SCIENCE-BASED RISK GOVERNANCE
OF NANO-TECHNOLOGY

RISK GONE

Documented protocols, data capture, metadata templates for revised OECD tests, & pe-validated alternative test methods

DELIVERABLE 6.3

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Abstract

The aim of Task 6.1 was to assess selected OECD ecotoxicology test guidelines and other well-developed test methods for their applicability to environmental hazard assessment of engineered nanomaterials (ENMs) and identify if any adaptations were necessary to reduce uncertainty in human risk assessment approaches. A key aspect of this activity was the development of refined data capture templates to support the harmonisation of the data collected and its streamlined analysis. Deliverable D6.1 reported on the updated experimental procedures for the three main assays that were worked on in WP6, namely the daphnia reproduction assay (OECD 211), the Comet assay for genotoxicity assessment applied to fish cell lines from rainbow trout gills and from zebrafish embryonic cells, and impedance assays for assessment of cytotoxicity and oxidative stress in high throughput using fish cells and daphnids (in development – see also Deliverable report D6.2).

The present deliverable, D6.3, describes the work undertaken to update the templates for capture of background data on lab facilities and procedures (e.g., running cultures and day-to-day and seasonable variability in assay performance as baseline quality control and quality assurance data), and from the assays themselves. These amended or newly generated data capture templates have been implemented also into the RiskGONE Template Wizard tool by Idea Consult as part of the overall RiskGONE data management activities and our efforts to ensure that all RiskGONE data is presented in accordance with the FAIR (Findable, Accessible, Interoperable and Re-usable) principles.

We present also the Instance Map approach, developed and extended in NanoCommons as a means to visualise the various assay steps and thus to map out the full set of data and metadata that needs to be captured to support re-use of the data by others in the future.

The resulting data capture templates and visualisations of the datasets via the InstanceMaps will form part of the amended Test Guidelines (TGs) proposals that will be prepared for the test methods Daphnia reproduction, Comet Assay for fish cells and whole daphnia and Zebrafish embryo, and for impendence testing of ecotoxicity in fish and daphnids, to be submitted as standard project submission form (SPSFs) to the OECD Working Party on Manufactured Nanomaterial (WPMN) for onward development and validation.

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List of Abbreviations

AOP - Adverse Ooutcome Pathway

API - Application Programming Interface

CEINT - Centre for Environmental Implications of Nanomaterials

CI - Cell Index

D – Deliverable

DO - Dissolved Oxygen

EC - Effective Concentration

ENM - Engineered nanomaterial

FAIR - Findable, Accessible, Interoperable and Reusable

Hpf - hours post fertilization

ICP-MS - Inductively coupled plasma mass spectrometry

ID - identifier

ISATab - investigation/study/assay tab-delimited (metadata capture template)

JRC - Joint Research Centre

MIE – Molecular Initiating Event

MIRCA - Minimum Information for Reporting Comet Assay

MWCNTs - multiwalled carbon nanotubes

OECD - Organisation for Economic Cooperation and Development

PBPK - Physiologically based pharmacokinetic

QA/QC - Quality Assurance / Quality Control

REST - Representational State Transfer

RR – Round Robin

SOP - Standard Operating Procedure

SPSF - standard project submission form

TEM – Transmission electron microscopy

TH - Total Hardness

TG – Test Guidelines

WP – Work Package

WPMN - Working Party on Manufactured Nanomaterial

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1. Introduction

1.1 Role and importance of data capture templates

The FP7 NanoREG project developed a set of templates for harmonisation of data logging, based on structuring the information on assays widely used in engineered nanomaterials (ENMs) safety testing for regulatory purposes, which they presented as a set of MS Excel templates, developed by JRC and released under the Creative Commons Share-Alike license (Totaro et al., 2017). While not strictly following the investigation/study/assay tab-delimited (ISA–Tab) and ISA–Tab–Nano specifications, the templates have been designed around "ISA–Tab logic", i.e., structuring the data in investigation–study–assay-related groups. As summarised in Kochev et al. (2020), the decision to create new templates instead of using ISA–Tab/ISA–Tab–Nano ones is the perceived low applicability of the latter in a more "lab-related" data logging due to lack of user-friendliness and experimentalists familiarity with, and preference for Excel formats. The NanoREG/JRC templates are used for data entry in a number of EU funded projects, including RiskGONE, which has been updating several of the templates based on the updates to the assay procedures (standard operating procedures, SOP) developed in WPs 4, 5 and 6, on physico-chemical, toxicology and ecotoxicology, respectively.

A high-level overview of the structure of the NanoREG data logging templates includes:

- The sample information group describes the ENM (including names, identifier (ID), supplier, vial number and replicate number, as well as dispersant). The reporting organisation, operator, and date of the experiment are also in this section;
- An unnamed group listing the module (physicochemical, in-vitro, or in-vivo), the endpoint (e.g., cell viability), and the assay name (e.g., "Alamar blue");
- Method and instrument information;
    - A subgroup "size distribution", providing placeholders for size distribution measured for the sample (including details of the dispersion protocol and dispersion medium). These fields are (almost) constant across all templates;
    - A set of parameters describing the experiment, including cell lines, instrument, controls, time points, concentrations. These differ widely across different experiments.
- Results group: several columns to specify measured outcomes, along with measurement uncertainty;
- SOP (reference to the protocol).

This deliverable report focused on the updates to data logging templates identified as necessary as a result of the research performed in RiskGONE WP6.

1.2 Metadata as the basis for data re-use

Metadata is defined as data about data, and are necessary to make a dataset meaningful for others (Papadiamantis et al., 2020) in order to enable other researchers to use the data for other proposes, such as in development of models or during meta-analysis to enable identification of trends or patterns in data. Studies to investigate the intrinsic properties and conditional behaviours of ENMs, i.e., their transformations in the environment and during interaction with living systems, can have many purposes and designs, and the purposes and designs of those studies will influence the formulation of appropriate metadata to describe the data that were or will be created.

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Indexing the metadata makes them findable (much like the table of contents or index in a textbook). Probably, the most well-known metadata capture template is the investigation/study/assay (ISA) tab-delimited (TAB) format discussed in Section 1.1 above, which has been extended to nano also (ISA tab nano), although this is not widely implemented by researchers or nanosafety databases in practice as it is complex and has quite a steep learning curve to understand what is required.

The FAIR principles—Findable, Accessible, Interoperable, and Reusable (meta)data— were developed in order to allow experimental data to be used beyond its origin (Wilkinson et al., 2016), and are not specific to ENMs. The FAIR principles are intended to enable re-use of (nanosafety) data for solving scientific problems, data gap filling, reading across applications, material and property modelling and supplying tools for other needs of science, industry, and regulators. The ubiquitous use of non-FAIR data entry formats (e.g., Excel spreadsheets) can be a stumbling block towards FAIR data, thus requiring steps to ensure compliance with the FAIR principle—a FAIRification workflow. A generic FAIRification workflow includes several steps: gaining access to the data, analysis of the data and metadata, defining a semantic model for the data and metadata, making data and metadata linkable, and finally, deployment (hosting the FAIR data and providing human- and machine-readable access), as described in Kochev et al. (2020).

