [185].

A standard OECD/ISO test extension where *E. crypticus* were exposed to graphene oxide (GO) NMs during a full life cycle test (from cocoon, during 56 days) it was shown that GO was ca. 3 times more toxic than its reduced form (rGO) ((rGO caused nearly no toxicity up to 1000 mg/kg [186]). Further, the effects observed at early life stages (hatching success) were good predictors of latter effects (survival and reproduction) for GO. The toxicity of Nano Carbon Black (NCB) [proposed as remediating agent for metals after surface modification, e.g. oxidation] is also higher in its oxidized form (surface modification), probably related with oxidative stress [187,188]. Thus, careful attention should be given to the application of modified (oxidized) NCB in soil remediation.

Iron (Fe) based nanomaterials

Iron (Fe) based materials (being the most common nano zerovalent iron (nZVI)) are often proposed as remediating agents, although it has been shown it can be toxic.

Standard OECD/ISO test with *E. fetida* exposed to several Fe based NMs (FerMEG12, Carbo-Iron, Magnetite, Nano-Goethite, and Trap-Ox Fe-zeolites) showed no toxicity occurred up to 100 mg Fe/L [189], but nZVI induced acute toxicity on filter paper (72h_LC50 ~ 750 µg/cm^2) [190]).

Results from chronic tests in soils corroborate the tendency that nZVI induce toxicity to soil invertebrates (e.g. for *E. fetida*, reproduction is affected at 100 mg/kg [191]; for *E. fetida* and *L. rubellus* mortality occurred at 300 mg/kg in a sandy loam soil, and at 500 mg/kg in OECD soil [192]; for *F. candida* reproduction is completely inhibited at 1000 mg/kg [193]. On the other hand, Fe_2O_3 NMs were not toxic to *F. candida*, based on standard OECD/ISO test [143] nor to *E. crypticus*, even when based on standard OECD/ISO test extension [58].

Earthworms (*E. fetida* and L. *rubellus*) avoided soil spiked with nZVI [190,192], but only above concentrations that impact reproduction (≥500 mg/kg) [192]. Toxicity of nZVI to *E. fetida* and L. *rubellus* did not increase with 30 days aging time (increased mortality around 250–500 mg/kg) [192]. nZVI induced toxicity via non-specific stress mechanisms, e.g. initial induction of Reactive Oxygen Species (ROS) scavenging enzymes (e.g. Superoxide Dismutase (SOD) and Catalase (CAT) [194], followed by impairment in oxidative stress response [190] with consequent Reactive Oxygen Species (ROS) formation and oxidative damage to lipids and DNA [195]. The strategy of nanoremediation of metal contaminated soil with nZVI was evaluated with *C. elegans*, showing that nZVI added to soil reduced the toxicity [in terms of survival, growth, and reproduction] of metal (Pb, Zn and Cd) spiked soil up to 30 days after incubation (out of 120 days), but not at 120 days post-incubation [26]. Microarray analysis results showed a transcriptomic profile of increased biosynthesis of defensive enzymes responsive to oxidative stress at time 0 (soil collected immediately after the 120 days of incubation with nZVI), consistent with the higher remediation capacity. At day 14, metal responsive genes were down-regulated (corresponding to the time at which lower bioavailable metal concentrations were detected in soil). As the effectiveness of nZVI as remediator decreased (120 days after incubation) also the transcriptional oxidative and metal-induced responses were attenuated [26], showing that the gene expression profile of *C. elegans* was a good indicator of stress response.

Other nanoparticles/nanomaterials

The major findings for NMs with less abundant studies available are described in the following section.

Other metal based NMs (Al2O3, SiO2, ZrO2, CeO2, Co3O4, WCCo, MoO3, TiSiO4, Ni, Au, Quantum dots)

Aluminium oxide (Al2O3) NMs. Based on standard ISO/OECD tests, Al_2O_3 NMs caused low to no effects to *E. fetida* (no effects on survival up to 13.000 mg/kg, reduction on reproduction at 10.000 mg/kg [196]) and to *C. elegans* [no effects on survival, growth or reproduction up to 5 g/L [197]). Again, avoidance was a much more sensitive endpoint, with an EC50 of 5000 mg/kg for *E. fetida* [196].

Silicon dioxide or silica (SiO2) NMs. Based on standard ISO/OECD tests SiO_2 NMs were not toxic to *E. fetida* [73] or *E. crypticus* [198], up to 1000 mg/kg.

Alternative tests, via aqueous exposure (ISO reconstituted water, or soil:water extracts) followed by post-exposure in clean soil to *E. crypticus* [198] did not show toxicity. However, when the SiO_2 NMs were encapsulated in europium polyoxometalates (Eu-POMs, investigated as nanocarriers in drug delivery), the Eu-POM/SiO2 NMs were toxic to *E. crypticus*, based both on the standard ISO/OECD test [reproduction EC50 = 232 mg/kg], and based on aqueous exposure via soil:water extracts (but not in ISO reconstituted water) and posterior transference to clean soil [198]. The results indicate that the presence of organic matter or other soil particles (present in the soil:water extracts but not in the ISO reconstituted water) interact with the Eu-POM/SiO2 NMs, and increased toxicity. Again, the testing of different exposure routes [increasing complexity in a stepwise method] provide a good alternative to assess and distinguish the contribution of soil components for chemical toxicity.

