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	EXECUTIVE SUMMARY D 1.4
DELIVERABLE TITLE	**SOP on (alternative) data completeness for regulation.**
RESPONSIBLE AUTHOR	**Mónica Amorim (UAVR)**

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This document outlines aspects to be considered for the criteria and strategy for alternative test data inclusion (omics, modelling, read/across, grouping).

Category formation, grouping and read across methods are broadly applicable in toxicological assessments and may also be used to fill data gaps for safety assessment and regulatory decisions on NMs. There are two major aspects of any read-across application, namely assessing similarity and uncertainty. The use of alternative test methods such as subcellular functional assays can improve the interpretation beyond and deserve further exploration. Some examples are implemented and used (e.g. US-EPA) for risk assessment, although much less than conventional methods. We revised and summarised the state of the art and provide recommendations for alternative test data inclusion towards regulation. This included two major aspects of the use of alternative data for the environment:

1. current state of the art regarding alternative test data, by reviewing literature and scoping the main aspects, including 2 main alternative data: A - omics, molecular (UAVR); B - read across, grouping, modelling (UFZ, Fraunhofer).

2. Approaches and recommendations for alternative test data inclusion towards regulation. A list of criteria for alternative (modelling, read/across, grouping) data and pilot data sets for verification of tools elaborated by WP2 and WP3.

ALTERNATIVE TEST DATA

NAMs: Omics, Molecular

Novel materials challenge the adequateness and fit-for-purpose of OECD (Organisation for Economic Co-operation and Development) standards, as these were developed to assess hazards of “conventional” chemical substances and not advanced materials (e.g. materials that may deliberately change behaviour). 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).

Tests can be 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 overall governance of NMs, is being widely discussed and considered under NANORIGO. 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 NAMs to establish “Alternative Tests” both in EU, USA, Canada and Australia, Japan, South Korea.

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.

Results from the review on soil invertebrates (Fig.1) show a large % of data comes from alternative tests, here including a selection of gene/cell level endpoints, in vitro testing.

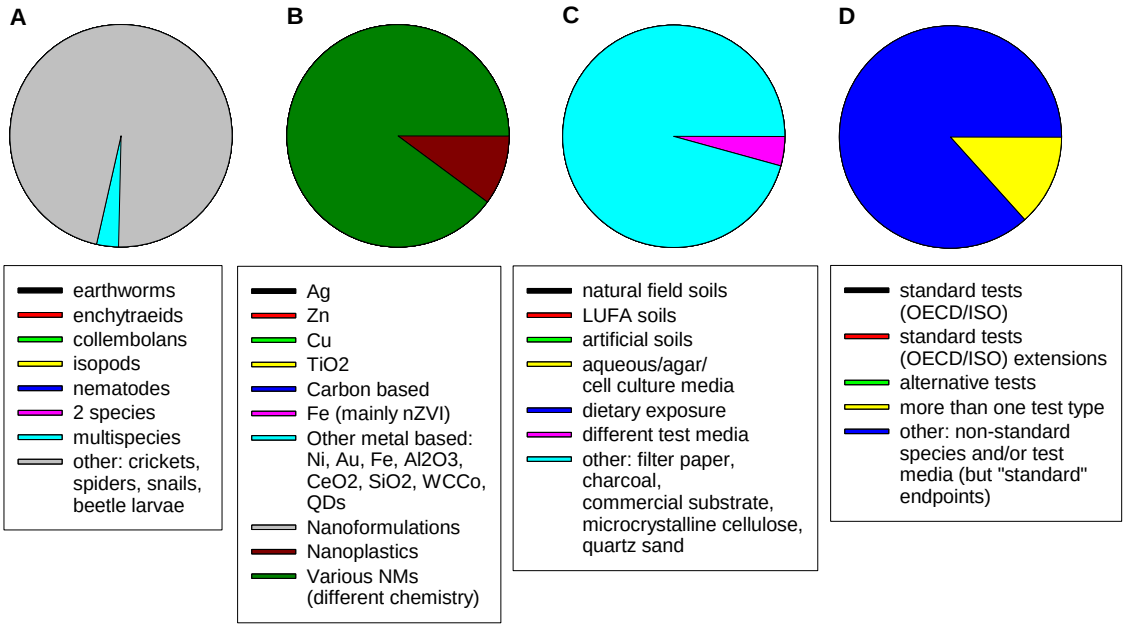

Figure 1. Distribution of the 188 papers analysed for the revision, by A) group of organism used as test species (single species test, otherwise considered in the sections “2 species” and “multispecies”), B) chemical identity of the tested NMs (single chemical tested, otherwise is considered in the section “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. From https://doi.org/10.1016/j.nantod.2021.101242

The use of standard tests alone, e.g. where endpoints like survival and reproduction are assessed, do not inform about the in-between period and sources of the outcomes. It is a typical black box concept (Figure 2), we don’t know when, how or why it happens.

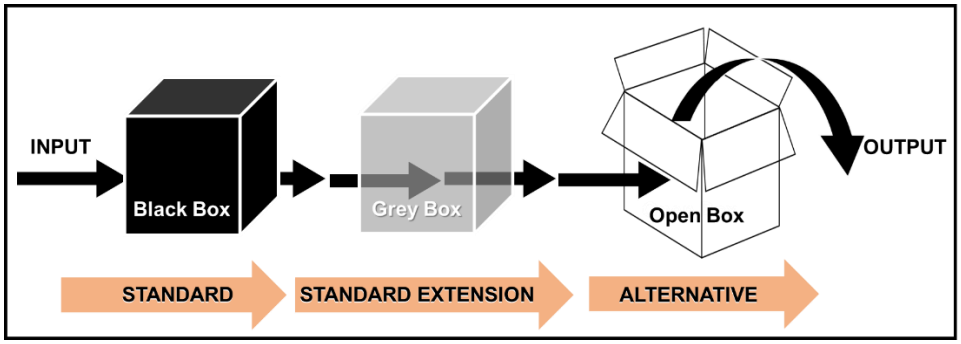

Figure 2: Schematic representation of the concept analogy for testing and boxes: standard test – black box, standard extension test – grey (see through) box and alternative test – open box.