The eNanoMapper/Ambit data model has been designed and implemented in order to accommodate the challenging features and attributes required to produce suitable chemical substance and ENM databases, namely the handling of:

(i) Physicochemical identity (different analytic techniques, manufacturing conditions, batch effects, mixtures, impurities, size distributions, differences in the amount of surface modification, etc.);

(ii) Biological identity (a wide variety of measurements, toxicity pathways, effects of ENM coronas, modes-of-action, interactions, cell lines, assays, etc.);

(iii) Support processes requiring information (raw data, study summaries for regulatory purposes; linking with experimental protocols; risk assessment; grouping, safety-by-design) and

(iv) support for data analysis (via an application programming interface (API) and various types of views or interfaces such as “spreadsheet” or matrix, etc., merging multiple values, conditions, or similar experiments into a matrix).

The ENMs information workflow and data processing life-cycle poses many challenges due to the wide diversity encountered in its data sources, the many data input formats used, the wide range of data organization methods and the huge variety of modelling tools used on the data (Kochev et al., 2020). The eNanoMapper/Ambit database, as used in RiskGONE, enables **mappings** (i.e., eNano**Mapper**) between the different data formats and data presentations. The eNanoMapper/Ambit data model is a flexible and general data structure that can describe measurements, with dynamic (not fixed) fields for endpoints, experimental conditions, protocol application, and several more metadata fields, annotated by ontology entries. The eNanoMapper data model is a generic description of any measurement and does not enumerate predefined fields for recording the results of a particular experiment. The latter (what are the essential parameters to describe an experiment?) is a domain specific scientific question: how to represent the selected “aspects of the reality”.

The workflow to make Excel spreadsheets FAIR involves a multistage process of aligning data and metadata with domain specific ontologies and data formats. The data is human-accessible through a web user interface and machine readable through a REST API with authentication and authorization.

Within RiskGONE, a Template Wizard was developed to automate and harmonise the data capture process across the consortium and during the Round Robin (RR) exercises where numerus partners were generating similar data that needed to be integrated for analysis. The Template Wizard, developed

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by Idea Consult (RiskGONE's partner), integrates the specific ENMs being used in RiskGONE into a dropdown menu (and now extended to other projects), and shows the options for end-points for which templates have been developed in a web form, and based on the users selections generates the template customized with the selected options. Each template has a set of common options (project, partner, work package, SOP) and a set of template specific options. Some of the options set the values of specific fields, while other options affect the data layout.

In order to generate the metadata / data capture template, the user is requested to:

- Specify the exact number of experiments and the number of replicates
- Enter a comma separated list of ENMs (and control) test concentrations, time points and labels for positive and negative controls
- The actual controls can be specified in the spreadsheet
- The layout of the raw data and results sheet depends on the number of the experiments and replicates, the concentrations and time points
- There is no need to select the material(s), this could be done once the template is generated
- Please use **decimal point** "." as decimal separator when entering numbers!
- Please do not rearrange and move fields around; if you need to adapt the template, contact the project data manager!

This template allows data entry for multiple materials. Please specify the number of materials in the relevant web form field. Figure 1 shows an example of an in vitro data assay and the template specification sheet.

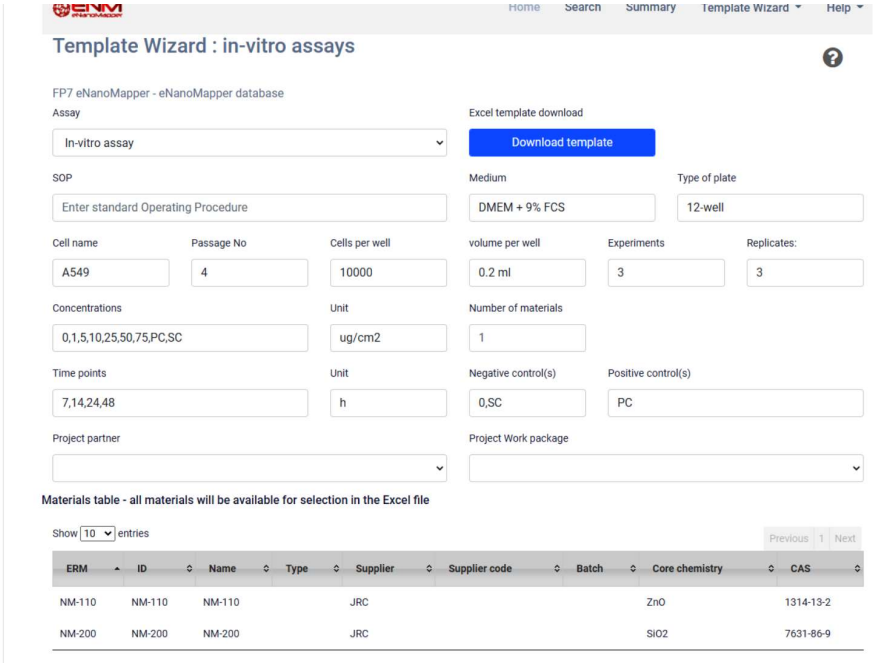

Figure 1: The RiskGONE Template Wizard showing the metadata required (e.g., assay type, SOP, cell type, passage number of number of cells per well, the Medium used, the number of replicates etc.).

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1.3 Lack of and gaps in existing templates for ecotoxicity testing

Within EU funding of nanosafety research over the last decade or more, research addressing nanoecotoxicology has been much more limited than that addressing human toxicology, given the human-centric nature of regulation. Indeed, it has been reported in the literature that ecotoxicology researcher lags toxicology research by about 10 years, and the same holds true for nanoecotoxicology (Kahru and Dubourguier, 2010). Indeed, a recent review on the role of the ENMs protein corona in the environment showed that the major focus of template development has also been directed towards ENMs with potential biomedical applications: the ENMs whose coronas have been evaluated that have use in biomedical applications are more diverse, including proportionally many more studies of gold, iron and lipid particles, while environmental corona research has addressed ENMs considered to be highly toxic (for example, quantum dots), that are widely released (for example, CeO_2) and biocidal materials (for example, Ag/Cu). Clear gaps exist in the dataset for corona studies on iron or iron oxide particles, and on graphene and other carbon-based materials, despite their widespread application in environmental remediation, and in terms of biocidal ENMs and others under development for agricultural applications (Wheeler et al., 2021). Thus, not surprisingly, far less emphasis has been placed on development of nanoecotoxicology templates in previous and ongoing projects than on those for toxicology assays.

Within NanoREG, there was some ecotoxicology work, but this was limited by the small number of ecotox-partners in the project (NanoREG D4.12). The major effort was devoted to the development of methods to prepare dispersions of ENMs for toxicological studies in aquatic systems, in order to address the reproducibility of such studies, through RR-style testing. Existing OECD and ISO standard methods for ecotoxicity assessment were adapted specifically for ENM testing and developed into defined SOPs. Once the dispersion and ecotoxicity SOPs were defined, NanoREG assessed the acute ecotoxicity of the NanoREG core ENMs (multiwalled carbon nanotubes (MWCNTs) and Ag, ZnO, SiO_2, TiO_2 and CeO_2 nanoparticles (NPs), in species representing different trophic levels (*Pseudokirchneriella subcapitata*, *Daphnia magna*, *Caenorhabditis elegans*, *Chironomus riparius*, *Danio rerio* and *Salmo trutta*).

