SCIENCE-BASED RISK GOVERNANCE
OF NANO-TECHNOLOGY

RISK GONE

Report on harmonised ecotoxicology SOPs used to propose amendments to the existing OECD TGs

DELIVERABLE 6.1

Due date of Deliverable: 31.12.2021
Actual Submission Date: 31.12.2022
Responsible partner: UoB, United Kingdom

Report Author(s): Katie Reilly, Zhiling Guo, Laura-Jayne Ellis and Iseult Lynch (UoB), Mihaela Roxana Cimpan, Ying Xue and Ivan Rios-Mondragon (UiB), and Maria Dusinska, Elise Rundén Pran, Eleonora Longhin, Naouale El Yamani (NILU)
Reviewed by: Eleonora Longhin and Maria Dusinska (NILU)

Nature: R (Document, report)
Dissemination Level: PU (Public)

Call: H2020-NMBP-13-2018
Topic: Risk Governance of nanotechnology
Project Type: Research & Innovation Action (RIA)
Name of Lead Beneficiary: NILU, Norway
Project Start Date: 1 January 2019
Project Duration: 50-Months

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

RISK GONE

Document History

Version	Date	Authors/ who took action	Comment	Modifications made by
0.1	22-10-2021	Iseult Lynch, Katie Reilly (UoB), Mihaela Roxana Cimpan (UiB), Elise Rundén-Pran, Naouale El Yamani, Eleonora Longhin, Maria Dusinska (NILU)	Outline of contents shared with WP6 partners	Iseult Lynch based on feedback from WP6 partners
0.2	15-01-2022	Complete 1st draft	Shared with all WP6 partners	Iseult Lynch based on feedback
0.3	30-12-2022	Iseult Lynch (UoB)	Sent to PMO (NILU)	Maria Dusinska, Eleonora Longhin (NILU)
1.0	31-12-2022	PMO (NILU)	Submitted to Commission	

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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. In this task, round robin (RR) interlaboratory comparison (ILC) exercises were not possible due to the small number of partners with the different methods / biological systems available, but we are able to report on the repeatability, reproducibility and internal transferability between team members in the host labs for each of the test systems. The data and methods are also feeding into NanoHarmony for further evaluation and into ILCs, to support the activities of the OECD working Party on Manufactured Nanomaterials (WPMN) and other standardization bodies.

Available standard operating procedures (SOPs) were critically evaluated to determine their suitability for ENMs environmental hazard assessment and the need for potential assay adaptations. Evaluation of the assays was performed, and on the basis of this updated and complete SOPs for the respective endpoints were developed, as follows: i) colony forming efficiency (CFE) assay, ii) comet assay and iii) im

pedance assays on fish cell lines; iv) acute (immobilisation) and chronic (reproductive) toxicity in Daphnia magna adapting the OECD 211 test guideline, and v) Alamar Blue assay. A total of 5 ENMs were tested, TiO_2 and ZnO in round 1 and CuO, multiwalled carbon-nanotubes (MWCNTs) and Tungsten in round 2. As there were insufficient partners in WP6 to perform Round Robin test, an alternative approach was utilised whereby the assays were performed by several researchers at the same institute and/or the equipment was moved to nearby labs for confirmatory assessment (e.g., UiB equipment transported to NILU). Data templates for data and metadata entry into the RiskGONE instance of the eNanomapper database were developed (see Deliverable Report D6.3 for details) and the resulting data was / is being uploaded.

Amended Test Guidelines (TGs) have been prepared in WP5 for the test methods CFE and CA, and the further minor tweaks for use with fish cell lines are reported here, and will be included in the validation efforts if the WP5 SPSFs submitted to the OECD Working Party on Manufactured Nanomaterials (WPMN) for onward development and validation are selected for further work. A draft SPSF was prepared for updating TG211 for the *D. magna*, and is being further developed and documented during the remaining months of RiskGONE for submission. Similarly, the impedance work is being presented initially to the Norwegian delegation to the OECD WPMN, and based on their advice additional progress will be made to develop the method towards a test guideline.

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List of Abbreviations

AB – Alamar Blue

AC - Alternating current

AOP - Adverse Outcome Pathway

ATCC - American Type Culture Collection (a supplier of cell lines)

CFE – Colony Forming Efficiency

CI - Cell index

D – Deliverable

DLS – Dynamic Light Scattering

EC - effective concentration

ECIS - electric cell-substrate impedance sensing

ENM - Engineered nanomaterial

FBS - Foetal Bovine Serum

ICP-MS - Inductively Coupled Plasma Mass spectrometry

ILC – Interlaboratory Comparison

LOEC - Lowest observed effect concentration

M – Month of the project timeline

MWCNTs - multiwalled carbon-nanotubes

NOEC - No observed effect concentration

NOM – natural organic matter

OECD - Organisation for Economic Cooperation and Development

RR – Round Robin

SOP - Standard Operating Procedure

TEM – Transmission Electron Microscopy

TG – Test Guidelines

WP – Work Package

WPMN - Working Party on Manufactured Nanomaterials

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1. Introduction

In the context of regulatory testing of chemicals and nanomaterials, a standard operating procedure (SOP) is a set of step-by-step instructions on how to perform a specific assay or test, compiled by experts based on a round robin validation or inter-laboratory comparison to ensure repeatability and reproducibility of the assay. SOPs aim to achieve efficiency, quality output, and uniformity of performance, while reducing miscommunication and failure to comply with industry regulations. In regulatory toxicology, the most widely used SOPs are those developed and approved by the Organisation for Economic Cooperation and Development (OECD), which are the basis of the Mutual Acceptance of Data across jurisdictions. Most of the OECD Test Guidelines (TGs) were developed and tested for soluble chemicals, prior to the emergence of nanomaterials (ENMs) and thus there has been a concerned effort over the last 10+ years to evaluate their suitability for ENMs, and to update the TGs where necessary. RiskGONE is playing a critical role in supporting this activity, and is one of the few projects to have strong focus on the ecotoxicity TGs, and the assessment of the applicability of cytotoxicity and genotoxicity testing approaches developed for mammalian cells for use with fish cells also, as part of the ongoing commitment to the 3Rs principles of Replacement, Reduction and Refinement of animal tests, especially those using vertebrates.