Avoidance was a sensitive endpoint for SiO_2 NMs when testing different species [199]: avoidance response for *E. fetida* and *E. crypticus*, and no avoidance for *F. candida* (collembolan), *Porcellionides pruinosus* (isopod) and *Tenebrio molitor* (arthropod) larvae, showing different results depending on the species.

Zirconium dioxide (ZrO2) NMs. Based on the standard OECD/ISO tests ZrO_2 NMs caused no effects in terms of survival and reproduction in *E. fetida* [73] or *E. crypticus* [165] up to 1000 mg/kg. Alternative test in *E. crypticus* via aqueous exposure (in ISO reconstituted water) followed by post-exposure in clean soil [165] did not cause effects either.

Cerium dioxide (CeO2) NMs. Based on the standard OECD/ISO test, CeO_2 NMs, including the reference JRC materials NM212, NM211, NM213 [200] caused no effects in terms of survival and reproduction in *E. fetida* up to 10.000 mg/kg, [201]. Alternative tests, however, showed histological alterations (cuticle loss from the body wall and some loss of gut epithelium integrity) in worms exposed to the highest concentrations (10.000 mg/kg) suggesting that CeO_2 NMs can induce long-term toxicity [201].

Cobalt oxide (Co3O4) NMs. Based on the standard OECD/ISO tests, Co_3O_4 NMs caused no effects in terms of survival or reproduction in *E. andrei* and *F. candida,* up to 1000 mg/kg. Further, *E. andrei* avoided soil spiked with Co_3O_4 NMs at 5000 mg/kg [202].

Alternative tests in the same species, assessing sub-cellular level endpoints (biochemical oxidative stress related), showed Co_3O_4 NMs caused effects at much lower concentrations (e.g. increase in LPO levels at 269 mg/kg), suggesting oxidative damage that can induce further long-term effects [202].

Tungsten Carbide Cobalt (WCCo) NMs. Based on standard OECD/ISO test, WCCo NMs showed relatively low toxicity, with no effects on survival up to 6400 mg/kg and a reproduction EC50 of 1500 mg/kg to *E. crypticus* [57].

The standard OECD/ISO test extension, using an additional 28 days (56 days instead of standard 28) showed higher impact than predicted based on the standard [57]. Again, evidences support the need to extend the exposure period to assess the risks of NMs [203,204]. Further, WCCo NPs toxicity in *E. crypticus* (EC50 = 1500 mg WCCo/kg soil DW) was higher than the equivalent toxicity of Co alone (via $CoCl_2$) or WC alone, and hence toxicity must be due to a nanospecific effect or a combined effect of WC and Co (mixture toxicity).

A multigenerational exposure to WCCo NMs showed that toxicity did not increase (higher reproductive performance was reported) [205], but the analysis of epigenetic markers showed an increase in global DNA methylation [206], which occurred also in unexposed generations – indicating transgenerational effects - and in association with the reported phenotypic effect (increase in reproduction) [206].

Molybdenum oxide (MoO3) NMs. Based on the standard OECD/ISO tests, MoO_3 NMs were acutely toxic to *E. fetida* in OECD artificial soil, in a time (7 versus 14 days) and concentration (40 and 500 mg/kg) dependent manner, with significant effects at day 14, in both concentrations, [207]. Using a surrogate media (microcrystalline cellulose), higher toxicity was reported at day 14 (73% versus 53% in soil), but not at day 7. That was related with high levels of Mo in worms tissues, in comparison to those exposed in soil [207], possibly due to higher bioavailability of Mo in the surrogate media. Alternative tests (biochemical, oxidative stress related) from the same study, showed that MoO_3 NMs reduced glutathione reductase (GR) and catalase (CAT) activities, at 500 mg/kg in microcrystalline cellulose media, and reduced GR activity at 40 mg/kg in soil [207], indicating that MoO_3 NMs can induce oxidative stress to *E. fetida*, but not bellow concentrations causing effects at organism level.

Titanium silicon oxide (TiSiO4) NMs. Based on the standard OECD/ISO tests $TiSiO_4$ was overall not toxic in terms of survival and reproduction to *E. andrei* and *F. candida,* up to 1000 mg/kg, [208]. Again, avoidance was a more sensitive endpoint, with *E. andrei* avoiding significantly at 1000 mg/kg [208].

Nickel (Ni) NMs. Based on standard OECD/ISO tests, Ni NMs induced low to no toxicity to *E. fetida* up to 1000 mg/kg [73], while *E. crypticus* showed a significant reduction in reproduction at 600 mg/kg [54]. A standard OECD/ISO test extension, the FLC in *E. crypticus*, showed that Ni NMs toxicity was lower compared to the ERT, which could be due to increased resistance of the organisms when the exposure starts from cocoons stage (as done in the FLC test, whereas the ERT starts with adult organisms) [54]. Further, Ni NMs did not follow a monotonic dose-response curve, i.e., the lower concentration 100 mg/kg induced the same effect as higher concentrations (1000 and 1800 mg/kg) [54]. This non-monotonic response (also observed for Ag NM300K [53] and Au NMs [209]), is associated with a higher dissolution of NMs at the low concentration window, carrying obvious implications when assessing the risks of NMs.