An example of the type of data generated within an acute *D. magna* toxicity test using the OECD 202 test guideline, wherein immobilisation (lack of movement) of the daphnids after 15 seconds of gentle agitation is used as a proxy for mortality, is shown in Table 1. The NanoREG project showed that <24h and 4-day old daphnids were adequate to perform assays with JRCNM01000a (TiO_2 NPs), since no mechanical issues were observed. Neonates (<24h) were more sensitive to JRCNM01000a than 4-day-old organisms, during exposure periods of 48 and 72 h, as expected. The researchers observed agglomeration of NPs at all test concentrations and all measured time intervals. However, Dynamic Light Scattering analysis results obtained showed Z_{ave} of ~240 nm for stock dispersions, which were outside the determined for JRCNM01000a in the benchmarking study. From the acute daphnid data, there was no immobile animal after 24 hours of exposure (100% mobility of the daphnids), whereas after for 72 h of exposure, percentages of mobility of the animals were 90, 90 and 65% at 1, 10 and 100 mg/L of TiO_2 NPs, respectively, as shown in Table 1.

Table 1. Total number of mobile animals (< 24 h old) following exposure to TiO_2 NPs for 24 h and its percentage of mobility for each tested concentration after an exposure period of 72 h. R = replicate

Concentrations (mg/L)	R1	R2	R3	R4	Total mobile animals	% Mobility
control	5	5	5	5	20	100
0.01	5	4	5	5	19	95
0.1	5	5	5	5	20	100
1	5	4	4	5	18	90
10	4	4	5	5	18	90
100	2	3	4	4	13	65

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The OECD 202 test guideline specifies a set of environmental parameters that need to be recorded during the test, to ensure that any effects on the organisms observed during the test arise from the chemical exposure and not from a deterioration in quality of the medium, i.e., due to pH changes or lack of dissolved oxygen (DO). Within NanoREG these parameters were recorded at each timepoint, as shown in Table 2. However, neither these environmental data, nor the details of the dose-response curves that are generated from the data shown in Table 1, are reported in the ecotoxicity data capture template used in NanoREG, a portion of which is shown schematically in Figure 2, where pH and DO are captured but only in the final medium (i.e., after 72 hours in this case), while only the summary data of Effective Concentration (EC) 10, 20 and 50 which correspond to 10, 20 and 50% of the total organisms being immobilised, are reported in the data capture template. This means that if the materials are not very toxic, and thus don't reach an EC_{50} value there is no way to report the data easily. Indeed, this led to the well-documented problems in re-using the NanoREG data in nanoREG2, Gov4Nano and other projects (e.g., Bossa et al., 2021), where huge additional effort was needed to curate and quality assure the NanoREG and other data, as a result of inconsistencies in reporting even within the NanoREG templates.

Table 2. Chemical and physical parameters obtained from acute toxicity test with TiO_2 NPs using as test organism 24 h old *Daphnia magna* individuals. The parameters were collected at 0 and 72 h. T (temperature in °C); DO (dissolved oxygen in mg/L); TH (total hardness in mg $CaCO_3$/L).

Concentrations (mg/L)	pH		T		DO		TH	
	0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
0	7.90	8.57	19.5	20.1	6.57	5.39	220	220
0.01	7.90	8.06	19.6	20.1	6.81	4.56	220	220
0.1	7.90	7.92	19.8	20.2	6.76	4.29	-	-
1	7.90	7.98	19.7	20.1	6.75	4.56	212	212
10	7.90	8.13	19.9	20.1	6.69	5.11	-	-
100	7.90	7.93	19.8	20.2	6.72	4.32	204	204

The total hardness (TH) was only measured at the low, intermediate and high tested concentrations.

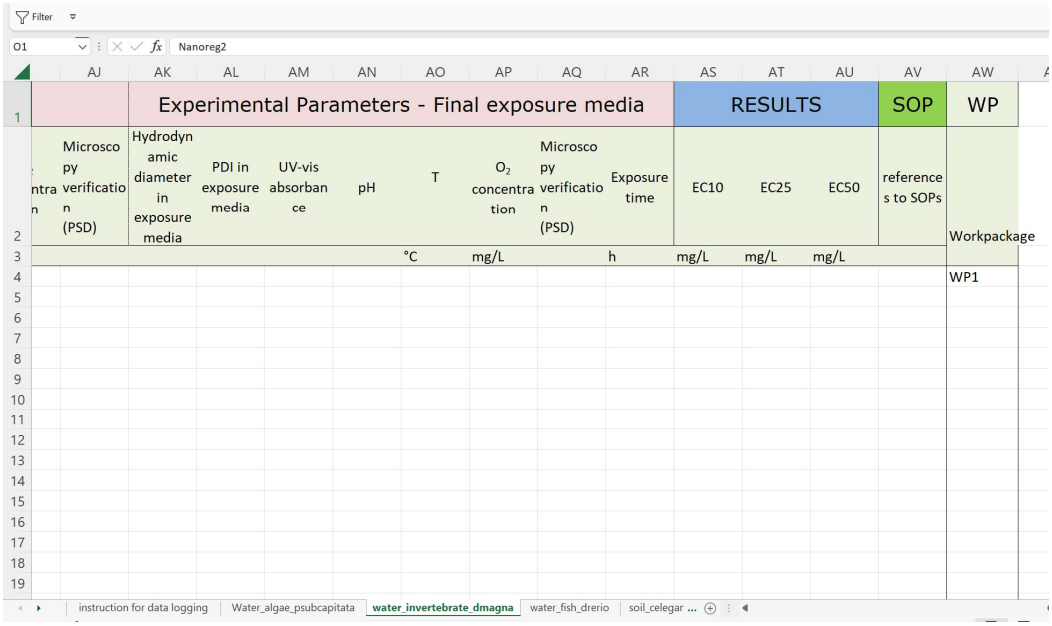

Figure 2: A portion of the ecotoxicology data capture template for *D. magna* developed in NanoREG. The focus in NanoREG was very much on the ENMs dispersion stability so most of the columns of

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metadata relate to this, and there is nothing on the biological system itself – i.e., to indicate if neonates (<24 h) or 4 day-old organisms were used, nor to collect the TH or other environmental parameters.

The existing NanoREG ecotoxicology template does not include any information at all on the biological organism. Even assuming that the OECD 202 or OECD 211 tests were followed, some information on the species (*D. magna*, *D. pulex* or other) should be included, as well as the age of the organism at the time of exposure (<24 hours old, 1-3 days or 4 days as used in many of the NanoREG tests, 7 days, adults etc.). Other information that is important to include to allow understanding of the impacts of the surroundings on the ENMs dispersion stability is:

(1) the amount and type of algae (food) the daphnids were exposed to and how long prior to starting the test;

(2) the medium composition and whether or not there was conditioning (and if so by what age daphnids) or other additives (e.g., serum proteins etc)., and for a reproduction test

(3) as part of the overall quality assurance / quality control (QA/QC) parameters, it would be really important to give a typical range for the number of offspring / daphnid / brood over the past 3 months, as this can vary and provides additional strength to any significant change in offspring numbers. This is especially important for the work within RiskGONE in developing the revised chronic toxicity assay which looks at number of offspring, as well as changes such as induction of males in response to the presence of ENMs.

Based on this analysis, it is clear that there is some significant work needed to improve and develop the metadata and data capture templates to ensure the correct documentation and storage of RiskGONE ecotoxicity data, and to maximize the potential for re-usability of the acute and chronic data generated for daphnia, as well as updating of the existing RiskGONE templates for Comet Assay (genotoxicity) and for Cytotoxicity using Impedance developed in WP5 for *in vitro* testing using human and animal cells for use with fish cells and other ecotoxicity relevant cell lines.

