DISCUSSION

Environmental hazard testing of nanobiomaterials

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Abstract
The European Medicines Agency (EMA) regards the potential risks of human medicinal products to the environment and their impacts are assessed, as well as management to limit this impact. Hazard assessment of novel materials, which differ from conventional chemicals, e.g. nanobiomaterials, poses testing challenges and represents a work-in-progress with much focus on the optimization of required methodologies. For this work-in-progress, we here highlight where changes/updates are required in relation to the main elements for international testing based on OECD guidelines, supported by knowledge from the nanotoxicity area. The outline describes two major sections, nanobiomaterials and environmental hazards, including its challenges and learned lessons, with recommendations for implementation in OECD guidelines. Finally, the way forward via a testing strategy is described.

Keywords: Medical devices, Advanced therapy medicinal products, Nanomedicine, OECD standardization, Nanobiomaterials

Background
The European Medicines Agency (EMA) defines Nanotechnology “as the use of tiny structures—less than 1000 nm across—that are designed to have specific properties”; this includes mentioning structures and not only substances; the structures are designed on purpose and not just happened to be at nanoscale; the structures have specific properties not obtainable in isolation with the individual components of the nanostructure [1]. This is an example of how specific applications, i.e. medical, may require refined definitions for the same constituent materials. The accuracy of definitions is critical for regulation purposes (see, e.g. the recent guidance for nanoforms [2]) for which materials’ regulation depends upon. Regarding the potential environmental risks of human medicinal products, the EMA [3] outlines that “In accordance with Article 8(3) of Directive 2001/83/EC, as amended, the evaluation of the potential environmental risks posed by the use of medicinal products shall be submitted, their environmental impact shall be assessed and, on a case-by-case basis, specific arrangements to limit this impact shall be considered.” There is no legal definition for “nanomedicine” but like for nanomaterials, REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) [4] or CLP (Classification, Labelling and Packaging) regulations’ [5] provisions apply [6].

Nanobiomaterials (NBM) can present a wide array of shapes, forms and status, e.g. powders, fibres, scaffolds, emulsions and the nanoparticles can present different functionalization groups. Hazard assessment of NBM should cover all of materials’ life cycle stages, as also discussed for nanomaterials [7]. In the production stage, predominantly human health hazards are envisaged via occupational exposure, whereas when usage/application stage, exposure is mainly to the user (the target in this case). However, all materials may be released during production, and all have an end-of-life and ultimately all go to waste, reaching the environment. Depending on the kind of NBM, emission may be direct or via sewage, for example reaching the environment via wastewater treatment plants, playing a significant role. The diversity of the environment, including atmosphere, aquatic to terrestrial compartments, including a variety of target
species and physical chemical factors creates a complex challenge. The terrestrial compartment has been recognized not only as one of the major entry paths but also the sink for NMs [8], e.g. although 90% CNT (Carbon Nano-Tubes) production is accumulated in landfills it can be discharged depending on the technical design of the dump, 10% end up directly in soil [9] and less than 1% is found in sediments and air.

For the development of an intelligent testing strategy (ITS) and integrated risk management (IRM) framework, we need a battery of robust (standardized or validated) ecotoxicity tests. The tests should be fit-for-purpose with the ability to include the test of a variety of novel materials yet to be developed. The aim is to cover realistic worst-case scenarios in a broader sense, to reflect the need for environmental sustainable solutions, and setting increased environmental safety criteria for risk assessment.

We will here go through the general concerns when testing hazard of NBMs. International environmental hazard guidelines follow a general OECD outline and the main elements are selected and discussed (Table 1) (for detailed see Additional file 1):

We describe where changes are required and why, this supported by individual studies methods and results. The outline describes two major sections: (1) nanobiomaterials and (2) environmental hazards, including its challenges, learned lessons and recommendations.

**Novel materials: the case of nanobiomaterials (NBM)**

Since an official definition for nanobiomaterials does not exist, we for the purpose of this paper use this term to cover a wide variety of materials. Depending on their use they can be classified as Medical Device (MD) or Advanced Therapy Medicinal Products (ATMP), amongst others. This includes a range of applications, e.g. tissue regeneration, drug delivery, in vivo imaging/biosensing to coating of implants/wounds. Such materials can be provisionally allocated to 1st-generation (Bioinert), 2nd-generation (Bioactive) or 3rd-generation materials (Biomimetic/Bioresorbable/Stimulating specific cellular responses at molecular level) [10] (Additional file 2: Table S1). These cover particulates, fibres and larger scaffold materials; hence, this also covers the variety of forms that will be assessed for potential environmental risks.