The ecotoxicity protocols using for example *Daphnia magna* were originally designed for bulk chemicals though have been deemed broadly acceptable for ENM testing, despite ENMs existing as suspensions rather than dissolved chemicals (see Figure 1) (Nasser and Lynch, 2019). These protocols fail to account for key exposure features of ENMs such as the fact that the natural clearance processes in these organisms require food to push out previously accumulated matter, and that under realistic exposure scenarios, ENMs will have acquired a biomolecule corona that changes their identity, stability, uptake and excretion. Thus, the lack of biomolecules added to the medium and lack of feeding can lead to significant over or underestimation of the amount of ENMs taken up by, or retained within, *D. magna* leading to uncertainty of dose and ultimately miscalculation of ENMs toxicity and the risks posed by these materials. Herein we present evidence to support the call for revised guidelines for *D. Magna* acute and chronic toxicity tests for hazard and risk assessment of ENMs. Within RiskGONE, WP6 partners LIST and UoB are contributing to the NanoHarmony ILC on updating OECD 202 (as well as 201 on algae and 203 on preparation of ENMs for fish toxicity testing) which was planned for 2022 but will now take place in January and February 2023.

Currently, the ecotoxicity test guidelines, such as OECD 202 and 211, do not take into account that natural waters contain biomolecules which can bind to ENM surfaces creating an eco-corona which can cause stabilization/destabilization of ENMs dispersions (Xu et al., 2020). Even in biomolecule-free medium, *D. magna* themselves secrete proteins and carbohydrates from their guts and via their moulting fluid, causing the surrounding medium to constantly be ‘conditioned’ (Nasser & Lynch, 2016). Similar phenomena have also been reported for fish, mussels, earthworms and other environmentally relevant test species (Wheeler et al., 2021). The interactions of these biomolecules with ENMs can lead to stabilisation, re-dispersion or destabilization of the ENMs, and means that the toxicity experiments are themselves dynamic and that the ENMs after 24 hours and 48 hours of exposure, and their interactions with the organism, will be very different from those in the first couple of hours, which confounds the interpretation of the toxicity.

In cases where the biomolecules causes agglomeration and an increase in ENM size, the result is often that these larger ENMs are a more attractively sized food source for *D. magna* than the individually dispersed ENMs, thus enhancing uptake (Nasser & Lynch, 2016). ENMs with an acquired corona may

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get taken up to a higher degree than pristine ENMs (due to the above-mentioned agglomeration), but also have an enhanced retention within the gut of *D. magna* (Nasser & Lynch, 2019) making *D. magna* feel full for longer, potentially leading them to delayed development and, if sustained, to starvation which also impacts on their ability to reproduce. Ultimately, the OECD standard protocols may not be adequate for use with ENMs on several grounds, as depicted in Figure 1, as they were originally designed for solutions and are based on chemical potential driven equilibrium distributions and not for ENM suspensions which have a high surface area that bind to biomolecules, are potentially taken up through active processes rather than simply diffusing into the gut, and indeed may be further internalised by receptor mediated process from the gut.

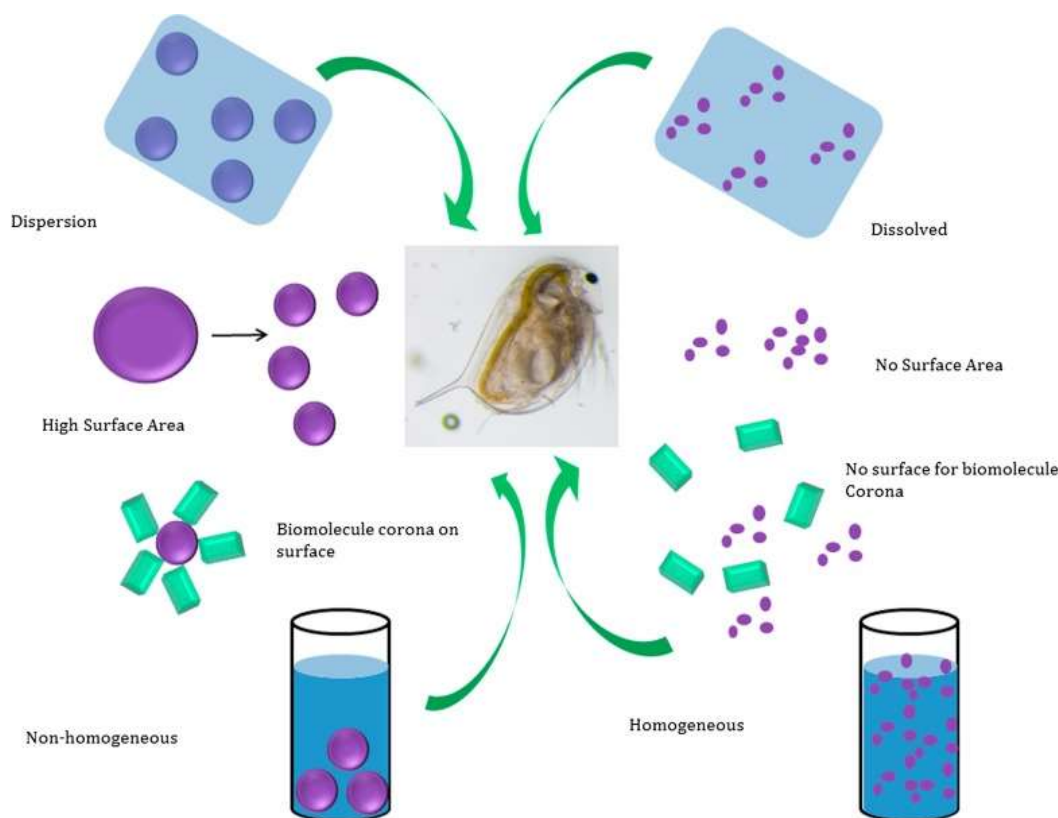

Figure 1. Differences between ENM suspensions (left of *D. magna*) and dissolved chemical solutions (right of *D. magna*) and how they interact with *D. magna*. The large surface area of ENMs leads to the acquisition of a bio-molecule corona which affects the ENM properties (e.g., stability, agglomeration) and may make them a more attractive food source. Additionally, ENMs are potentially taken up through active processes (driven by their acquired corona) rather than simply diffusing into the gut and may be further internalised by receptor mediated process from the gut. Reproduced from Science Direct.