Alternative tests using HTP omics showed that the mechanisms of toxicity of Ni NMs were common to Ni salt, namely increase in proteolysis, apoptosis and inflammatory response, suggesting that likely more than 7 days of exposure are necessary to capture a Ni NMs specific response [23].

Gold (Au) NMs. For Au NMs, known to be a highly stable element, no toxicity was reported to *Enchytraeus buchholzi* up to 37.5 mg/kg [210], while for *E. fetida,* a significant reduction in reproduction occurred at 20 mg/kg (non-monotonic response) and at 50 mg/kg for different size Au NMs, probably not related with ions release (because, unlike observed for the $HAuCl_4$, Au NMs did not affect MT gene expression) [209].

Quantum dots. Quantum dots (QDs) are semiconducting nanoparticles, for which properties (and toxicity) are depending (aside from the well-known factors, such as size, shape, etc.) on its

composition. In soils, toxicity studies with QDs were performed on Cadmium Selenide QDs (CdSe QDs) [211], and Cadmium Telluride QDs (CdTe QDs), with various coatings [212]. Both studies [211,212] have shown that Cd was released from the QDs, and was accumulated by earthworms (*E. andrei* and *E. fetida*, respectively). Further, based on standard OECD/ISO tests, it was shown that soil aging (6 months) increased reproductive toxicity of CdTe QDs to *E. fetida* [freshly spiked soils EC50 of > 2000, 108, 65, 96 mg CdTe/kg for bulk, PEG-, COOH- and NH4 + -coated CdTe QDs, respectively; and aged soils EC50 of 165, 88, 78 and 63 mg CdTe/kg for bulk, PEG-, COOH- and NH4 + -coated CdTe QDs, respectively) [212].

Nanoformulations (e.g. nanopesticides)

Nanoformulations (nanoagrochemicals or nanopesticides) have the potential to support the necessary increase in global food production in a sustainable way, however, as reviewed in Grillo et al. [213], much of the research has so far been carried out in a haphazard and random manner, mostly looking for beneficial aspects (e.g. enhancement in efficacy) with little attention to the potential adverse effects and impacts.

Studies have shown that nanoformulations of "traditional" pesticides can alter the fate and bioavailability of pesticides, for instance increasing its bioaccumulation by earthworms [214,215]. The toxicity of an antimicrobial compound (glycerol monolaurate) or different fungicides (Eugenol, Mancozeb, and their mixture) to *F. candida*, was eliminated or reduced when they were tested as nanoformulations (nanocapsules or nanoemulsions) [216,217].

Based on standard OECD/ISO tests, a nanoformulation of atrazine was more toxic than the pure active ingredient (atrazine) and its commercial formulation (Gesaprim®) to *E. crypticus* [reproduction EC_{50} of 114, 161, and >400 mg atrazine/kg, respectively) [55]. When testing via a standard OECD/ISO test extension, in the FLC the toxicity patterns changed: nanoformulation [EC_{50} = 276 mg atrazine/kg] ≥ active ingredient (a.i.) [EC_{50} = 236 mg atrazine/kg] > Gesaprim® [EC_{50} = 436 mg atrazine/kg].

For inorganic Cu based pesticides, the nanopesticide Kocide 3000® was more toxic to *F. candida* than non-nano formulations (Cu $(OH)_2$ solutions) [218]. This is a case where the impact to non-target soil organisms was higher using the nanoformulation.

There are still many gaps/needs in the field of nanopesticides hazard assessment/risk assessment, as recently pointed [213], including a better mechanistic understanding of nanopesticides (and degradation products) hazards, that would help to predict long-term effects and promote safer-by-design production/modification of nanopesticides. This is a field under expansion and maturation, where much more data is needed. Additionally, regulation issues remain unresolved so far, given the infancy of the nanopesticide field, although this will have to be discussed and needed adaptations implemented in a way that both nano and pesticide aspects can be ensured, for a detailed overview see e.g. the section of Regulatory frameworks of nanopesticides in [213].

Important output: Regulation issues for nanopesticides remain unresolved so far, given the infancy of the field. Revisions are needed to ensure both nano and pesticide aspects are covered in the implemented regulation.

Nanoplastics

In the last few years, the increased awareness that part of the tremendous amount of plastic debris will turn into micro- (<5 mm) and nanoplastics (NPls, <1 μm), this due to various forces including mechanical abrasion, has significantly increased the research on the environmental effects of those materials.

Polystyrene NPls, a representative material widely used in personal care products and ubiquitous in marine samples, was studied in soil environment, using the standard species *E. crypticus* [219]. Based on the standard OECD/ISO test, the reduction in survival and reproduction reported at 1200 mg polystyrene NPls/kg was caused by the dispersants present in the suspension used.

Alternative test method, exposing *E. crypticus* to polystyrene NPls through spiked food induced an increase in reproductive output (number of cocoons produced in 7 days) [220]. However, there was a decrease in organisms' weight (at 10% polystyrene NPls) that was linked to alterations in the enchytraeids gut microbiome [220]. This study shows that even without significant effects on reproductive output (organism level standard endpoint), the exposure to polystyrene NPls inhibited key bacteria in the organisms' microbiome (alternative test method) which can further affect not only the organism and populations, but also the soil ecosystem.