**Challenges**

**Information on test substance**
The lack of proper characterization equipment and the high dynamic nature of NMs, i.e. transformation, remain the main challenges. Please see ahead “The need

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**Table 1 Information regarding the main aspects addressed in OECD guidelines, where “NO CHANGE”, “CHANGE” or “TO DEVELOP”, “TO STANDARDIZE” (partly developed but needs standardization) are recommended for the hazard assessment of NBMs**

| Subjects addressed in the OECD environmental in vivo hazard protocols | No change | Change | To develop | To standardize |
|---|---|---|---|---|
| Principle of the test | No change |  |  |  |
| Test acceptance/validity criteria | No change |  |  |  |
| Information on test substance |  | Change |  |  |
| The need for reference test substances |  |  | To develop |  |
| Reference substance—ORGANISMS |  |  | No change |  |
| Reference substance—MATERIALS |  |  | To develop |  |
| Description of the test |  |  |  | To standardize |
| The need for adaptations to test NBMs |  |  |  | To standardize |
| The need of instruments and techniques for environmental and biological media characterization |  |  |  | To standardize |
| Preparation of media |  |  | No change |  |
| Selection and preparation of test animals |  |  | (No)change |  |
| Preparation of test concentrations |  |  | Change |  |
| Mixing the test substance to the media—the need for spiking and dispersion consensus |  |  | Change |  |
| Performance of the tests |  |  |  | To standardize |
| The need of testable materials and worst-case scenarios |  |  |  | To standardize |
| Test groups and control |  |  | No change |  |
| Test conditions and feeding |  |  | No change |  |
| Test design—The need to cover whole material life cycle and The need of relevant test duration |  |  | Change |  |
| Data and reporting |  |  | Change |  |
| Treatment of results |  |  | Change |  |
of instruments and techniques for environmental and biological media characterization”.

The need for reference test substances
The need for representative NBMs for testing purposes is very important, also in the context of standardization and validation for regulatory purposes [11]. Depending on the nature of the materials (solid, particulate, fibrous, suspension, etc.) different testing methods are necessary. e.g. testing of nanomaterials requires adaptations to the standard testing methods as developed for chemical substances [12, 13]. Whether one reference material (or several) should be recommended to cover these differences is not yet clear. For NM the European Commission (JRC, Joint Research Center) created a repository for a list of representative NM [14–16]. What is currently key for environmental safety of NBM is to ensure that such a working list is representative for reference NBMs. This is currently addressed, e.g. in European Union projects such as the H2020: BIORIMA: BIoMaterial RIsk MAnagement. The work focuses on covering a wide variety of materials representing different forms, usages, and potential fate and effect scenarios. The development of references can clearly only progress within an iterative process between scientific evidence and exchange with all involved stakeholders [11]. Further, as well-known, factors such as shelf-life/-stability are important aspects for NMs when it comes to repository reference materials, i.e. less-stable materials will have to be tested within a short time after synthesis. There should be a correspondence between the shelf-life and when actual testing is performed, with a clear indication of the expected time for usage that is fit for purpose.

Description of the test

The need of instruments and techniques for environmental and biological media characterization
Characterization, especially in complex media like in solid environmental samples [17], e.g. soil or biological tissues, is not always possible due to difficulties and limitations in the analytical techniques available. This well-known issue hampers the interpretation of results considerably and needs attention.

Performance of the test

The need of testable materials and worst-case scenarios
Materials which cannot be homogeneously distributed in the test media to maximize exposure, e.g. scaffolds, are unsuitable for testing purpose when following the current paradigm for environmental standard hazard guidelines. One of the strategies and main challenge for such materials is the milling for hazard testing purpose. The milling or grinding is recommended to allow testing (e.g. testing impact on small organisms [18]) as this will represent a closer look at a worst-case scenario, i.e. when materials are released in the environment, these undergo ageing, weathering and fragmentation sometimes over many years [19, 20]. This is especially prominent for NBM, where scaffolds may be composite materials, e.g. polymers with embedded nanomaterials. Even it may seem straightforward to obtain a powdered version of, for example, a bone implant material, it has been shown not to be so because the powder obtained suffers from wide size distribution range or may not represent worst-case nanosize particles. Further, the milling process is often yielding too low amounts compared to testing requirements.

Test design
The need to cover whole material life cycle
Once the material has been fully characterized and the stability known, it must be ensured that all aspects of the materials’ full life cycle are covered [21] for the hazard assessment. The form, fate and exposure of each material can differ along the various life cycle stages and at the end of life. This may have a large impact in terms of effect assessment. For instance, a highly durable/persistent material (e.g. like many plastics [22]) may cause little to no toxicity when tested as synthesized/pristine material and using current hazard test systems. However, as the material degrades along the life cycle it may become more reactive, e.g. by releasing embedded compounds or by degradation to smaller particles that are easier taken up, bio-accumulated/-transferred. Such material information should ideally be provided from the materials’ producers and the functions and this in turn should be used to derive the best testing strategy.

Learned lessons and recommendations
Information on test substance
The test substance, i.e. test material in this case, should as a minimum contain the information equivalent to what is required in a regulatory context for nanomaterials (see ECHA Annex 6, 7a,b,c [23–25]), e.g. size, shape and surface chemistry. A better approach would be to follow the suggestion by OECD WPMN, see OECD ENV/JM/MONO(2016)2. Obviously only reporting minimum requirements will limit a refined hazard assessment and the possibility to use read-across information.