2. Background on the ecotoxicity tests being optimized in RiskGONE WP6

2.1 Daphnia reproduction assays – induction of males and multi-generational testing

Deliverable report D6.1 provides a detailed background on the use of daphnia as a test species for environmental monitoring and ecotoxicity assessment, and laid out the major challenges identified in RiskGONE that need to be addressed through updating of the test guidelines. *Daphnia* are a well-established and widely used model organism for freshwater toxicity testing due to their keystone status in the environment, rapid parthenogenetic reproductive cycle and sensitivity to a range of xenobiotics which they are exposed to as a result of their filter feeding behaviour. A broad set of behavioural and morphological changes can be observed in *Daphnia* when exposed to environmental stimuli, which forms the foundation of defined and standardised protocols for chemical toxicity testing, such as the OECD Test Guideline (TG) 202 (Acute toxicity) and TG 211 (Reproduction) tests.

In particular, the chronic (reproduction) assay has the following features:

- Test duration: 21 days.
- The total number of living offspring produced per parent which does not die accidentally or inadvertently during the test and the number of living offspring produced per surviving parent animal at the end of the test are reported.
- The study report includes:
  - daily counting of offspring,
  - daily recording of the parent mortality,

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- weekly measurement of oxygen concentration, temperature, hardness and pH values and the determination of the concentrations of test substance.
- Optionally, other effects can be reported, including the sex ratio of the offspring.

As reported in detail in RiskGONE D6.1, the proposed extensions to the current 21-day chronic (reproduction) assay (OECD 211) to account for the additional complexities introduced by ENMs with their intrinsic and extrinsic properties, include:

1) incorporation of ENMs specific considerations that affect the relevance and reproducibility of the results, including:

a. Use of conditioned medium to account for ENMs corona formation

b. Comparison of freshly dispersed versus medium-aged particles

c. Extension of the assay duration to 28-days as ENMs can delay maturation & reproduction (Figure 3a)

d. Inclusion of videos of ENM internalisation / depuration to support toxicokinetics modelling

e. Imaging of the daphnia during exposure to enable assessment of phenotypic and morphological changes to the daphnids. Such images can also be used in machine learning algorithms to predict toxicity, as shown in our previous work and described in RiskGONE D6.1 (see also Figure 3b).

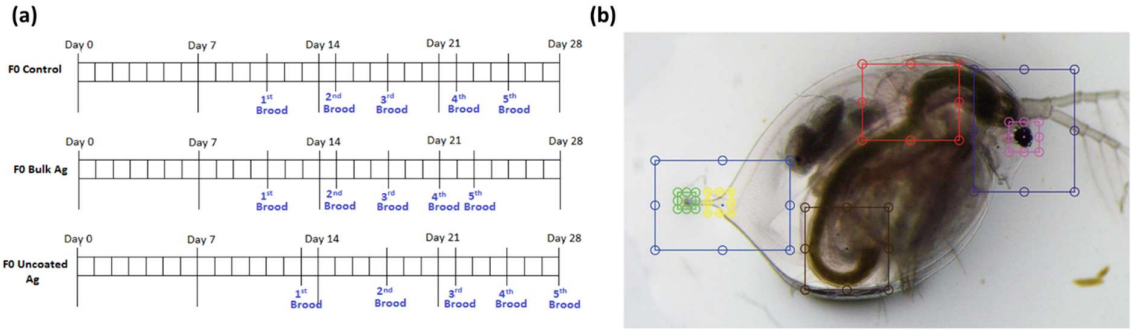

Figure 3: (a) Schematic showing the delays in time to first and subsequent broods resulting from exposure to uncoated Ag ENMs compared to Bulk silver and untreated control daphnids. (b) Light microscopy image showing a daphnia image and the areas that are measured using the machine learning algorithm developed in the H2020 NanoSolveIT project including changes in tail length, morphological changes such as loss of eyes or appendages, and absence / presence and intensity of lipid droplets (red box), which are used to determine the degree of toxicity of the ENMs applied to the daphnids. From Karatzas et al., 2020.

2) inclusion of additional ENMs-related endpoints to facilitate mechanistic insights including assessment of dissolution, ENM internalisations and morphological changes to the daphnids (could also include genotoxicity assessment in same assay if of interest). A minimum set of end-points should include:

f. Transmission electron microscopy (TEM) analysis of accumulation and localisation of ENMs in the gut over time & damage assessment

g. Inductively coupled plasma mass spectrometry (ICP-MS) analysis of particle loading following depuration for 24 hours (at 1, 3, 7, 14, 21 and 28 days) - internalised concentration needed for physiologically based pharmacokinetic (PBPK) modelling

h. Phenotypic changes – quantification of lipid deposits, loss of tail length etc.

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i. Potential assessment of genotoxicity at different timepoints via extraction of haemolymph and application of Comet assay (for example).

3) extension of the reproduction assay to include 1-3 additional generations using a paired approach of parent only exposure plus recovery versus continuously exposed (see Figure 4) to allow assessment of sensitisation versus adaption to low-level pollution.

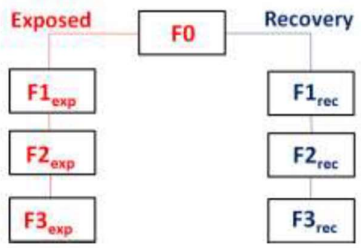

Figure 4: Multigenerational design showing the recovery and exposed generations after the F0 parental exposure. Note, the F1rec generations are born into exposure and then removed (within 24 hours post birth) to assess the recovery in the following generations.

4) Consideration of the inclusion of a temperature as an additional stressor as a means to future-proof the TG and allow also consideration of threat to populations under climate change.

The data capture templates will need to capture information on the daphnids, the envrironmental conditions and the traditioanl and additional end-points being captured. To support this, we utilise the approach of instance mapping (see Results section) developed initially by the Centre for Environmental Implications of Nanomaterials (CEINT) and further developed in NanoFASE and NanoCommons H2020 projects, as a means to capture the complexity of environmental experiments using ENMs, whereby the ENMs inteactions with their surroundings lead to transformations of the ENMs (physical, chemcial, biological and macromolecular interactions, as described by Lowry et al., 2012) and consequently a need to report the physico-chemical properties of the ENMs at different points during the experiment (instances of where the ENM might change its characteristics at a specific moment in time). Importantly, the instance map also captures the information at the medium for each instance, and a physical or chemical change to the ENM that alters the chemical or biological properties of the medium is also described as a new instance. For further details of instance maps, see the NanoCommons User Guidance Handbook: https://nanocommons.github.io/user-handbook/data-management/instance-maps/.

2.2 Genotoxicity assessment of fish cells via the Comet Assay

Deliverable report D6.1 provides a detailed background on the use of fish and fish cell lines as a test species for environmental monitoring and ecotoxicity assessment, and presents the major challenges identified in RiskGONE that need to be addressed through adaption of the existing SOPs for Comet Assay for use with fish cell lines. Indeed, a recent Nature Protocol presented a Consensus Statement for the Minimum Information for Reporting Comet Assay (MIRCA) providing recommendations for describing comet assay conditions and results (Møller et al., 2020, Azqueta et al., 2023 in press). These recommendations differentiate between 'desirable' and 'essential' information: 'essential' information refers to the precise details that are necessary to assess the quality of the experimental work, whereas 'desirable' information relates to technical issues that might be encountered when repeating the experiments.