Exposure of *E. crypticus* to Acrylic, Polyethylene, Polypropylene and Epoxy microparticles in LUFA 2.2 soil showed no effect in terms of survival or reproduction, both via the standard OECD/ISO test and its extension up to 60 days [58]. This is a relevant case study not only because of the thorough coverage (25 OECD tests), but also because it compares the toxicity of each component in real materials, where NMs were embedded in a plastic matrix, hence compares the matrix (plastics of different sources), the NMs and the NMs embedded in the matrix (product). The authors [58] conclude that the standard guidelines are not adequate to test plastics, recommending adaptations such as prior aging/weathering of the plastics, and testing nanoplastics instead of microplastics, e.g., via grinding. For most PLs that are like very persistent and very bioaccumulative (vPvB) substances, extending the test duration will not help.

Important output: Testing in biological systems should be adapted depending upon expected durability: (1) highly durable materials should be tested after ageing and weathering, and (2) highly degradable/changing materials should be tested as synthesized/pristine.

The results also support the recently proposed REACH [221] restrictions, which state that micro- and nano-scaled plastics behave like PBTs and vPvBs [although the REACH proposal only targets deliberately manufactured and intentionally added micro- and nanoplastics].

Important output: Regulation issues for unintentionally released/formation of nanoplastics remain unresolved. Although the recently proposed REACH restrictions (micro- and nano-scaled plastics behave like PBTs and vPvBs) it only targets deliberately manufactured and intentionally added micro- and nanoplastics.

Other microplastic studies focused on its ability to act as carriers of other chemicals such as pesticides or antibiotics. Alternative test methods (effects on gut microbiome community) showed that the combination of microplastics (polyamide and polyvinyl chloride) with the antibiotic tetracycline increased the diversity of antibiotic resistance genes (ARGs) in *E.crypticus* [in comparison to the antibiotic or the microplastics alone, and although the microplastics did not increase the accumulation of tetracycline in the organisms] [222]. Another study showed that low-density polyethylene microplastics size (1 and 5 mm) influenced the sorption and desorption of the pesticide chlorpyrifos and its consequent release to soil, with the soil spiked with 1 mm-microplastics having more than 100 times the concentration of chlorpyrifos, in comparison to the soil spiked with 5 mm-microplastics [223]. However, as the earthworm *E. fetida* seem to avoid the spiked soil (alternative method) by burrowing to the bottom of the test vessels in the presence of microplastics + pesticide (behaviour not recorded in controls) there was no clear vertical transportation of the microplastics and the pesticide to deeper layers of the soil. Nevertheless, alternative tests results (AChE inhibition, biomarker for neurotoxicity) showed that *E. fetida* was exposed to

microplastics´ released chlorpyrifos, and that chlorpyrifos reached concentrations high enough to cause a neurologic response [223].

Important output: Nano-micro-plastics can act as carriers of various toxicants and increase toxicity. The role of nano-micro-plastics to vertically transport other toxicants in soil requires further investigation.

Challenges, gaps and opportunities – recommendations and way forward

Available data

Data availability for NMs concerns not only quantity and quality per NM, it concerns a coverage of sizes, shapes, surface coatings and many other combinations. While the range of options is endless, there are probably more obvious gaps, where the need to understand the consequence of variations exists. For example, although Ag, ZnO and Cu/CuO are among the most studied NMs in terms of ecotoxicological effects, their numerous applications lead to the production of NMs with all sort of variations in size, shape and coatings, that are certainly not covered in terms of effect assessment.

There is perhaps an important distinction to make in terms of data fit for purpose, data can be produced for regulatory purpose or produced for research, often based on distinct requirements and aims. Hence, while progress in terms of research is driven by many different questions, regulation requires results from experiments with a targeted aim, standard, optimized and comparable across the labs in the world, usually a dose-response based paradigm, the standard OECD/ISO tests type.

Alternative tests data, e.g., high-throughput omics data, carries considerable data analysis burden, requiring more advanced techniques and computing power, generating a less friendly and popular feel compared to simpler/known statistics data type. The solution lies in two fronts, learning and improving the accessibility of software.

Open data and transparency

The multiple particle parameters in play when dealing with nano and the complex data when dealing with alternative testing, combined with the need for read across, puts an extra emphasis on having open data to verify or develop models across data. The free availability of data, meaning free from permission barriers such as copyright, embargo, etc., that would allow the data not only to be public but also to be re-used - the Open Data (OD) - emerged as global movement that began with the call for Open Science. The advantages of OD include facilitating scientific collaborations, enriching research and advances in analytical capacity to inform decisions. The last is particularly important in the field of human and environmental health, as the ability to access and combine diverse data can advance early signal detection, improve analysis and evaluation, inform program and policy development, increase capacity for public participation, enable transparency and improve accountability [224].

The European citizens' initiative to ban glyphosate and protect people and the environment from toxic pesticides has clearly set the case for the future. This stands as one example of data availability deficit also involving transparency issues, with further consequences for timely regulation, which raised much social awareness. Due to “erroneously omitted data” EFSA was forced to amend the existing maximum residue levels for glyphosate (https://www.efsa.europa.eu/en/efsajournal/pub/5862). The good thing is that OD and transparency issues became a priority and been followed to implementation via many actions.