The need for reference test substances
To build a repository for representative NBMs, as done for NMs [14], is not trivial and there is a need for many representative materials, i.e. to include the variety of key characteristics. To select one/few representative materials will obviously not capture the potential test limitations. If a set of representative materials is available, then
it is possible to choose the NBM matching assessing needs.

An example of a successful repository for representative materials are the JRC (Joint Research Centre) representative NMs, i.e. a list of 33 NMs [14]. This has strongly supported test comparison between labs [26], and further supported the analysis of variations within one NM, e.g. TiO₂ NM (NM103, NM104, NM105) [27], without which the guideline update recommendations would not have been possible. The experience from pure nanomaterial science illustrates the problem of representativeness, when it comes to shape and form. For example, a single size or aspect ratio variation in the same particles could mean toxic to non-toxic variations [28]. So in terms of NM representative references, it was important to have both one NM and a suite of different sizes, e.g. custom-designed NMs’ libraries [29] and a range of different NMs [30]. Hence, the recommendation is to study a wider range of NBM, as opposed to one or few NBM, and inspect their potential impacts.

Description of the test

The need of instruments and techniques for environmental and biological media characterization: There is a need to quantify and characterize materials, discriminate between the confounding factors in complex matrices such as the environment or media, including biological tissues. Hence, good methods and instruments need to be developed and made widely available for the research and regulatory community. Although there has been significant progress [31], the techniques and related information from the methods and instruments’ developments need to be facilitated also to non-experts. At the same time, given the complexity of the characterization task, efforts and progress made on the measured biological effects (ecotoxicity) should be given higher recognition and weight to support regulation. In essence, if we were to wait with risk assessment until the in situ characterization is fully effective then innovation would be far away.

Performance of the tests

The need of testable materials and worst-case scenarios: Since the NBM come in various shapes, sizes and forms, there is a need to transform these to testable materials to meet the hazard testing requirements. If NBM are solid fibres, discs, bricks etc., we recommend to have these grinded/milled to the nanoscale for hazard testing purpose. We are aware that the approach of grinding the materials, aiming to test a worst-case scenario for the environment, may not capture effects based on the morphology (e.g. aspect ratio dimensions of fibres). The relationship between morphology behaviour and bioavailability has not been investigated and is an additional concern.

For materials with a structural dimension such as scaffolds, a homogeneous distribution in the media and maximization of the exposure can be achieved if the material is grinded to a powder (particles), suitable for spiking directly in solid media or disperse in aqueous media. However, the relevance of these results will be limited unless an ageing of such particles, to represent the full life cycle coverage including end of life.

State-of-the-art standardization hazard procedures foresees mixing of the test material with the test media. Done wrongly this may lead to high unknown variability in exposure concentrations amongst replicates (see “spiking and dispersion issue” below). Further, as pointed out some materials (or primary fragments from these) “may be the same size as or even larger than many important test organisms” this becoming the equivalent of “measuring the toxicity and uptake of rocks from mountains on cows” [18]. Given this, it is important that the grinding or milling is standardized and adequate (nanometer scale), within a small size distribution, i.e. as homogeneous size as possible amongst particles, and provided in sufficient amounts (up-scaling) for hazard testing procedures.

Test design

The need to cover whole material life cycle

It is very important to consider that many of these NBMs are persistent materials, made to last, and will only cause an impact after years of wear and tear or degradation. The biological systems have a limited test duration and even novel longer term testing, e.g. 60 days, will not necessarily capture effects of the material end of life [32]. Hence, test NBMs need to have representative samples from each of the various stages in the full life cycle, e.g. as synthesized, in use, end-of-life [19, 33]. For each step, the shelf-life (persistency/stability) of the sample should be accounted for before testing. To normalize such differences for materials’ toxicity ranking (see, e.g. [34]), an ITS should be implemented, namely testing in biological systems based on durability, e.g. (1) highly durable materials shall be tested after ageing and weathering, and (2) highly degradable/changing materials shall be tested as synthesized/pristine. Hence, for very persistent NBMs, we recommend to age and weather these prior exposures in biological systems. The right methodology to obtain such ageing is yet an additional challenge but work is ongoing in this area [21, 35, 36].

Environmental hazard assessment of NBM

Ecosystem services in a wide environmental diversity should be covered, including functions and species in the terrestrial (bellow and above ground), aquatic and waste water treatment plants. There is currently a range of guidelines that were developed for hazard assessment
of chemicals, which have been challenged for testing of other materials like nanomaterials (NMs). The present concerns NBMs and its specific characteristics, aiming to cover worst-case scenarios. We hereby summarize the available tools for hazard assessment in the key environmental compartments (soil, water, wastewater treatment plants) and NBM recommended.