In environmental ecotoxicity, fish are the most utilised vertebrate for environmental hazard and risk assessment, representing an indispensable component of integrated toxicity evaluation in the aquatic environment (Braunbeck et al., 2015). Zebrafish (*Danio rerio)* has been a popular laboratory non-mammalian model for more than 20 years, especially for its ability to spawn huge amounts of eggs across the whole year, its high fecundity, rapid development, and an extensive literature base (Strähle et al., 2012). The rapid development and transparent features that embryo, juvenile larvae, and adult

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stages of zebrafish display during their cell division, fertilization, and final morphogenesis, makes them suitable for microscopic screening for agents that disrupt normal development. Furthermore, zebrafish share around 70% similarity with human genomes, including major developmental and physiological processes, such as the digestive, nervous and cardiovascular systems, features that can potentially mimic the development of human diseases both genetically and phenotypically facilitating development of detailed understanding of the disease processes and therapeutic strategies (Zhang et al., 2003). Rainbow trout gill cells (RTgill-W1) are one of the most common types of fish cell species used for toxicity studies, and indeed are the basis of the recently published (June 2021) OECD Test Guidance 249 on fish cell line acute toxicity.

Despite the benefits of research on zebrafish embryos, they are still not frequently used with the Comet assay. Most studies have been conducted with adult fish and during the embryo–larval stage, with only a small number of studies have been performed on embryos (Canedo and Rocha, 2021).

Within RiskGONE we have used both Zebrafish cells as well as Zebrafish embryos with the Comet assay. For Zebrafish embryos whole body squashing method is used. After the treatment with ENMs the embryo is submerged in a minimal volume of fresh medium supplemented with pronase E (2 g/L) for 4 min to soften the chorion. Then the embryo is rinsed with fresh medium (without pronase E) and placed directly in a drop of LMP agarose, covered with a coverslip and gently squashed to obtain single cells. The cells will spread all over the microscope slide, remaining embedded in the agarose. For Zebrafish cells the cells are isolated as described in Figure 5.
Detailed protocols for the cell preparation and the comet assay are described in our joint paper in the Nature methods (Azqueta et al., 2023 in press). A key difference that needs to be captured in the Template Wizard is the culture temperature, since lower temperatures (22-28 ºC) are required to resemble the temperature of the donor habitat (Castaño et al., 2003). ZF4 cells are commonly maintained under 5% carbon dioxide (CO_2), despite the recorded physiological effects on fish (e.g., acid-base regulation).

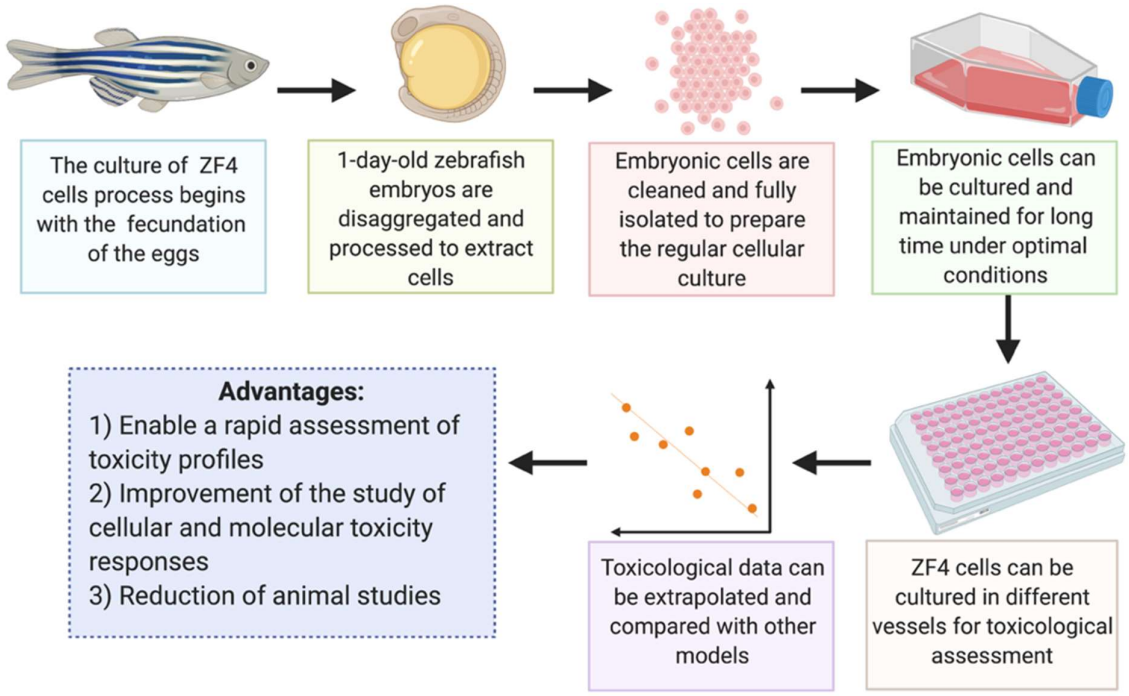

Figure 5. Zebrafish embryonic cells as an alternative toxicological model.

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The next stage of activity within WP6 will be to update the data capture template further for use with Zebrafish embryos also, including reporting on details such as the age at which the embryos were harvested, and the duration of exposure (maximum up to 96 hpf in the absence of ethical approval and an animal handling licence. It is possible to freeze (at −80 °C) up to 2 weeks freshly harvested cells isolated from embryos in physiological buffer containing 10% (vol/vol) DMSO, without a significant increase of DNA damage, but this would need to be noted in the reporting template (Azqueta et al., 2023 in press).

2.3 Impedance-based ecotoxicity assessment – metadata capture for method acceptance

Deliverable report D6.1 provides a background on the use of label-free impedance-based measurements for assessment of the cytotoxicity (Cimpan et al., 2013), including of fish cell lines, and D6.2 provides additional insights into the potential of label-free impedance flow cytometry for nanotoxicity screening (see Figure 6) which enables rapid and multiparametric analysis of single cells, making it a promising future candidate to assess cellular nanotoxicity (Ostermann et al. 2020).

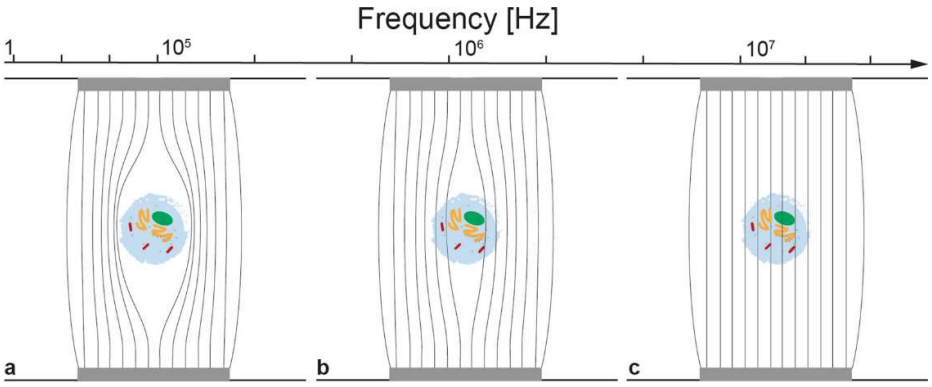

Figure 6: Behaviour of a cell in an electric field. (**a**) At low frequencies, the cell membrane constitutes a significant barrier to the current flow and information about the cell size is gained. (**b**) At intermediate frequencies, information of the membrane properties is revealed. (**c**) At high frequencies, the current can cross the cell membrane providing information about the cell interior. (Figure adapted and used with permission from Amphasys AG).