Despite the last decade progress towards OD [e.g., H2020 mandatory open access, or for microarray experiments, the Minimum Information About a Microarray Experiment (MIAME), that describes the minimum information required to ensure that microarray data can be easily interpreted and that results derived from its analysis can be independently verified, has to be available to the public prior publication, via Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) website, a public database], there are still many challenges to overcome. These challenges include, among many other, the significant resources needed not only to set up but also maintain databases for public use and combinability (e.g., data standards to ensure transparency regarding the source, how the data are generated). There is also some resistance to share data based on the legitimate concern that open data could be used inappropriately, if the purpose for which the data was collected and the limitations of the data are not well-understood, and to make sure that there is equity in data access.

Technical test aspects

Test designs

While there are many advantages of implementing a variety of test designs because it will create knowledge beyond, the lack of supporting comparable designs will limit the possibility for read-across. The solution is not necessarily easy to achieve but goes through harmonization of e.g. descriptors, endpoints, test duration, etc., as increasingly recommended for standardization [2,3,4,114–116]. Certainly, the creation of a repository of NMs (e.g. the JRC Nanomaterials Repository [225]) had a fundamental role in facilitating data production for read-across.

Standard OECD/ISO tests cover the biological side for comparable results, although up to now there has been much ongoing work on the adaptations of guidelines to assess hazards of NMs from the ones developed originally to assess hazards of chemical substances [3].

The importance of alternative tests added value has been shown, probably more than ever before [5,9,65], but it has been clear that there are several challenges here. One challenge is the standardization level/maturity varies widely, from little to quite matured. Nevertheless, they may in first instances act as supporting evidence.

Test materials

Read-across and the periodic table. As well known, NMs can have an endless number of combinations of shapes and sizes, making it impossible to test all combinations. However, this should not be confused with that except from small NMs (i.e. NMs in the very low nm range may show unconventional behaviour), most NMs behave as can be derived from the periodic table information, e.g. possible oxidation or chemical structure. This may be especially important with omics studies where one of the assumptions of key events is that there is a direct link between the NMs physio-chemistry and the molecular biological response. This is also why atomistic modelling of NMs is of interest. And it is obviously also important for read-across and grouping.

Referential type NMs, designed for benchmark. In line with this, one of the main obstacles to derive general conclusion on the toxicity of NMs based on the literature data, is the large variety of NMs tested in hand with the lack of thorough characterisation. To overcome this problem, the European Commission's Joint Research Centre (JRC) created a repository of Representative Test Materials that hosts samples of a number of commercially available NMs: the 'JRC Nanomaterials Repository' [225,226]. The initial aim of the JRC NMs repository was to provide “the same NM” to different laboratories [the NM samples are obtained by subsampling from a single batch following well defined standardized operation procedures] promoting better reproducibility and reliability in safety testing of NMs. These NMs have been explored in projects under the EU framework programmes for research, e.g. FP7 and H2020 (e.g. NANO- GENOTOX [http://www.nanogenotox.eu/],

MARINA [http://www. marina-fp7.eu/], NANoREG [http://www.nanoreg.eu/], SUN [http://www.sun-fp7.eu/], BIORIMA [https://www.biorima.eu/]), which greatly improved their further characterization [225] and production of a gross volume of biological toxicity data to the same source NMs. The use of similar and well-characterized NMs by the scientific community, is of extreme importance for the generation of comparable and reliable experimental results and datasets in support to regulatory research. Good progress is also made with the JRC NMs repository which contains NM models that can be used for comparison. The repository for instance, among others contains, TiO_2 NMs [NM100, NM101, NM102, NM103, NM104, NM105 [170]] and CeO_2 NMs [NM211, NM212, NM213 [200]] which has been and can be used.

Libraries of NMs, designed for modelling. NM libraries [compositional libraries: group of NMs of related but different chemical composition; or combinatory libraries: group of NMs of the same chemical composition but with individual physicochemical property (e.g., size, shape, aspect ratio, crystal structure, dissolution rate, and surface charge) systematically altered [227]] represent an alternative solution to the screening of specific NM descriptors, allowing the control of descriptors, tuning e.g. size, while keeping the other constant. Examples of such libraries include Fe doped TiO_2 NMs [118], Fe doped ZnO NM [117], Fe doped CuO NM [120]. This means that for instance the TiO_2NM library yields materials with size of 11, 10, 8, 5 nm when doped with Fe at 1, 2, 4, 6%, respectively. These libraries are designed to provide the base material, e.g. TiO_2, with altered functionalities e.g. a change in band-gap due to Fe doping.

Specific NMs, designed for functionality. Material specific properties should be considered in a case by case, to meet worse case scenarios. For instance, the known photocatalitic properties of TiO_2 should be included in the experimental test design by including UV exposure. The exposure of TiO_2 combined with UV has shown significant differences compared to non-UV exposed (e.g. [24,165]), this representing a relevant exposure scenario.

Materials of different sizes, structures and shapes, e.g. nanobiomaterials (NBM) (medical devices and advanced therapy medicinal products) can be disks (bone chirurgic), meshes (wound dressing), gel-type emulsions (tissue engineering), or individual particles such as spheres, rods (photothermal therapy). This carries important considerations for the testing of hazards to the environment such as the mixing in the test media and exposure issues. There is a need to meet a common testing strategy, e.g. it has been recommended that grinding to the nanoscale would be a closer representation of worse case scenarios in the environment for real world materials [228] or NBM [3]. This is also supported by probabilistic modelling of NBM release into the environment, where the type of application and associated waste treatment is a very important aspect [229]. NBMs with wound dressing application are predominantly incinerated, whereas NBMs in bone tissue application will remain in the body and are buried or cremated. For instance, sewage sludge application (including hospital sewages) can be an important source of e.g. Ag to soils (Ag NM is increasingly used in the medical industry due to antibacterial, antifungal, antiviral, anti-inflammatory, and osteoinductive properties). Hence, it would make sense that the testing strategy prioritises the most probable application and release scenario besides tonnage.