**Soil: invertebrates**
There are a number of key species with standardized guidelines, which represent a selected start priority to assess novel chemicals and materials see, e.g. REACH. An overview of the most commonly used species in soil is given (Additional file 2: Table S2). Testing should cover as many species as possible, aiming to include representatives of the different life traits and soil functions provided for ecosystem services. Preferentially the number of species should allow for distribution-based approaches (SSD: Species Sensitivity Distribution), and although such an approach may at first seem more costly, it will ensure a much better coverage of major global concerns such as sustainability, including biodiversity, and generally have a benefit (more test results reduce uncertainty). Further, various endpoints should be covered, specially chronic and longer term [37, 38], and besides, bioaccumulation is also of concern for nano-enabled products; hence, trophic transfer is a further issue [39]. As recommended by the European Chemicals Bureau [40], the effect assessment for the terrestrial compartment should include data on (1) primary producers (plants), (2) consumers (e.g. invertebrates that represent an important group in the soil compartment), and (3) decomposers (comprising microorganisms). In this way, spectra should cover various species from different taxonomic groups to ensure a much better coverage of different sensitivity of ecological indicators, e.g. also representing fungivores, herbivores and predators amongst consumers. A well-known highly relevant aspect is species interactions; this can be assessed using, e.g. multispecies testing systems assembled in the laboratory [41, 42] or in higher tier as mesocosms’ type [43]. There are several standard guidelines (OECD, ISO) available for soil (Additional file 2: Table S2).

**Wastewater treatment plants: microorganisms, including soil microorganisms**
Microorganisms have a key role in terms of degradation and turn-over of many substances and materials. These organisms act as a fully integrated community and the measurement of the impact is on the biological function/quality (as opposed to effect of individual species).

Waste water treatment plants (WWTPs) are suitable to simulate complex environmental processes in a laboratory scale applying standardized conditions. Originally developed to test the degradability of organic substances, the results of WWTP test show that they can be used to assess the ecotoxicological impact of materials with low or partial degradability. With this WWTP tests, the environmental fate and effect of materials released via sewage can be assessed. Sewage sludge and effluents containing degraded materials can be used in terrestrial and aquatic ecotoxicological tests providing additional information. In contrast to the test design for the approaches with aquatic organisms and soil fauna using single species, populations are used for the assessment of impacts on microorganisms in WWTPS. An overview of selected recommended test guidelines in the scope of registration/notification of chemicals is given (Additional file 2: Table S3). There are many standardized methods [44], an ISO overview is presented by Philippot et al. [45] and an updated version is presented (Additional file 2: Table S4). The methods and results are used as indicators of soil functions [46]. Some approaches and guidelines address one specific microbial function (e.g. ISO 23753-1 (2019); ISO 17155 (2012)) whilst others are designed to address the functional diversity by measuring several functions in one approach (e.g. ISO 20130 (2018); ISO 22939 (2010)). In addition, microbial structural diversity is determined, e.g. common approaches are the analysis of extracted phospholipid fatty acids (PLFA) [47] or of extracted DNA and RNA [48].

**Water: algae, invertebrates and vertebrates**
Representative species of three different taxa: algae, invertebrates and vertebrates (fish), should be considered. Further, toxicity varies between species and with water conditions. Hence, the selection of the species should be representative of different scenarios, including freshwater and marine, differences in pH, temperature. There is a number of key species with standardized guidelines to assess hazard in the aquatic compartment. An overview of the most commonly used is given (Additional file 2: Table S5). Although the NBMs are generally not pelagic, there should be a focus on identifying which species and which exposure duration are relevant for particular exposure scenarios.

**Challenges**

The need for adaptations to test NBM
The current testing performance paradigm was developed for chemical substances testing, and hence it is not sufficiently adequate for many novel materials, such as NBM. This has been shown for NMs [49] which contain relevant properties needing specific design. For NMs, size is known to be a key distinct feature. Although the hazard assessment and characterization lag far behind
the materials’ technological developments, there are currently available recommendations [26] to reduce the limitations and uncertainty of results obtained with NMs. Recommendations for NBMs should be in line with acquired knowledge from common aspects of NMs.

**Description of the test**

**The need for spiking and dispersion consensus**

One of the main challenges relates to well-known and well-characterized exposure during ecotoxicological testing, i.e. the lack of homogeneous dispersions of NBM. Depending on the material’s and the environmental media properties, at least two kinds of spiking are recommended, as liquid dispersion and as dry materials. Obviously, both may be used depending on the characteristics that best represent realistic scenarios.

**Performance of the test**

**Test design and the need of relevant test durations**

It is clear that the standardized test durations and test setup are not adequate for elucidating the importance of many aspects of new materials, e.g. the importance of durability and shapes for the longer term biological effects [50]. Hence, test designs should be adapted to mimic most relevant and up-to-date scenarios, e.g. the test duration should relate to the likely exposure time. From a biological point of view the test design cannot be a one size fits all, but must depend on the test species and endpoint of concern [51].