Similarly, the tests developed for cytotoxicity and *in vitro* genotoxicity testing using mammalian cell lines need to be assessed for their applicability to ecotoxicity-relevant cell lines. Currently not all species of interest are available as cell lines, but fish and worms are good examples of test species where well-established cell lines are available commercially. For example, rainbow trout and zebrafish cells are commonly available, including gut, gill, and embryonic cells, allowing translation of the existing cytotoxicity / genotoxicity tests, such as colony forming efficiency (CFE) and Comet assay to ecotoxicity testing once appropriately tested and validated, and development of new higher throughput assays such as the impedance based assays as described in further detail below.

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2. Background information

2.1 A high throughput Colony Forming Efficiency (CFE) assay for cytotoxicity

To cope with the high number of nanomaterials manufactured, it is essential to develop high-throughput methods for *in vitro* toxicity screening. At the same time, the issue with interference of the ENM with the read-out or the reagent of the assay needs to be addressed to avoid biased results. Thus, validated label-free methods are urgently needed for hazard identification of ENMs to avoid unintended adverse effects on human health. The CFE assay is a label- and interference-free method for quantification of cytotoxicity by cell survival and colony forming efficiency by CFE formation. The CFE has shown to be compatible with toxicity testing of ENMs. Within WP5 of RiskGONE, an optimized protocol for a higher-throughput set up of the CFE assay (Rundén-Pran et al., 2022), and in WP6 the team have assessed its applicability to fish cells as an example of its use in ecotoxicity testing also.

The CFE assay was optimized and standardized some years ago for ENMs testing by the JRC's Nanobiosciences Unit and validated in a interlaboratory comparison study (Ponti et al., 2014), and is applicable for most adherent mammalian cells in culture, with stable cell lines mostly being used. Individual cells are exposed, and each surviving cell will divide and form a colony. This allows for the quantification of cell survival/cell death, and also for the detection of cytostatic effects by evaluating the size of the colonies. Reduced colony size will reflect slowed cell proliferation and growth. The test method has similarities with the plating efficiency assay (part of the OECD TG 476), however, for the plating efficiency assay exposure is performed on a confluent cell population grown in a monolayer, whilst for the CFE assay cells are considered as individual colonies (spots, as shown in Figure 2).

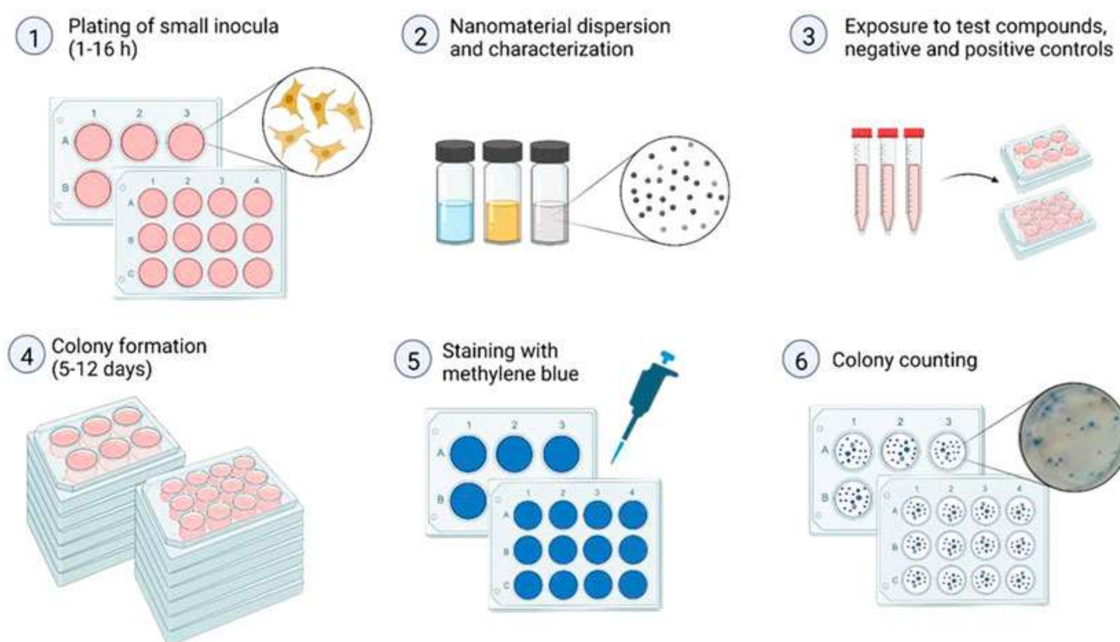

Figure 2: Graphical design of the colony forming efficiency (CFE) assay (Created with BioRender.com). 1. Trypsinize and count the cells. Seed the cells in correct density. It is important to mix the suspension prior to plating to ensure an even suspension of cells, as well as to spread the cells evenly in the wells. Remember to label both lid and the plate properly to avoid mix-up. Keep the cells in the incubator. 2.

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Prepare dispersion of ENMs. Perform proper particle characterization. 3. Dilute ENMs and controls in culture medium and add to the plates. Remember to make 2 × concentration since there is already half of medium in the well. 4. Leave the plates with the cells in the incubator to form colonies, normally 5–12 days 5. When colonies visible by eye are formed in negative control plates, the colonies should be stained in 1% methylene blue. Add 20 µl of methylene blue into each well and leave for minimum 30 min. Remove the staining solution into waste bottle. To reduce background staining, the plates can be rinsed carefully with water after staining. Leave the plates to dry. 6. Count the colonies. h, hours; d, days. Reproduced from Rundén-Pran et al., 2022.