Materials like most plastics are designed to be durable, behaving similarly to Persistent, Bioaccumulative, and Toxic (PBT) or very persistent and very bioaccumulative (vPvB) substances. Such materials should undergo aging/weathering processes before testing and grinding to the nanoscale [58]. This is the case of highly durable plastics, where testing of pristine materials will mostly induce a physical impact, e.g. by disrupting the guts. Although this is an important and hazardous aspect, the long-term impact should be covered, i.e. the one corresponding to its degradation to nanoscale.

Test level (standard, standard extension, alternative). Standard tests have an unquestionable place and have been developed for all good reasons. These should be performed and act as a benchmark for validation, let aside that adaptations have been deemed necessary for materials like nano. At the same time, progress should be made in what concerns the toxicity level, the role of acute survival type tests is no longer regarded relevant for setting safety values (unless if as a range-finding), and the need of chronic type tests has merited consensus long ago. This should be clearly recommended and implemented at the regulatory level, supported by policy makers. There is a need to update testing requirements to ensure increased safety level, e.g. making chronic tests mandatory in first tier, replacing the acute. For example, it is time to revisit the extensive biological/ecological knowledge available to include the alternative testing into a biological framework [it is acknowledged that a biological framework was also used to create the standard test]. For example, regarding extension of the standard – a full life cycle test will provide information that is beyond the standard EC10/50 reported, and from an ecological point of view this can be directly included in more extensive population modelling which will give a better risk evaluation.

Standard extensions represent clearly show-cases of the importance of longer term exposure. The process towards implementation should be facilitated via addition to current guidelines as annexes. Good proof of concept includes the mentioned case studies for standard OECD/ISO test extension for a full life cycle with Cu [52], standard OECD/ISO test extension of the reproduction test (56 days instead of 28) with WCCo [57] and MWCNT [58].

The main advantage of the use of alternative test endpoints is the in-depth mechanistic understanding obtained, which can be used across materials and across species. Additional advantages are considered, e.g. molecular and (sub)cellular level (e.g. gene, protein) is an expected earlier response, which usually precede the effects at higher level of biological organization (e.g. tissue, organism). This has the potential to predict effects at an earlier stage. One of the challenges is the selection of time points at which effects are monitored, this may or may not capture the key events, hence thorough time course experiments are important to establish a basis before criteria can be established for routine use.

One of the major benefits lays on the integration of the information coming from the various levels, a perfect example for the Adverse Outcome Pathways (AOPs) concept as outlined and recommended by OECD [204,230]. The establishment of AOPs, linking the initial events to the adverse outcomes [98], with further input from toxicokinetic and toxicodynamic models should also provide more information for a better prediction of biological responses over time. Current limitations for a full systems toxicology implementation include the lack of enough advance in methods to grasp and integrate results from the various layers given the sheer complexity. There is nevertheless a lot of work and progress expected from ongoing projects (e.g. H2020: NANOINFORMAtix) and the increased use of machine learning techniques should soon deliver improved approaches.

Because described test levels are relevant, the developed databases should be inclusive and open to integrate novel data endpoints, as also necessary for future materials. There is a need for criteria for data quality and completeness, especially for novel data like from alternative tests. Based on systems biology, guidance on best practices and test design should be given focussing on the specificities of testing NMs, delivering SOPs (Standard Operating Procedures) on (alternative) data completeness for regulation, as further outlined in e.g. [3].

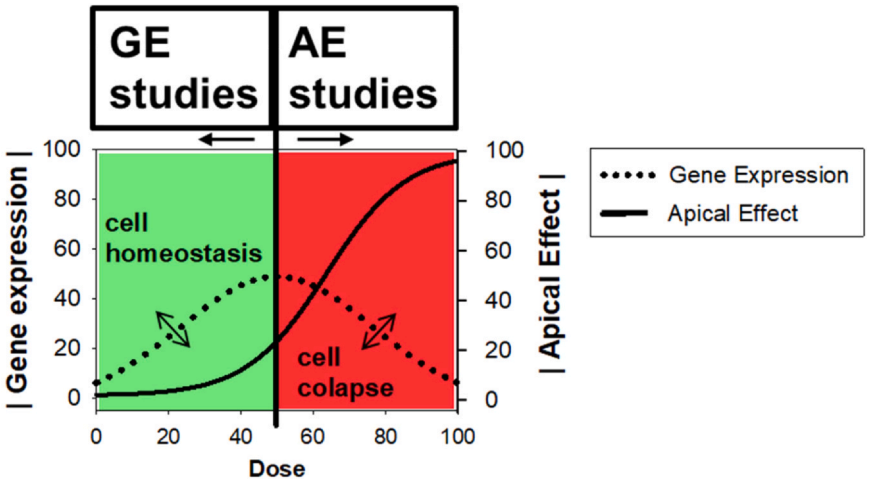


Fig. 5. Cell gene expression dynamic status and apical effect dose response. The cell strives for a homeostasis state, and when exposed to a certain dose of a stressor, this can be achieved by increasingly inducing gene expression (GE). Because there is a maximum level of dose (stressor) after which toxic effects are higher than supported by the cell, an actual shut down of GE occurs. The apical effect (AE), e.g. at the phenotype level such as reproduction reduction, will occur after cellular homeostasis failure. The arrows seen in the GE line indicate that the line shape can be more or less skewed. The doses range for GE and AE research are indicated.