**Learned lessons and recommendations**

**The need for adaptations to test NBMs**

Adaptations of the current guidelines to testing NBMs include all referred components to be either integrated or subject to new guidelines/guidance documents as currently done to NMs, please see Table 1 for a list of recommended adaptations. The most important aspects are detailed below.

**Description of the test**

**The need for spiking and dispersion consensus**

Hazard testing procedures require that the best method is implemented towards a maximum exposure of the biota to the test material. Given the difficulty of mixing solids homogenously into many environments, spiking should be done replicate by replicate. This procedure ensures a known amount of test material in each replicate, which is lost sometimes when first mixed as a stock solution into a batch medium and subsequently split onto replicates. Although the homogeneity of the mixture of the material in the test media cannot be ensured for all materials and media, this technical update improves the reproducibility of amount of material per replicate and hence exposure. Further, the use of electronic microscopy with EDS analyses can help to determine the homogeneity of distribution in various media. The importance of concentration variation within each replicate depends on how much (in time and space) the test organism penetrates the full media core. This methodology can be used for systems where initial disturbance (mixing) is not a problem. There are also test media-specific issues and recommendations.

**Soil media** For terrestrial test systems focusing on invertebrates and plants, non-dispersed (dry) NBMs should be mixed as dry constituents prior to soil moistening and before adding the biota [13]. If the test material is a liquid dispersion, it is recommended to add this directly onto pre-moistened soil; further, a control for the liquid without the NBMs should be run.

**WWTP media** For WWTP testing of NBMs materials for which stable stock suspensions can be prepared and which do not sorb significantly to the tubes, stock dispersions should be used. Every WWTP requires a separate vessel with stock dispersions to avoid uneven distribution across replicates. The synthetic sewage, tap water and material’s stock dispersion can be mixed within a tube system (concentrated synthetic sewage is mixed with water and then with the test material). In the case a WWTP with denitrification and nitrification chambers being simulated, the mixture is applied into the denitrification section. If a WWTP with just a nitrification chamber is investigated, the mixture is applied into the nitrification section. For materials for which no stable stock suspensions can be prepared, which significantly sorb to the tubes or have other issues, direct addition to each replicate is recommended. The test material can be added directly into the media without making a stock suspension, e.g. denitrification (two-chamber WWTP) or nitrification (one-chamber WWTP) chamber in small amounts at several times per working day (e.g. five times). At the weekend the frequency can be reduced.

For instance, for materials which are primarily released into wastewater and sorb to sewage sludge in wastewater treatment plants the materials can in a test be added to soil as a mixture with sewage sludge, simulating realistic environmental conditions and pathways. Using model wastewater treatment plants for spiking of sewage sludge (OECD Guideline 303A, 2001) considers additionally potential transformations of the material. To measure the impact on terrestrial microorganisms, the treated soil and a control soil are incubated over several weeks and with the microbial activity or the composition of the microbial population is determined at predetermined time-points.
**Aquatic media** For aquatic testing [12], dispersions should be directly mixed in the aqueous exposure media. Powders can be dispersed following the NANOGENOTOX protocol [52]. When the previous options are not viable or food exposure is required, administration via food is recommended [53]. An approach for food spiking may be to use water and olive oil as a possible vehicle, although other solvents may be more relevant for various NBMs [54].

**Performance of the test**

**Test design and the need of relevant test durations**

Concerning the test design and its duration, the two main considerations regard the test organism and the material. The test duration should by default relate to the test species life cycle duration and associated endpoint(s). Nevertheless, most of the standard tests covers only a fraction of the species life cycle, at most only one generation, and favour shorter life cycle species.

We here outline recommendations on adaptations to standard tests illustrating where extension of test duration and optimization of endpoints are possible, circumventing many of the present hurdles of novel materials (see summary in Table 2).

For *E. crypticus* recommendations are to detail and extend the exposure period as much as practical possible. Regarding detailing, additional detail is possible if using an FLC test instead of an ERT, meaning that a number of endpoints and information can be obtained.

A full life cycle test (46 days, see [57, 61, 62]) represents an added value compared to the standard (28 days). Amongst others, it provides the power to discriminate mechanisms between metal salts and the related metal nanomaterials, and gives an understanding of the sensitive life stages’ toxicity. Regarding the extension of the exposure period, e.g. we have beneficial results with monitoring along and up to 84 days instead of 28 days as in the standard guideline. Our results showed that for *E. crypticus* counting (i.e. through additional sampling) the total number of organisms (hence estimating survival and reproduction) at days 0–7–14–21–28–60–84 allows to observe amplified effects of WCCo NMs at days 60 and 84, not identified at day 28 [56]. Further, the additional counting at days 7, 14 and 21 provides detection of effects at earlier life stages such as delayed embryogenesis and time to reproduction. Finally, a multigenerational test is optimized [32, 38] and recommended [32, 38], especially to assess the potential epigenetic [37].