In this deliverable, we provide the protocol for an optimized and miniaturized version of the CFE assay for higher through put, moving from Petri dishes to 6-well plates and further to 12-well plates (Rundén-Pran et al., 2022). The assay is easy to perform, and time- and cost-efficient, and found to be very suitable for cytotoxicity testing of ENMs. As part of this deliverable, we also include data related to the application of the CFE assay to fish cell lines, specifically the Rainbow trout gill cell line (RTgill). The complete SOP is given in **Appendix 1**.

2.2 Comet assay for assessment of genotoxicity (*in vitro* and *in vivo* formats)

The *in vitro* comet assay is a widely applied method for investigating genotoxicity of chemicals including ENMs. A big challenge in hazard assessment of ENMs is possible interference between the ENMs and reagents or read-out of the test assay, leading to a risk of biased results. Here, we describe both the standard alkaline version of the *in vitro* comet assay with 12 mini-gels per slide for detection of DNA strand breaks and the enzyme-modified version that allows detection of oxidized DNA bases by applying lesion-specific endonucleases (e.g., formamidopyrimidine DNA glycosylase or endonuclease III) (El Yamani et al., 2022). We highlight critical points that need to be taken into consideration when assessing the genotoxicity of ENMs, as well as basic methodological considerations, such as the importance of carrying out physicochemical characterization of the ENMs and investigating uptake and cytotoxicity. Also, experimental design—including treatment conditions, cell number, cell culture, format and volume of medium on the plate—is crucial and can have an impact on the results, especially when testing ENMs (El Yamani et al., 2022). Toxicity of ENMs depends upon physicochemical properties that change depending on the environment. To facilitate testing of numerous ENMs with distinct modifications, the higher throughput miniaturized version of the comet assay is essential.

The standard alkaline comet assay procedure has been described in various papers, and in exhaustive detail in a recent Nature Protocols paper (Azqueta et al., 2023). Here we emphasize the particular considerations that need to be taken into account when applying it to ENMs, with a brief outline of the overall procedure. The principle of the assay is that strand breaks release the supercoiling in DNA loops and allow the DNA to extend towards the anode under electrophoresis, forming comet-like structures; the proportion of DNA in the tail represents the frequency of DNA breaks. A summary of the comet steps is presented in the Figure 3 below.

In this deliverable, we provide the protocol for an optimized and miniaturized version of the *in vitro* Comet assay for higher through put, moving from Petri dishes to 6-well plates and further to 12-well plates (Rundén-Pran et al., 2022). As part of this deliverable, we also include data related to the application of the in vitro Comet assay to fish cell lines, specifically the Rainbow trout gill cell line (RTgill) and the Zebrafish embryo cell line (ZF4). The complete SOP is given in **Appendix 2**.

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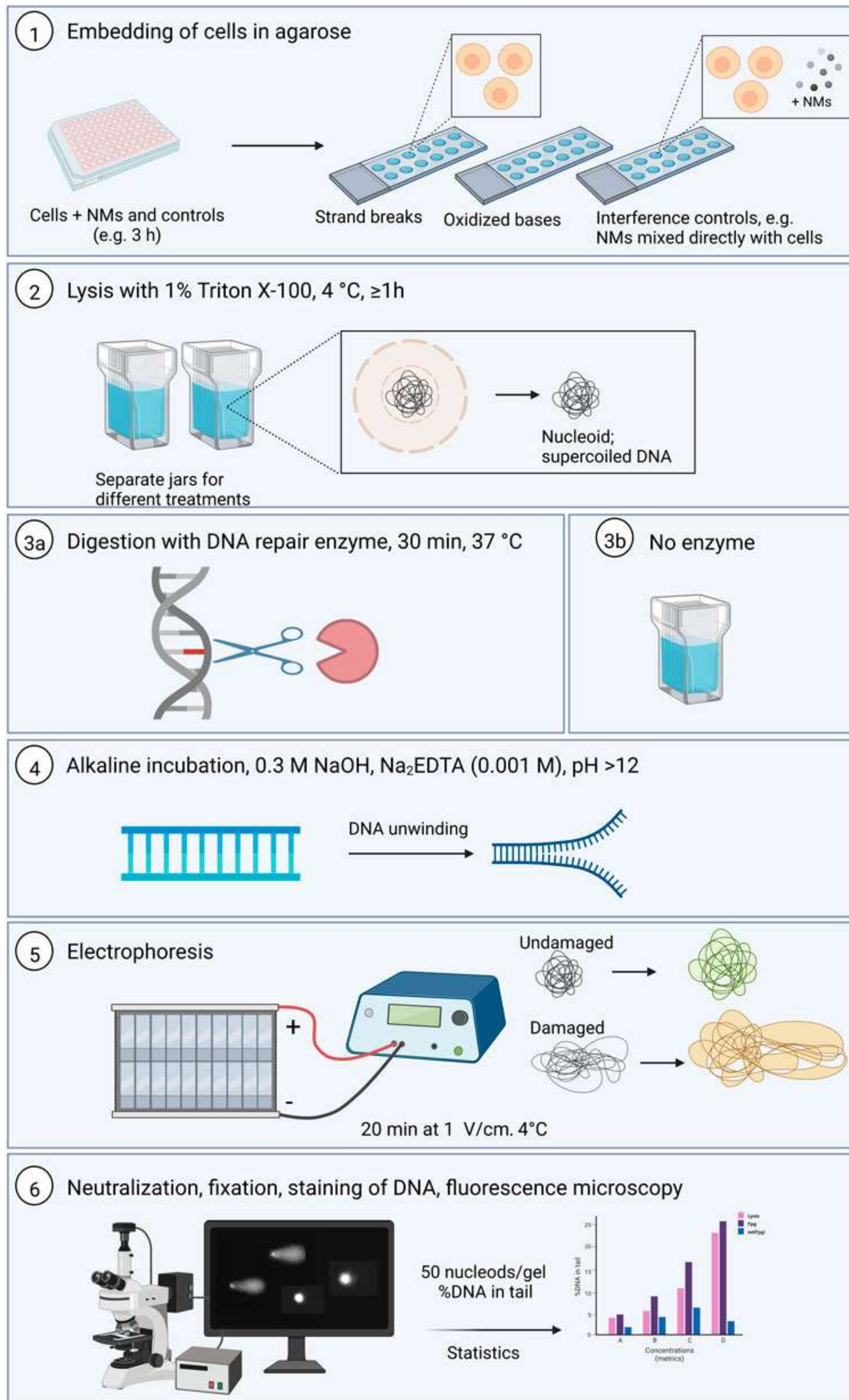