Detailed protocols have been developed, as described in these deliverable reports, and here we focus on the key aspects of metadata and data to be captured in these experiments. Impedance-based measurements of fish cells have been used to determine cellular growth and viability during particle exposure: the cells were seeded on gold-plated electrodes before an alternating current was applied using an xCELLIgence system (Agylent Technologies, USA).

Viable adherent cells impede the electron flow, and the results are displayed as Cell Index (CI), a unitless parameter, where CI = (impedance at time point n—impedance in the absence of cells) / nominal impedance value. A high CI indicates cell proliferation and thus viability, while a low CI indicates loss of cell adherence, cell death, and a reduction in the cell number. The increase of the CI after cell seeding indicates an increased adherence and cellular proliferation after which a plateau is reached. The method has been widely tested on a range of human and animal cell lines in WP5, and in rainbow trout gut (RTgutGC) cells, and zebrafish embryo (ZF4) cells. After 25 h of incubation, the cell culture media were replaced by either particle dispersions in complete cell culture media or control (complete cell culture medium without particles). The exposure time is visible as a peak in the CI due to the temperature change.

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In collaboration with Idea Consult, data capture templates have been developed for the label-free impedance-based methods, which are presented below, in general and with any necessary adaptions for use with other cell lines or even daphnia neonates (for example).

3. Results and Discussion

3.1 Development of a data capture template for Chronic daphnia studies and multigenerational studies

D. magna are a well established test organism in toxicity studies and have a range of chronic toxicity end points, such as growth (eye-tail length), reproduction (total offspring and time to first brood), induction of males and resting egg production, which are are all well established end points. These can be further complemented with sublethal markers such as lipid deposits, morphological defects, delays in moulting and changes to kairomone signalling. With increasingly complex experimental designs, such as chronic, pulsed and multigeneration toxicity studies, there is a range of data that can be used to further the understanding of molecular initiating events (MIE)) and how this can lead to establishement of Adverse Ooutcome Pathways (AOPs) for *Daphnia* toxicity – see RiskGONE Deliverable report D6.4 for the intial draft of the initial draft of Dynamic Energy Budget based AOP for chronic ENMs ecotoxicity.

SOPs are critical to assay performance and provide a good basis from which to build metadata and data capture templates. However, they often miss out on key pre-steps, or in the case of assays based on TG make a lot of assumptions, which can hamper data re-usability. For example, prior to running any daphnia assay, whether acute or chronic, there is a need to maintain running cultures of organisms between broods 3-7, and then to start new running cultures; scale-up to ensure the appropriate numbers of neonates prior to performing experiments is required. Indeed, keeping records of the normal behaviour of the organisms / cells in each individual lab in each specific assay is also vital for regulatory testing, but is not something that is formalised in most academic laboratories. Through complete metadata capture, as facilitated also by the experimental process visualisation as an InstanceMap will facilitate this also, increase data quality, data completeness, data reproducibility and data re-usability.

Figure 7 shows an example of an instance map approach to document the running cultures of daphnia to capture and documents the small seemingly insignificant steps that different individuals culturing daphnia might do unconsciously and thus that don't always appear in the SOPs, and as a basis for evaluation of the impacts of these steps on the daphnia culture performance. These form part of the provenance and quality assurance metadata that helps to demonstrate the trustworthiness of (hazard) data to others who may wish to re-use the data, for example in modelling or as part of a risk assessment. The instance map shows that the daphnia are cultured in a high hardness medium, which is aerated for a minimum of 8 hours prior to use in culturing and the dissolved oxygen content is measured every 2-3 days to ensure it stays within the acceptable range. The pH of the medium is also measured and moderated to within the defined parameters for the specific medium before use for the ongoing culturing of daphnia. The running cultures are typically in large (1L) beakers with 900 mL medium and can contain 10-15 adults, with the medium being refreshed three times per week. All cultures are fed the same daily algal ration of *Chlorella vulgaris* (7.5 mg C days 0-7, 11.25 mg C days 7 onwards, with double rations on Fridays to cover the weekend) and kept in a 20 °C laboratory under a 16:8 hour light: dark cycle. The steps involved in maintaining the daphnia, and the algae on which they feed, are shown in the instance map of Figure 7. Tracking of the number of offspring per brood is one of the essential quality control measures to record, using the template shown in Figure 8. Third brood daphniids are used for all experiments (e.g., acute or chronic toxicity testing) to ensure optimum genetic health of future cultures.

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The application of the instance map to the acute daphnia toxicity standard OECD test (OECD 202[1]) has also been demonstrated, as per Figure 9. An intentional feature of the OECD test guidelines is that they leave some flexibility for the user, in that they recommend a specific medium, but it is not essential (and indeed many labs use tap water or bore hole water), and thus the lab needs to prepare its own detailed SOP that underpins the experiment.