Omics – low versus high-throughput. High-throughput (HTP) omics have key advantages compared to low-throughput. Probably the first to mention is the fact that it is an open method, it provides a (close to) full screen of responses without prior decision, it is a creator of hypothesis and novel knowledge. The second is that the high level of information provides more advanced analysis type with more robust interpretations. For instance, in gene expression studies (RNAseq or microarrays), the analysis progressed towards affected pathways (groups of genes) instead of individual gene changes. One of the drawbacks are that data are often semi-quantitative and may require confirmation of specific targets (e.g., via low-throughput). Another is the data analysis requirements which carry higher complexity and expertise.

Low-throughput (LTP) omics studies, besides providing a lower scan, are biased, e.g. when studying target genes (by qPCR) there is a pre-selection of which genes to confirm, it is hypothesis driven and based on previous knowledge. Additionally, it is less robust than HTP studies, and variable results can be reported on similar gene-targets (e.g. as the related with metal homeostasis – metallothionein (MT), oxidative stress – catalase (CAT), and general stress responses – heat shock proteins) (e.g. [102–104,107]).

Low-throughput techniques (e.g., qPCR of a number of target genes, biochemical analysis of oxidative stress biomarkers, etc.) are quite useful as confirmatory tools at lower scale and specific targets, being quantitative and important to provide mechanistic confirmation, as long as not over-interpreted. Further, being simpler in terms of requirements they can be used broadly in many laboratories and create a larger data bank.

Ideally, a combination of low and high throughput should be used. Starting with HTP, e.g. analysing the entire (or a large portion) of the transcriptome/proteome/metabolome of a cell/organism, a snapshot of everything that is changing (hypotheses free) is obtained. This reveals several mechanisms (pathways) of toxicity which in turn require confirmation of selected points – this is where and when LTP techniques have a key role. This is in line with the AOP structure, for which the HTP omics techniques can contribute to inform on the most significant mechanisms involved in response to stressors, and LTP techniques can confirm the hypothesized molecular initiating events.

In vitro to in vivo extrapolation

In vitro tests provide key mechanistic input. In vitro testing from immortal cell lines has many advantages, given its maturity and larger reproducibility, but not without caveats (cells that do not keep the exact phenotype when immortality is established; epigenetic changes contributing to the establishment of cell immortality; cellular crisis – role of proportion of proliferative and apoptotic cells - before the arrival of immortality [231]). In vitro testing from primary cells, they do not live forever, undergo senescence processes and have limited potential for self-renewal and differentiation, but can be much more realistic and preferred to indicate in vivo impacts.

For invertebrates, although without established cell line cultures, one of the main advantages is the possibility to perform both in vivo and in vitro testing, given the existence of an acquired and optimized method (this is not the rule for most species and is not trivial) [154,232], but also the fact that the in vitro may be obtained from cells of exposed whole organisms [101,233,234] this represents a gold model for in vitro-in vivo extrapolation.

Last, because stress response mechanisms are among the most conserved across animals, including vertebrates and invertebrates, more invertebrate models should be developed to become in vitro models and focus should be given to this research field.

Considerations for risk assessment

Dose response paradigm. Several studies have reported that NMs can cause effects via non-monotonic responses, i.e., higher effects occur at low(er) than at higher doses. Proof of concept case studies exist for Ag [53,61], Ni [54], and Au NMs [209], as discussed in previous sections. This is a very important aspect to be considered, as the effects of high concentrations cannot predict for lower. Further, given the usually low PECs (Predicted Environmental Concentrations) for NMs this can represent a serious underestimation of risks based on the current dose-response paradigm for chemical substances.

The reason for these observations lay on the fact that, although NMs tend to agglomerate in media, there seems to exist an optimal concentration range where dispersion is maximum, and when this occurs toxicity can be maximal too, when it corresponds to maximal reactivity of particles and bioavailability [this should not be confused with *hormesis*, a positive biological response to a low stress level, a strategy observed in many organisms]. Nevertheless, there may also be biological explanations to the non-linear response.

Non-linear dose responses are not a novel phenomenon, e.g. markers of endocrine disruption are well-known to have a bell shape response pattern, where typically there is an increase of response by activation of molecular responses followed by a decrease after a

maximum level was reached. This is also the typical dose-response in gene expression, where linear dose-responses should not be the expected model, instead, a bell shaped curve response is the usual pattern aiming cell homeostasis. A detailed conceptual figure (Fig. 5), adapted from [24], shows how the cell gene expression dynamic status and apical effect dose response are linked: the cell always strives for a homeostasis state, and when exposed to a certain dose of a stressor, differential regulation of gene expression (GE) is observed. Because there is a maximum level of dose (stressor) after which toxic effects are higher than supported by the cell and an actual shut down of GE occurs, and eventually cell collapse. The apical effect (AE), e.g. at the phenotype level such as reproduction reduction, will occur after cellular homeostasis failure.