Figure 3. Summary of the comet assay protocol for both standard and the enzyme-modified version (Created with Biorender.com). 1. Cells are seeded in correct density using 96 well format and exposed

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to the ENMs and controls and after exposure time, the cells are embedded with 0.8% LMP agarose to make 12-gel format slides. 2. Lysis incubation at 4°C for at least 1 h 3a. the slide with samples to be incubated with DNA repair enzyme to reveal oxidative damage are incubated with the enzyme for 30 min at 37°C. 3b. The slides with samples for DNA strand breaks detection remain in the lysis solution. 4. All the slides are placed in the alkaline solution for DNA unwinding. 5. Electrophoresis is run for 20 min at 1V/cm. 6. At the end of the unwinding, all the slides are washed by the neutralization solution, fixed and then stained before visualization and scoring. 50 nucleoids are analyzed per sample or gel. %DNA in tail parameter is collected and statistical analyses performed. h, hours; d, days; LMP, low melting point. Reproduced from El Yamani et al., 2022.

The *in vivo* Comet assay measures the generation of DNA strand breaks under conditions in which the DNA will unwind and migrate to the anode in an electrophoresis assay, producing comet-like figures. Measurements are on single cells, which allows the sampling of a diversity of cells and tissues for DNA damaging effects. The Comet assay is the most common *in vivo* method for genotoxicity assessment of ENM. The Method outlined here includes a recommended step-by-step approach, consistent with OECD 489, taking into consideration the issues impacting assessment of ENM, including choice of cells or systems, handling of ENM test articles, dose determination, assay methods and data assessment (Cardoso et al., 2022). This method is designed to be used along with the accompanying “Common Considerations” paper, which discusses issues common to any genotoxicity assay using ENM as a test article (Elespuru et al., 2022). The *in vivo* Comet assay is currently being applied to *D. magna* in RiskGONE WP6 and minor adjustments are reported where needed.

2.3 Impedance-based high throughput assay for ENMs cytotoxicity assessment

A main priority in the assessment of putative toxic effects of ENMs is the development of new or adaptations of existing instruments and methods that recreate the biological environment and type of exposure to ENMs (Dusinska et al., 2015). An important source of confounding or conflicting data in nanotoxicity testing is represented by ENM interference with assay components and/or detection systems (Guadagnini et al., 2015; Kroll et al., 2012; Ostermann et al., 2020; Collins et al., 2016). Some of the most encountered interferences are seen in label-based assays as assay reagents can be adsorbed onto ENMs, which prevents markers from binding to cellular structures of interest and thus lead to distorted results. Methods employing fluorescent markers can be perturbed by ENMs that fluoresce or quench fluorescence; additionally, a general drawback is represented by the fact that a precise quantification of fluorescence is difficult to obtain. Another shortcoming of label-based assays is that they usually require multiple steps which can permanently modify or damage the cells (Cheran et al., 2008; Pliquett et al., 2010). Such assays are oftentimes time-consuming, labor-intensive, and complex. Many traditional methods can only evidence effects at a specific point in time and thus do not give an overview of the biokinetic behavior and of the real time interactions between biological structures and toxicants over time.

Reliable, time- and cost-efficient, eco-friendly methods and instruments for nanotoxicity testing that are not prone to ENM-induced interferences are urgently needed. Label-free detection methods have emerged as a more reliable alternative to optical- and reagent- based methods (Guadagnini et al., 2015; Kroll et al., 2012). Impedance-based assays that measure the electrical properties of cells are label-free and represent a promising alternative with the potential to become a method of choice for the initial screening of ENMs’ toxic effects (Ostermann et al., 2020; Hondroulis et al., 2010; Cimpan et al., 2013).

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Impedance-based monitoring makes use of the electrical properties of an object, composed of dissipative elements represented by as ohmic resistors and/or conservative elements represented by capacitances and inductances. It measures how much objects, such as cells, impede the flow of electrical current. Proliferation, growth, morphology, and adhesion can be evaluated by measuring the impedance of electrodes covered by cells (Cheran et al., 2008; Pliquett et al., 2010; Cimpan et al., 2013). Impedance-based methods have an additional advantage when compared to traditional methods because they not only allow "in-situ" real-time recordings without the use of markers, but also the monitoring of the dynamics of cell growth and viability (Cimpan et al., 2013).

Real-time cell monitoring

Real-time cell monitoring by electric cell-substrate impedance sensing (ECIS) is a label-free, non-invasive biophysical assay detecting dynamic cell responses. Impedance-based devices for assessing the behavior of adherent cells, such as the xCELLIgence system (ACEA Bioscience Inc., San Diego, CA), utilize golden-plated electrodes placed at the bottom of cell culture vessels (E-plates) (Cimpan et al., 2013). Cells seeded on the electrode induce changes in the measured impedance output due to the fact that cells, with their insulating bilipid membranes, act as dielectric objects (Hondroulis et al., 2010; Giaever & Keese, 1993; Xiao et al., 2002). An alternating current (AC) is applied through the electrodes and the extent to which the cells impede that current is measured (Cimpan et al., 2013). As the cells that attach to the electrode at the bottom of the culture vessel grow and multiply, the impedance increases, which provides information about the cell count, cell morphology, attachment to the substrate and viability. When cells die, they detach from the electrode surface, causing a drop in the recorded impedance, which indicates a reduction in the number of viable cells (Cimpan et al., 2013; Yeon & Park, 2013). Live nanotoxicity screening can be run in medium and high throughput, using 16-well and 96-well E-plates (Pan et al, 2013a, 2013b). The limitation of impedance-based devices is that they give little information about mechanisms behind the above-mentioned cellular responses. However, a significant advantage is that real-time observation facilitates the identification of key time-points and concentrations for further, more targeted, in-depth mechanistic studies (Kavlock et al., 2012). A summary of the protocol used is shown schematically in Figure 4.