For *F. candida* a multigenerational design is also recommended. Performing a test with an extended exposure period, the density in the test vessels may increase to unpractical numbers, i.e. too high density which would compromise the test viability. Hence, we recommend to perform an extension of the exposure time to 84 days (corresponding to three generations) by restarting of exposure of juveniles after every 28 days, one reproductive cycle [58, 59]. In addition, the addition of size as an endpoint is recommended to also be included in the test evaluation.

For *E. fetida*, which has a longer reproductive life cycle (56 days), the recommendation is to favour an added detail in one life cycle, this is instead of the time extension. For example, a full life cycle can be performed where organisms are exposed from the cocoons stage throughout the whole life cycle. This will still require a substantial amount of time and resources for output compared to other smaller species. On the other hand, being a larger species a clear advantage is that it is possible to assess tissue distribution (quantitative) and perform biochemical analysis and further molecular and omics, where the mass of one single worm is enough to perform all analysis. This opens a window for a highly detailed understanding of the mechanism of toxicity, a key feature sought for in novel risks assessment (see also New Approach Methodologies (NAM): Beyond standard testing).

According to OECD TG 216 and 217 (test guidelines on microbial N-transformation and C-transformation) for non-agrochemicals, the incubation period is 28 days. If agrochemicals are tested and the test parameters in treated and control samples differ by more than 25% on day 28, the test is continued until a difference equal to or less than 25% is obtained. The maximum test duration is 100 days. For the effect assessment of NBMs, we propose a general test duration of 100 days, the maximum test duration possible according to the test guidelines. Sampling dates after incubation periods of 7 days, 28 days, 56 days and 84–100 days (corresponding to one, four, eight and 12–14 weeks) are recommended. By the prolonged test duration, delayed bioavailability is considered [63].

For *Daphnia magna* [64], the standard reproduction test has a duration of 21 days. Multigenerational studies provide a more realistic exposure scenario and offer the opportunity to identify transgenerational effects, not covered by the standard and that may cause significant impact on the population dynamic [65, 66]. A multigenerational assay includes three generations (F0, F1, F2), each generation is exposed for a period of 21 days, monitoring survival, time of the first brood, and newly born offspring. All the generations may be maintained until the end of their life span (42 days), to test potential differences on the longevity in parental organisms, i.e. a total of 70 days (14 days’ sexual maturity F0, 14 days’ sexual maturity F1, 42 days’ life span of F2). The OECD TG 305 on bioaccumulation in fish
Table 2 Summary of selected standard tests where highlight is given to potential adaptations FOR THE TESTING OF MANUFACTURED NANOMATERIALS (including nanobiomaterials) of existing OECD/ISO guidelines FOR THE TESTING OF CHEMICALS regarding test duration and endpoints

| Test guideline                | Test endpoints                                      | Test duration (days) |
|------------------------------|-----------------------------------------------------|----------------------|
| **Enchytraeus crypticus**    | Survival and Reproduction                           | 21–28                |
| Standard (OECD/ISO): OECD 220; ISO 16386; OECD 317 Alternative 1: A1 Full Life Cycle test (adapted from standard [55]) | Kinetics, uptake, elimination, BAFs Hatching Growth Maturation Survival Reproduction | 14 + 14 11 25 46 46 |
| Alternative 2: A2 Extended test (adapted from standard [56]) | Survival, Reproduction, Population growth | 7, 14, 21, 28 60, 72, 84 |
| Alternative 3: A3 Multigenerational test (adapted from standard [37, 57]) F1–F4 spiked soil + F5–F7 clean soil | Survival and reproduction; epigenetics | 32, 64, 96, 128, 160, 192, 224 |
| Recommendation: Use A1, A2, A3 Implementation: Add A1, A2 and A3 in OECD/ISO as annexes. | | 46–84 |
| **Folsomia candida**         | Survival and Reproduction; 28                       |                      |
| Standard (OECD/ISO): OECD 232; ISO 11267; Alternative 1: B1 Multigenerational test (adapted from standard [58, 59]) F0, F1, F2: Survival, Reproduction, Size (adults, juveniles) | | 28, 56, 84 |
| Recommendation: Use B1 Implementation: Add B1 in OECD/ISO as annex | | All 28–84 |
| **Eisenia fetida**           | Survival                                               | 28                   |
| Standard (OECD/ISO): OECD 222 OECD 317 Alternative 1: C1 Full Life Cycle (adapted from standard) Alternative 2: C2 Tissue distribution and biochemical analysis | Reproduction Kinetics, uptake, elimination, BAFs Hatching Growth Maturation Survival Reproduction Tissue distribution biomarkers, omics | 56 21 + 21 21–28 14, 28, 42, 56 14, 28, 42, 56 56 3–7–14 (flexible) |
| Recommendation: Use C1 and C2 Implementation: Add C1 in OECD/ISO as annex Make use of C2 and proceed towards standardization Use A1–A3 as surrogate (oligochaete with shorter life cycle) | | All 28–84 |
| Alternative 3: C3 Earthworm in vitro test [60] | Cell viability—biomarkers, omics, corona formation | 1 |
| Recommendation: Use C3 for rapid mode-of-action understanding Implementation: Further develop this system to a higher TRL level | | All 1 |
| **Microorganisms**           | N-Transformation                                      | 28–100               |
| Standard (OECD/ISO): OECD 216/ISO 14238 Alternative 1: D1 Fixed prolonged test duration | | 28–100 |
| Standard (OECD/ISO): OECD 217/ISO 14239 | All microbial activities | 84–100 |
includes two exposure alternatives, aqueous media for soluble substances or dietary for non-soluble substances. For NBMs, a dietary exposure will be the most appropriate, meaning an uptake of 7–14 days and post-exposure (depuration) for up to 28 or 42 days, i.e. until the test substance can no longer be quantified in whole fish (OECD 2012b). According to previous experiments with ZnO NMs [67] depuration was not fully achieved in intestine and gills, hence larger periods of depuration should be considered and are expected needed for certain NBMs.