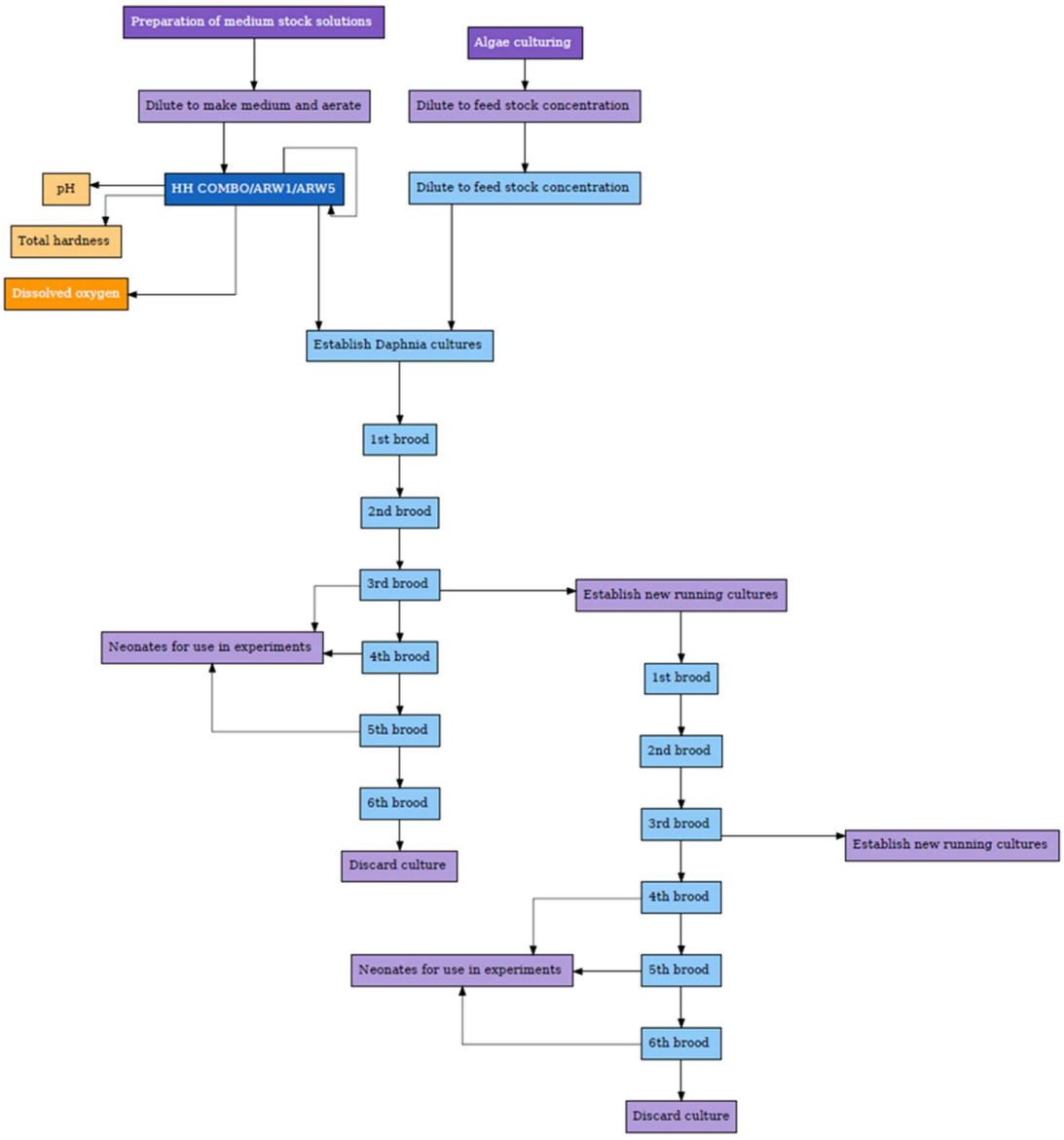

Figure 7: Instance map visualising the steps in maintaining continuous daphnia cultures. The colour coding is as follows: Purple: Key steps in the protocol; Dark Blue: Medium; Orange: medium parameters to measure; Light blue: daphnia cultures; in later InstanceMaps there will also be measurements recoded about the daphnids (in light purple) and about the ENMs at each step of the protocol. Daphnia typically produce broods from about 10 days old and roughly every 3 days thereafter, with the 3rd to 7th broods being the most genetically stable, and thus suitable for experiments. Tracking of the number of offspring

[1] https://www.oecd-ilibrary.org/environment/test-no-202-daphnia-sp-acute-immobilisation-test_9789264069947-en

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per brood is one of the essential quality control measures to record, using the template from the OECD 2011 TG is shown in Figure 8. Details such as organism species, strain, and culturing conditions (temperature, pH, dissolved oxygen, light:dark cycle) can be captured here as well as the specifics of the medium, culturing vessels etc.

Daphnia culturing record **Start date of record:**

Date	Day of Culture	Culture/Jar						Offspring						Food	Media Change	Observations/Comments
		1	2	3	4	5	6	1	2	3	4	5	6			

Figure 8: A simple data capture template for monitoring the health and performance of running daphnia cultures, wherein the amount of food and dates of medium changes are reported along with the numbers of offspring measured per running culture jar.

In the InstanceMap example shown in Figure 7, we have not (yet) linked to other aspects of an overall study that would be required, such as characterisation of the stock solution and assessment of the ENMs' stability in the test medium. However, the beauty of the instance map approach is that this linking of experiments / experimental steps is easy. The acute daphnia immobilisation test (OECD 202) has been mapped as shown in Figure 9, and a related data capture template has been developed and is currently being implemented into the Template Wizard. The OECD 211 chronic (reproductive assay) has also been mapped (Figure 10), including the proposed extension of the chronic test to cover multiple generations is being developed currently.

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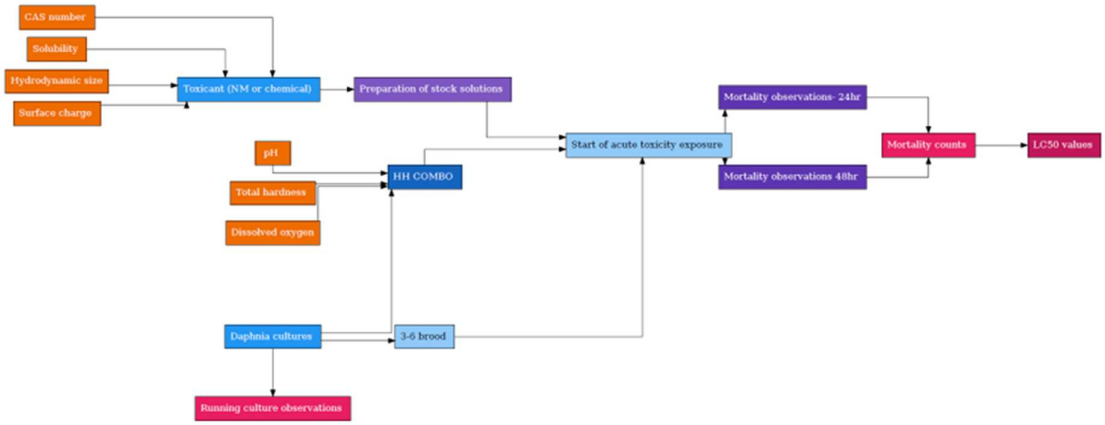

Figure 9: Representation of the OECD 202 Test Guideline for acute toxicity to daphnia as an instance map. For ENMs there would be a link from the stock solution to the range of characterisation studies needed, such as size distribution, surface charge, stability over time, etc.

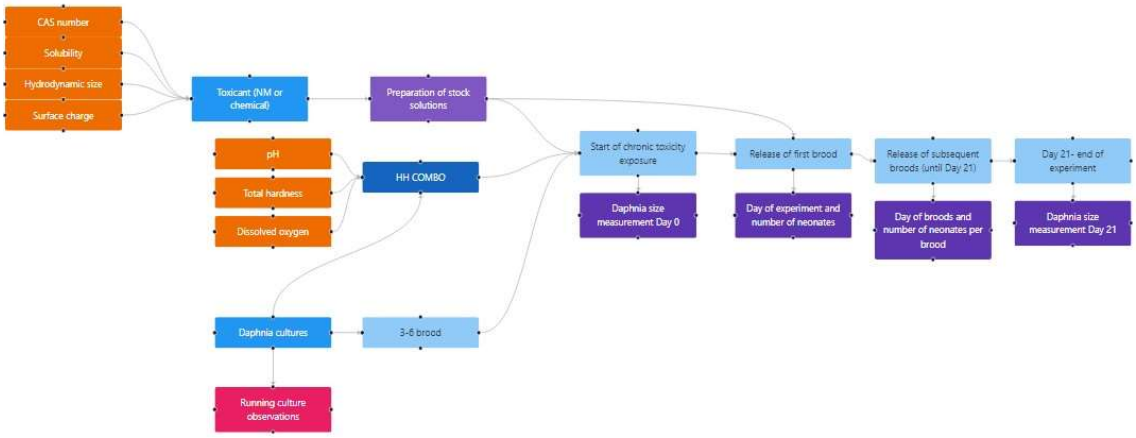

Figure 10: Representation of the OECD 211 Test Guideline for chronic reproduction toxicity to daphnia as an instance map. For ENMs there would be a link from the stock solution to the range of characterisation studies needed, such as size distribution, surface charge, stability over time, etc. This represents a single generation, and the data needed for the assay quality assurance also, and at each timepoint the number of offspring, ratio of males:females and other endpoints as indicated in section 2.1 are being added into the data capture and metadata templates sequentially, depending on the study design specifications.