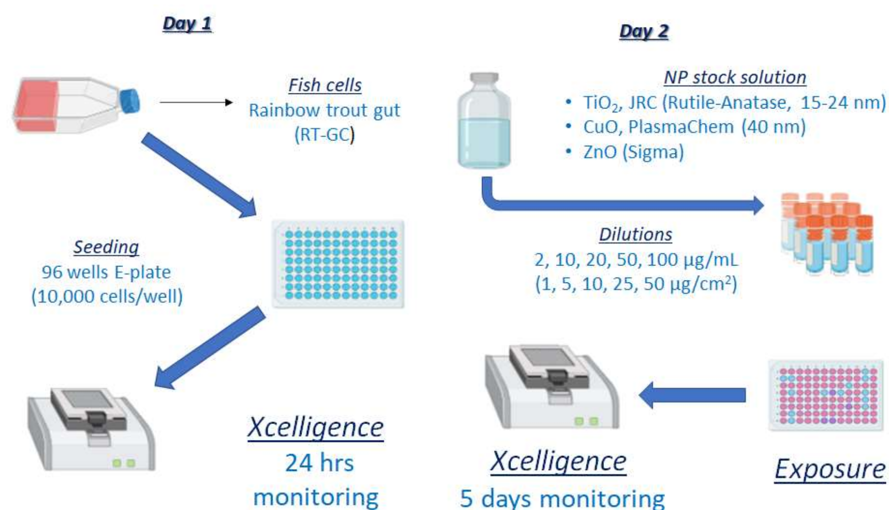

Figure 4: Impedance-based real-time monitoring (xCELLIgence, ACEA Biosyst.) of TR-GC cells. SOP: exposure to TiO_2 (JRC), CuO (PlasmaChem) and ZnO (Sigma) ENMs.

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Within WPs 5 and 6 of RiskGONE, impedance-based methods have been applied to assessing the cytotoxicity and ecotoxicity of a panel of mammalian and ecotoxicity-relevant cell lines, focusing in WP6 on fish cell lines from rainbow trout gut and zebrafish embryonic cells, and daphnia neonates. The complete SOP is given in Appendix 3.

2.4 Daphnia as a model organism for toxicity testing

As described in RiskGONE D2.4 on the adverse outcome pathway approach being developed in RiskGONE, tThe importance of the cladoceran Daphnia as a model organism for ecotoxicity testing has been well-established since the 1980s when it was standardised for regulatory testing of chemicals, due to its keystone status in the foodchain and sensitivity to pollutants which make it an essential indicator species. The mapping of the genome of its members *D. pulex* in 2012 and *D. magna* in 2017 further consolidated its utility for ecotoxicity testing by demonstrating the responsiveness of its genome to environmental stressors. Its short lifecycle and parthenogenetic reproduction make it hugely useful for assessment of development toxicity and adaption to stress. Under favourable environmental conditions (e.g., within the optimal range of parameters shown in Table 1), Daphnia reproduce parthenogenetically (Figure 5).

Table 1: Recommended conditions for optimal culture growth of Daphnia

Factor	Optimal Range
pH	7.0 - 8.6
Temperature	20 – 25 °C
Dissolved oxygen	> 6 mg/L
Water Hardness	160 – 180 mg $CaCO_3$/L
Light/ dark cycle	16 light/ 8 dark

Source: http://ei.cornell.edu/toxicology/bioassays/daphnia/culture.html

Parthenogenesis is a type of asexual reproduction in which the offspring develops from unfertilized eggs. Female Daphnia produce more female Daphnia without requiring a male to fertilize the eggs. Eggs are produced in the ovaries, and released via the oviduct into the brood chamber where they continue to develop, eventually hatching (within 1 to 3 days) into young neonates which are genetically virtually identical to the parent. The young neonates are then released to the outside at the next moult or shedding of the exoskeleton called a carapace. Usually, one clutch of eggs is produced for each adult instar (a phase between two periods of moulting in the development of an insect larva) with between 2 and 100 eggs per clutch. The newly hatched daphnids must moult, i.e., shed their outer carapace, several times before they are fully grown into an adult, usually after about two weeks. Each moult represents the transition from one instar to the next. Thus, the young are small copies of the adult; there are no true nymphal stages. Fully mature females are able to produce a new brood of young about every ten days under ideal conditions. The reproduction process continues while the environmental conditions continue to support their growth.