As detailed above there are many lessons learned from research in ecotoxicity of NMs, especially that more long-term information regarding hazard should be provided. As described, such information can be achieved in various ways, the most obvious options are by extending the test duration and by testing aged/weathered materials [21], whilst obtaining more detailed information, e.g. as additional endpoints.

**Way forward: a testing strategy**

**Mode of action: Standard testing in a strategic tiered approach**

The Hazard Assessment (HA) should comprise identification of the biological target (e.g. estimated specific endpoints or species affected) and the identification of the magnitude of the related biological response (e.g. estimated via a concentration–response analysis). If a biological mechanism is known for the NBM, then this should be considered in the choice of test systems, for example some materials are designed to be anti-microbial and this should prioritize microbial testing and the closest network associate in the trophic chain, e.g. microbial-dependent organisms. If specific concern exists based on NBMs’ transformation, e.g. partial transformation at anaerobic/aerobic conditions then the duration of the transformation should guide how long test durations are chosen. If a route to the environment is through WWTP and sorption of NBM to sewage sludge is expected, then

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**Table 2 (continued)**

| Test guideline | Test endpoints | Test duration (days) |
|----------------|---------------|----------------------|
| Alternative 2: D2 | Potential ammonium oxidation | 84–100 |
| ISO 15685 | All | 84–100 |
| Recommendation: Use D1 and D2 | | |
| Implementation: Add D1 in OECD/ISO as annex for particular materials | | |
| Add D2 in guidance for the testing of toxic ion releasing particular materials | | |
| Standard (OECD/ISO): OECD 303A | C-Transformation, N-Transformation | No fixed duration (plateau phase of min. 21 days) |
| Alternative 1: E1 | C-Transformation, N-Transformation; sewage sludge for soil spiking | Transformation: no fixed duration |
| Addition of denitrification chamber | Spiking: 10 days (mean sludge age in a wastewater treatment plant) | |
| Recommendation: Use E1 | C-Transformation, N-Transformation; sewage sludge for soil spiking | Transformation: no fixed duration |
| Implementation: Add E1 in OECD as annex for particular material | Spiking: 10 days (mean sludge age in a wastewater treatment plant) | |

**Daphnia magna**

| Test guideline | Test endpoints | Test duration (days) |
|----------------|---------------|----------------------|
| Standard (OECD/ISO): OECD TG211 | Survival Reproduction Growth (length) | 21 (daily monitoring) |
| ISO 10706 | | |
| Alternative 1: F1 | Survival Reproduction Growth (length) | 21, 42, 70 |
| Multigenerational (F0, F1, F2) | | |
| Recommendation: Use F1 | All | 70 |
| Implementation: Add F1 as annex to OECD TG211 | | |

**Fish**

| Test guideline | Test endpoints | Test duration (days) |
|----------------|---------------|----------------------|
| Standard (OECD/ISO): OECD TG305 | Bioaccumulation | Dietary: (10–14) + 28 or 42 |
| Alternative 1: G1 | Bioaccumulation | Dietary: (10–14) + 52 or more |
| Recommendation: Use G1 | All | depuration period of 52 or more days |
| Implementation: Add G1 as annex to OECD TG305 | | |

* Instrument and method that allows quantification of the specific material are required
sewage sludge tests are recommended. In various EU countries the sewage sludge is used as fertilizer and NBM can be released into the terrestrial compartment via this route. The simulation of WWTPs and the testing of sewage sludge added to soil are suitable as a higher tier approach in case of a specific concern. This is of course especially important to consider for NBM used in MD and ATMPs since these often have prior known target and function.

Despite the intelligent choice of testing based on materials’ characteristics and intended purpose, this is not standalone, because many human health-targeted materials (designed for a specific mode of action) can cause collateral or unintended environmental damage.