Next steps will be to implement the acute, chronic and multi-generation data capture templates into the Template Wizard including the recommended adjustments for ENMs to account for their dynamic nature and their impact on their surroundings, such that the instance (ENMs plus surroundings) are characterized at each timepoint.

3.2 Updates to the Comet Assay template for Fish cell lines

The data capture template for the fish cell Comet assay has been adapted by NILU from the WP5 template developed with Idea Consult for human hazard assessment tests. Originally, RiskGONE WP5 (NILU) provided layout specifications for the Comet assay on human cell lines. The data capture template has also been adjusted and refined after having been tested in the first RR exercise of RiskGONE.

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Specific adaptations for the use of the template in ecotoxicity testing (on fish cell lines) were addressed. The template was considered to be suitable with only minor changes needed in the description of the test conditions, cell line details and culture conditions. Here, the temperature for cell maintenance was added. This information was originally missing, as all human hazard assessment testing is done at 37 °C. However, fish cell lines are maintained at 19-20 °C, thus this condition needed to be specified. Figure 11 shows the updated Comet assay (meta)data capture template that has been implemented into the Template Wizard, showing the adaptions for fish cell lines.

For Comet assay with zebrafish embryos or daphnia, some additional adjustments to the (meta)data capture template are required, including indication of the organism being tested, the age at exposure (e.g., in hours post fertilization (hpf) for zebrafish embryo or in hours/days for daphnids (e.g., <24 h, <48 h) studies, the following adjustments are needed, based on the publication by Azqueta et al., 2023 in press.

43	CELL LINE DETAILS & CULTURE CONDITIONS						
44	Detailed cell type/line specification:	rainbow trout, epithelial gill cells					
45	Cell line short-name:	RTGILL-W1					
46	Supplier:						
47	Passage no	4					
48	Plate details as applic.:	96-well					
49	Gel format	2-gel format					
50	Number of cells per well	10000					
51	Total volume per well	0.2 ml					
52	Medium (Supplier/Lot No.):	Leibovitz`s L15 + 9% FBS					
53	Serum (inc. supplier/Lot No.):						
54	Serum concentration in culture medium:						
55	Serum concentration in treatment medium:						
56	Was serum heat inactivated? If app.:						
57	Number of gels	2					
58	Number of scored cells	50					
59	Temperature	19 C	←				
60	TREATMENT TIMELINE						
61	Time point unit:	h					
62	Time points labels	T1					
63	Time points	24					
64	TREATMENT CONCENTRATION						
65	*Treatment concentration series unit:*	ug/cm2					
66	*Treatment concentration series labels*	C1	C2	C3	C4	C5	
67	*Treatment concentration series (C):*	0	1	5	10	25	
68	*Treatment type series*	control_negative	sample	sample	sample	sample	
69							

Figure 11: Illustration of the changes needed for the Comet assay metadata capture template to account for use with fish cell lines: temperature was added since fish cells are not cultured at physiological temperature (37 ºC), but rather at the temperatures typical of river and ocean waters where the various fish are prevalent.

3.3 Development of a data capture template for impedance studies with fish cells and daphnids

The (meta)data capture template for impedance-based cytotoxicity measurements was developed in conjunction with Idea Consult and WP5, with modifications made for fish cells as noted also for Comet assay in Section 3.2 above to account for the non-physiological conditions (Figure 12).

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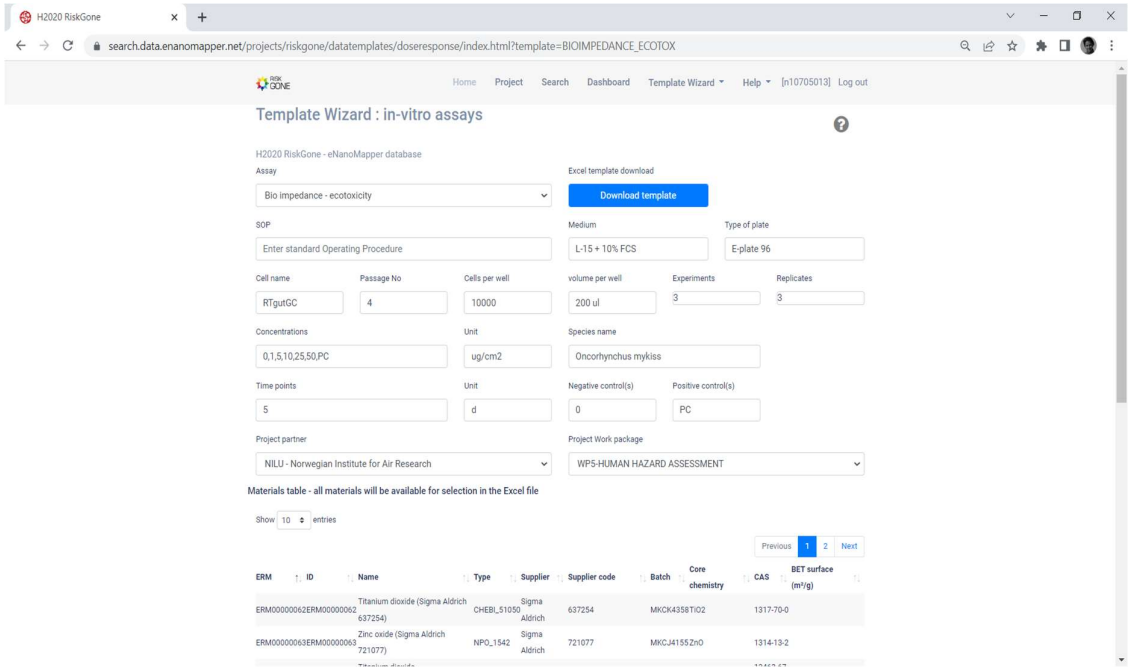

Figure 12: Template Wizard for impedance-based assay.

4. Conclusions

We presented here the development of refined (meta)data capture templates to support the harmonisation of the RiskGONE data collection and facilitation of the subsequent analysis, especially for RR data collected using the RiskGONE developed SOPs. The focus of the metadata capture template developed was on the updated experimental procedures for the three main assays developed and optimised within WP6, namely the daphnia reproduction assay (OECD 211), the Comet assay for genotoxicity assessment applied to fish cell lines from rainbow trout gills and from zebrafish embryonic cells, and impedance assays for assessment of cytotoxicity and oxidative stress in high throughput using fish cells and daphnids (in development – see also Deliverable report D6.2).

We presented the updated templates for capture of background data on lab facilities and procedures (e.g., running cultures and day-to-day and seasonable variability in assay performance as baseline quality control and quality assurance data), and from the daphnia acute and chronic assays themselves, based on the RiskGONE proposed updates to OECD 2011 and OECD 202. These amended or newly generated data capture templates have been / are being implemented also into the RiskGONE Template Wizard tool by Idea Consult as part of the overall RiskGONE data management activities and our efforts to ensure that all RiskGONE data is presented in accordance with the FAIR principles.

We presented also the Instance Map approach, developed and extended in NanoCommons as a means to visualise the various assay steps and thus to map out the full set of data and metadata that needs to be captured to support re-use of the data by others in the future.

The resulting data capture templates and visualisations of the datasets via the InstanceMaps will form part of the amended TGs proposals that will be prepared for the test methods Daphnia reproduction, Comet Assay for fish cells and whole daphnia, and for impendence testing of ecotoxicity in fish and daphnids, to be submitted as SPSFs to the OECD WPMN for onward development and validation.

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