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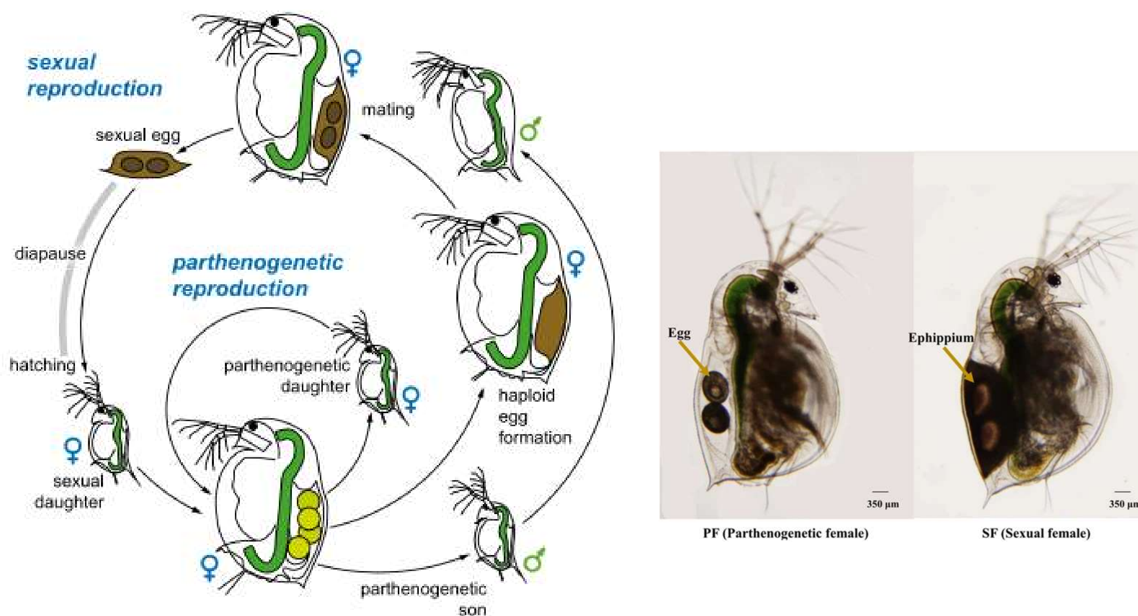

Figure 5: **Left:** The reproductive cycle of daphnia: under normal conditions daphnia reproduce asexually producing clonally identical offspring (so-called parthenogenetic reproduction). However, under stressed conditions males are produced and males and females mate to produce diapausing eggs that survive harsh conditions and hatch later when conditions improve. **Right:** Light microscopy images of parthenogenic reproduction with normal eggs versus sexual reproduction whereby females produce diploid resting eggs of ephippia, that are released, and can hatch at a later point regenerating the females.

Under harsh environmental conditions, or in response to some pollutants including some ENMs, production of new female generations ceases and parthenogenic males are produced instead (see Figure 5). However, even in harsh environmental conditions, males may make up considerably less than half the population. Males tend to be much smaller in size than females and are also distinguished by larger antennules. The males then fertilise the eggs forming ephippia or resting eggs, which can survive in the sediment for long periods of time (years) and then hatch when conditions improve, allowing the parthenogenetic reproductive cycle to begin again.

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 MIE) and how this can lead to adverse outcome pathways (AOPs) for *Daphnia* toxicity.

2.4.1 Reproductive test (OECD 211)

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

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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. Endpoints used for toxicity testing with *Daphnia* encompass acute responses, such as death (measured as immobilisation in the OECD TG 202), to changes in life history traits during the chronic test (OECD TG 211) including both reproductive changes, such as an increase or decrease in the number of neonates per adult daphnid or a delay between broods, in addition to delays or reductions in growth. Further to the standard test endpoints, phenotypic changes can be observed, such as additional spines on the helmet, variability in lipid deposits and behavioural changes, such as swimming activity.

The standard OECD reproductive test for *Daphnia magna* is over a single generation, and measures the time to the 1st and subsequent broods (noting any delays) and the number of offspring per brood (which is then divided by the number of adults to get the average offspring per adult). The test continues until the organisms have had 5 broods, or in cases where the adults died from the exposure it terminates when there are no further adults or no further offspring. Additional aspects can be also noted, such as numbers of aborted eggs (eggs that didn't hatch) and the presence of male offspring, which is a clear sign of pollution-related stress induced by exposure to the ENMs, as well as observing any morphological defects (e.g., loss of tail length, increased lipid deposits, change in body shape, delays in moulting etc). Indeed, using the images from the multigenerational studies, we were able to develop a machine learning model for prediction of ENMs' toxicity to daphnids across generations (Karatzas et al., 2020).

It should be noted that for the multi-generational approach that we have been developing at UoB, the parent (F0) generation is exposed and the offspring are removed within 24 hours of birth – the offspring are then split into two groups, one of which continues to be exposed to the same ENM in the same medium for the subsequent generations, while the other half are placed into medium only and are called the recovery group (see Figure 6). This parallel approach allows us to look at how the daphnids potentially adapt to the presence of ENMs (continuously exposed), and whether there are any epigenetic effects passed onto the offspring from the parental exposures. We note also that the subsequent generations are typically produced using the 3rd broods from the F0 (parent) generation, as there are considered the most genetically stable. The complete SOP is given in **Appendix 4**.

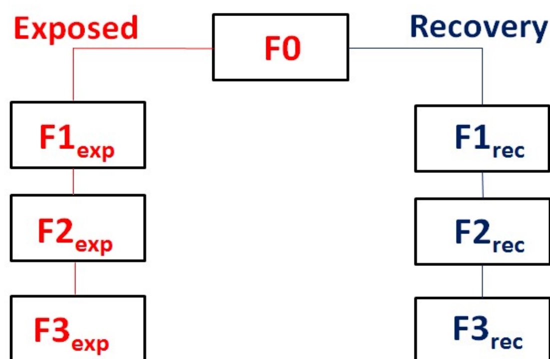

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Figure 6: Multigenerational design showing the recovery and exposed generations after the F0 parental exposure. Note, the F1rec generations are born into exposure and then removed (within 24 hours post birth) to assess the recovery in the following generations.

2.5 Almar Blue assay for cellular metabolic activity

The Alamar Blue (AB) assay is widely used to investigate cytotoxicity, cell proliferation and cellular metabolic activity within different fields of toxicology. The assay is based on the conversion of the fluorometric redox indicator resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide), a blue-coloured non-fluorescent compound to the fluorescent resorufin (7-hydroxy-3H-phenoxazin-3-one) following uptake into cells due to the reducing environment of the cytosol in cells (Longhin et al., 2022). Resorufin produces bright red fluorescence, with excitation range of 530–570 nm and emission range of 580–610 nm, that can be quantified (fluorescence intensity) and used as a measure of cell viability. The test can also be read on the basis of the absorbance at 570 nm, using 600 nm as a reference wavelength (the values need to be normalized on the reference wavelength). As resazurin is water-soluble, stable in culture medium, non-toxic and permeable through cell membranes, the AB assay has proven to be robust, simple to perform and relatively cheap, thus presenting many advantages compared with other cell viability and proliferation assays (Longhin et al., 2022).