**Long-term exposure testing—adapting standard testing**

As outlined above, test adaptations in regard to exposure duration for NBMs are still ongoing. However, it is clearly shown that by prolonging the test duration and detailing the sampling points, it is possible to identify effects not caught in the standard testing and also to identify especially sensitive life stages. Hence, an HA that takes into account long-term effects and added detailed endpoint series offers many advantages and is preferred for Risk Assessment (RA). In case of materials with potential long-term effects due to the resulting forms after ageing, long-term tests could better tackle hazards and avoid future claim for damages, this representing a clear advantage for the producer to go beyond the regulatory requirements, saving costs at a longer term perspective, but most importantly to increase safety for the environment.

**New approach methodologies (NAM): beyond standard testing**

Hazard identification evaluates the ecotoxicological data from a range of relevant species to assess the intrinsic hazard of a substance. Current ecotoxicological approaches to assess hazards of NBMs can either be based on methods adopted from classical ecotoxicology (OECD/ISO guidelines) or New Approach Methodologies (NAM) [68] including, e.g. mechanistic endpoints [69]. New Approach Methodologies usually refer to alternative methods, such as in silico and in vitro methods and may also include modelling, read-across and system biological outputs. These approaches can provide fundamental molecular–mechanistic understanding of the related toxicity, an aspect not caught in the classical regulatory ecotoxicology. This should obviously also be pursued for NBMs, with the additional benefit here that such approaches may also help to make the NBMs more effective in regard to their use, besides making them less of an environmental hazard. Assessments using NAM can be used both in relation to ECHA (European Chemicals Agency) and to TSCA (The Toxic Substance Control Act, US). For the environment, there are alternative models and endpoints, beyond OECD and ISO, which cover many relevant aspects and that fill in gaps of standard testing, especially for novel materials like NBM, and also that support the integration of omics as alternative tools for risk assessment [70]. Amorim et al. [69] show an overview of available tests in the terrestrial compartment and levels of detail that can be obtained (Additional file 2: Table S6).

Depending on the goal the Hazard Assessment strategy could proceed as a tiered approach (for a proposal see Fig. 1) moving from screening at the phenotypic organism level: (1) individual species chronic tests, to (2) individual species extended chronic tests; individual species multigenerational test, and add (3) multispecies extended test. The choice of test level will depend on the realistic worst-case scenario based on detailed evaluation of material properties. For an understanding of the underlying mechanisms of action screen at the non-phenotypical sub-organism level: (1) individual species cell viability assessment and multi-endpoint (in vitro short-term exposure), (2) multispecies in vitro tests (high-throughput multi-endpoint) and (3) omics. One
example of such full coverage for the standard test species *Enchytraeus crypticus* has been performed for a Cu NMs’ case study, showing the striking understanding obtained from combining in vitro testing [60], all omics (transcriptomics [71], metabolomics [72], proteomics [73]), epigenetics [37], full life cycle [57], full life span [50], multigenerational [38] and multispecies test system [41, 42]. Hazard understanding will not only fulfil the scientific community requirement for a better understanding of the world, but it will also serve as the basis and rational for regulators to decide upon the need for modifications of the current procedures for the testing of materials’ hazards.

Many of the described gaps in relation to NBMs come from the infancy of the NBMs’ research field compared to conventional chemicals and even to pure NMs. On the other hand, we are far ahead in terms of information access, knowledge and technology than the previous pioneer fellows [74], hence hold far larger responsibility to ensure not only the sustainability for future generations but also to make it a better world.

**Supplementary information**

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s12302-020-00369-8.

**Additional file 1.** Additional information regarding the subjects addressed in the OECD environmental in-vivo hazard protocols.

**Additional file 2: Table S1.** Representative bionanomaterials list types. **Table S2.** Overview of key terrestrial species and standard guidelines. **Table S3.** Overview of key microbial activity standard guidelines. **Table S4.** ISO Standard methods available in soil microbiology. **Table S5.** Overview of key aquatic species and standard guidelines. **Table S6.** Overview of available tests for effect assessment for soil invertebrates.

**Abbreviations**

ATMP: Advanced therapy medicinal products; BIORIMA: BIOMaterial Risk MAngement; CLP: Classification, labelling and packaging; ECHA: European Chemicals Agency; EMA: European Medicines Agency; EMEA: European Medicines Evaluation Agency; HA: Hazard assessment; IRM: Integrated risk management; ITS: Intelligent testing strategy; ISO: International standard organization; JRC: Joint Research Center; NAMA: New approach methodologies; NM: Nanomaterial; OECD: Organization for Economic Cooperation and Development; REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals; TG: Technical guidance; TSCA: The Toxic Substance Control Act; WWTPs: Waste water treatment plants.

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**Authors’ contributions**

MJBA drafted the first version of the manuscript, KHR, MLF, and JSF were responsible for specific sections. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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