Based on the standard OECD/ISO guidelines, there has been considerable development for a) additional endpoints, b) additional time points, and c) extended exposure period.

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.

READ ACROSS, GROUPING, MODELLING:

Engineered nanomaterials (ENMs) show great variation in size, shape, crystalline structure and surface modifications. According to the European Chemicals Agency (ECHA), grouping and read-across approaches can be applied to reduce the number of tests required for the risk assessment of ENMs (ECHA 2017). Physico-chemical (PC) properties are listed which can be suitable to identify similarities. They are differentiated in three categories: “What they are“ (chemical and physical identity), “Where they go“ (fundamental behaviour), “What they do“ (reactivity). A grouping hypothesis should be built on “What they are” and “Where they go”. Based on the endpoint under consideration the relevant ones have to be selected. Boundaries have to be specifically defined based on specific PC-properties.

Based on the criteria listed by ECHA, ECETOC developed a tool, to aid in identifying sets of similar nanoforms (https://www.ecetoc.org/tools/nanoapp/). Grouping regarding ecotoxicity is still in its infants compared to human toxicity (Schwirn and Völker 2019). Only in one approach besides human toxicity also the environment is addressed (Wohlleben et al. 2019). Currently it is not systematically assessed whether a grouping based on the selected criteria of the ECHA-list is working for grouping with regard to ecotoxicity. However, this information is required to justify read-across for specific nanomaterials. This is where nanoRIGO D1.4 steps in.

A selection of the PC-criteria listed by ECHA were applied to several which have been extensively characterised and tested with regard to their ecotoxicity. Based on the available information potential groups were defined. For instance, for TiO_2, crystalline structure, surface

functionalization and reactivity are considered as main properties defining different groups. The grouping is considered reliable if it corresponds to the ecotoxicity. Therefore, in the next step the materials are grouped using the EC50 values. For example, when studying data for algae and daphnia, grouping of materials do not group similarly in terms of ecotoxicity, few exceptions, e.g. ZnO. Further development of grouping regarding aquatic ecotoxicity suggest that some properties can improve the match, e.g. as identified empirically or by computational chemistry methods such as (Q)SARs ((Quantitative) Structure-Activity Relationships). Important properties include molecular polarizability, accessible surface area and metal-ion leaching.

It was obvious that some of the frequently discussed parameters are less important than others. An attempt to connect these findings was made in the so called SEG4nano tool. Although the tool is still in refinement, some basic conclusions can be drawn.

1. Approaches and recommendations for alternative test data inclusion towards regulation.

Available Data: Main priorities concern a coverage of sizes, shapes, surface coatings and many other combinations in hazard assessment, data fit for purpose - regulatory purpose, facilitating alternative tests data, e.g., high-throughput omics data analysis, e.g. improving the accessibility of software.

Open Data and transparency: 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. Despite the last decade progress towards OD there are still many challenges to overcome: 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).

Test designs: While there are many advantages of implementing a variety of test designs, the lack of supporting comparable designs will limit the possibility for read-across. The solution goes through harmonization of e.g. descriptors, endpoints, test duration, etc., as increasingly recommended for standardization. Adopt the alternative tests where standardization level/maturity is high.

Test materials: **Read-across and the periodic table:** Except from small NMs (i.e. in the very low nm range), most NMs behave as can be derived from the periodic table information, e.g. possible oxidation or chemical structure. This is also why atomistic modelling of nanomaterials is of interest. And it is obviously also important for read-across and grouping.

Referential type NMs, designed for benchmark: one of the main obstacles to derive general conclusion on the toxicity of NMs, is the large variety of NMs in hand with the lack of thorough

characterisation. The European Commission's Joint Research Centre (JRC) repository of Representative Test Materials: can provide “the same NM” to different laboratories 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.

Libraries of NMs, designed for modelling: group of NMs of related but different chemical composition or 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.

Specific NMs, designed for functionality: Material specific properties should be considered in a case by case, to meet worse case scenarios.

Test level (standard, standard extension, alternative): Standard tests should be performed and act as a benchmark for validation. Inclusion of standard extensions should be facilitated via addition to current guidelines as annexes. Alternative tests should be integrated, e.g. towards the Adverse Outcome Pathways (AOPs) concept. 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.

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.

Dose-response design: Alternative test methods that aim to understand mechanisms should be performed within a sub-lethal concentration range and a time-course.

Read across: less properties than those listed by ECHA (2019) are required for identification of similarity as basis for read-across regarding ecotoxicological endpoints, e.g.:

Algae: attachment is a relevant property. It can be used as surrogate for the unspecific multiple properties listed by ECHA and directly linked toxicity.

Daphnids: additional criteria solubility and reactivity for the alignment of groups formed according to EC50 values is material dependent.

Metal and metal oxides: Expansion to additional groups of ENMs needed.

In test media: characterization needed.

ECx needed: No effect (NOEC) data is inconclusive

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