This brings a follow up point that maybe calls for some update from risk assessment to a risk understanding designation when integrating mechanistic data. As mentioned, AOPs represent a way forward to integrate of omics/systems toxicology in risk assessment.

Stereotype myths

Dose-response design. Alternative test methods that aim to understand mechanisms should not necessarily have a conventional single-time point concentration-response design. It needs to be understood that the study items to prioritise are mechanisms, and this needs to be performed within a sub-lethal concentration range, a stage at which there are still mechanisms functioning and not a cell collapse stage. Further, a time-course experimental design is very important, aiming to capture the window where maximal activation of events occur (in relation to exposure and in relation to life-stage), and perhaps also when these stop. All too often there is a so called realistic exposure based on a relative short single-exposure duration (and with too few replicates) informing us that no toxicity took place – this is not fruitful to understand possible mechanisms not predictive of longer term hazards. This point is also especially well-known from conventional long-term (persistent) pollutants.

Environmentally realistic concentrations. The importance of including "environmentally realistic concentrations" (ERC) in the test design is well understood. Nevertheless, it should be clarified, it does not mean that the design should not include effect concentrations, i.e. a full dose-response curve should be used. The critics regarding studies performed at concentrations higher than ERC, reminds an old discussion about the usage of NOECs, on how it is "Well past time to stop using NOELc and LOELs" [235] or how "Bad habits die hard: The NOEC's persistence reflects poorly on ecotoxicology" [236]. Test designs focussing on ERC alone may provide NOECs without ECx, often a result of missing the high effect concentrations. To build models there is a need to get a full scale, and not a fraction of it (censored data), which will make modelling impossible and hence intrapolation unreliable. The problem extends further and holds true for the risk assessor who then needs a dose response that covers the high end of the model, i.e. high dosage and effect, and thus reduced uncertainty. The problem is not to extract information from a full dose-response but to have a fraction of it (censored data). Last, let us not forget that also PECs (Predicted Environmental Concentrations) are predicted averages, that vary and may be revised higher a few years later (especially with emerging materials), though they are an excellent resource to the lack of actual measurements and impossibility to do. Finally, knowledge regarding accidental risks is also considered highly relevant, as accidents require immediate response to avoid devastating consequences.

A vision. In a vision for a prosperous and sustainable society, risk assessment will strongly benefit from more and more in depth understanding of observed effects, one that promotes decision making beyond black box basis data (e.g. dead or alive worm, 50% reduction in reproduction, after a fixed period of time). Alternative tests offer additional key information, for instance at many time points between the standard test start and test end: was the hatching success decreased, was growth affected, was reproduction decrease related to the delay in reaching maturity, did we observe effects in the next generation, is there a change in epigenetic markers? Further, was there gene mechanisms being activated and able to revert the initial events? And what about resilience of the system, is it increased if we have a multispecies test set-up? Or is impact higher in a multispecies environment? All this can aid to make decisions because it sheds light on population and ecosystems modelling, and on future propagation of events.

This is not a general scenario of data availability, yet a large string of information has been made available for key environmental species. It would be wise to use this as it is also to use the start key information from standard tests. It is simply possible to support the decision with much more detailed input, either knowing longer term multigenerational exposure effects or what mechanisms are underlying the observed response. The potential is for a reduced uncertainty (better precision) and more accurate prediction of what is happening (closer to truth), or at least more knowledge based decisions, in forecasting future impact of manmade materials and hence protect the environment and human health with increased accuracy. Such alternative testing will require novel advanced data-storage and modelling (including statistics) approaches, approaches which are not standardized (e.g., as are NOEC/LOEC or ECx approaches). This is inevitable, but there will have to be some kind of agreement on approaches and an emphasis on that risk regulators are able to handle and understand these approaches. It is very likely that automated processes will be further developed, e.g. through machine learning, which will help the risk assessor in standardization of how to deal with alternative data. Obviously, science communication is key and the educational aspect should be emphasized as novel technologies require expert knowledge.

To go further, and aiming regulatory preparedness, because such progress can indeed seem scattered in science, not designed fit-for-RA-purpose, there should be a call for specific development of the tools, coverage of species, target endpoints and implementation in a standard procedure. This is all attainable as long as the resources are allocated, with defined needs, and aligned designs, instead of sporadically obtaining random information.

For a successful implementation of NAMs it is important to have a rapid and continuous communication between the science and policy partner. This is already happening to some extent (see e.g. [5]), but there is a continuous education both ways, i.e. both for scientists to better understand regulatory constraints and for regulatory to understand the scientific background for the developed tests.

The RA paradigm can move forward adding the risk understanding (RU) extra factor to the equation (RA=RA+RU). This will also help industrial relevance very much, allowing innovation to occur in hands with more sustainable solutions towards a circular safety, envisaging a zero-pollution environment.

CRediT authorship contribution Statement

SILG: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing - original draft, review & editing. **MJBA:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, review & editing. **JJSF:** Conceptualization, Data curation, Funding acquisition, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nantod.2021.101242.

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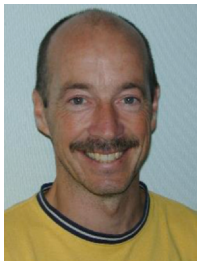

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