The use of the assay with ENMs entails specific aspects including the potential interference of ENMs with the test. The procedure of the AB assay applied for testing ENMs is described in detail and step-by-step in the SOP (Appendix 5), from ENM preparation, cell exposure, inclusion of interference controls, to the analysis and interpretation of the results, as shown schematically in Figure 7. Provided that the proper procedure is followed, and relevant controls are included, the AB assay is a reliable and high throughput test to evaluate the cytotoxicity/proliferation/metabolic response of cells exposed to ENMs (Longhin et al., 2022).

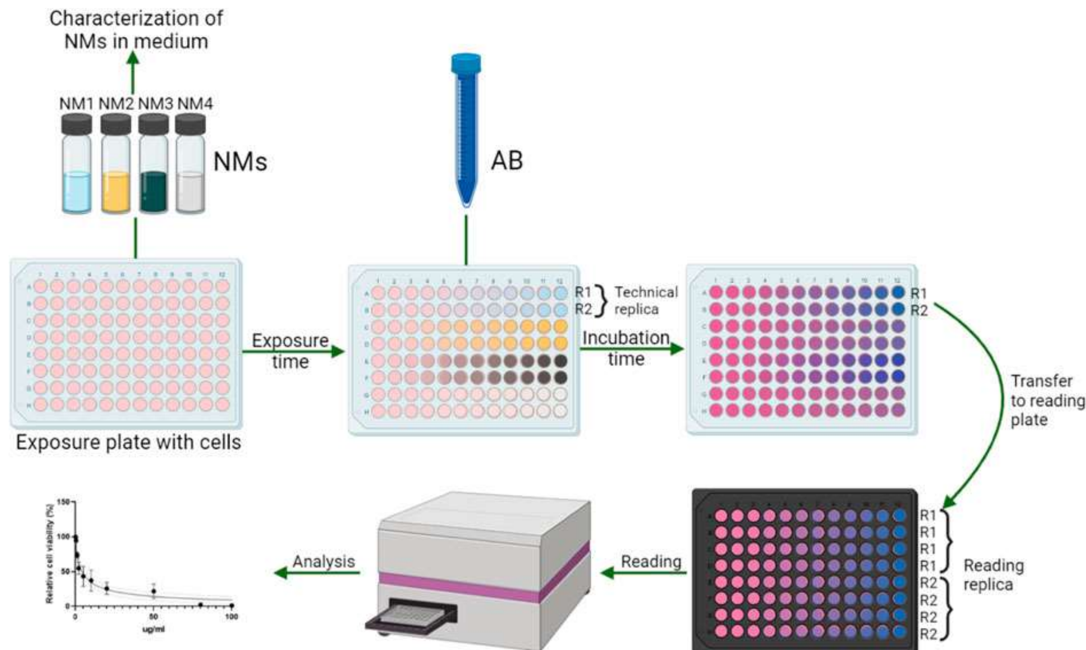

Figure 7: Schematic illustration of the steps in the Alamar Blue assay for cell proliferation / cellular metabolism. Reproduced from Longhin et al., 2022.

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Within WP6, the high throughput assay developed within RiskGONE was assessed using fish cell lines to assess its suitability as an ecotoxicity assay. The full SOP is reported in Appendix 5, with any adjustments needed for fish cells noted.

3. Results from applying the RiskGONE SOPs

Here some exemplar results from application of the various SOPs to the RiskGONE ENMs are presented, supplementing the results shown in the various periodic reports, while the main content of the deliverables is presented in the Appendices 1-4, that is, the RiskGONE SOPs for each of the 4 assays worked on extensively in WP6, namely the CFE and Comet assays, impedance-based assays and chronic reproduction assays in *D. magna* including the extension to multiple generations. Table 2 provides an overview of the ENMs used in the various studies.

Table 2: Overview of Nanomaterials and Cells used in the ecotoxicity screening. All particles were also assessed in *D. magna* chronic exposures.

Particle	Round robin (RR)	Stock concentration (mg/ml)	Dispersion protocol	Exposed cells AB / CFE / Comet assay	Exposed cells Impedance
ZnO (Sigma-Aldrich) (#721077)	RR1	5	Vortex	RTgill	RTgutGC
TiO_2 (JRC) (#JRCNM01005a990582)	RR1	5	RiskGONE WP4 Dispersion	RTgill	RTgutGC
CuO (PlasmaChem) (#PL-CuO)	RR2	5	RiskGONE WP4 Dispersion	RTgill C, ZF4	RTgutGC, ZF4
WC-Co (NanoAmor) (#5561HW)	RR2	2.56	NANOGENOTOX -protocol	RTgill, ZF4	RTgutGC, ZF4
MWCNT (Nanocyl, NC7000™) (#AQ0303)	RR2	5	Vortex	RTgill , ZF4	RTgutGC, ZF4

3.1 DLS measurements

Dynamic Light Scattering (DLS) measurements were carried out on the ENM stock dispersions, as well as the ENM dispersions in cell culture medium at the highest concentration used for exposure, i.e., 100 µg/mL.

The measurements took place at T0 (Time of exposure) and at the end of exposure, i.e., T120 (120 h) for RR1 ENMs and T24 (24 h) for RR2 ENMs. The cuvettes containing the ENM dispersions were kept in the same incubator as the RTCA station, replicating the environment of the cells in the E-plate during exposure.

Figures 8 and 9 display the DLS measurements for RR1 ENMs in DMEM/F-12 (10% Foetal Bovine Serum (FBS)).

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Figures 10 to 12 show the DLS measurements of RR2 particles in DMEM/F-12 (10% FBS) and L15 medium (10% FBS).

RR1-particles

ZnO (Sigma-Aldrich)

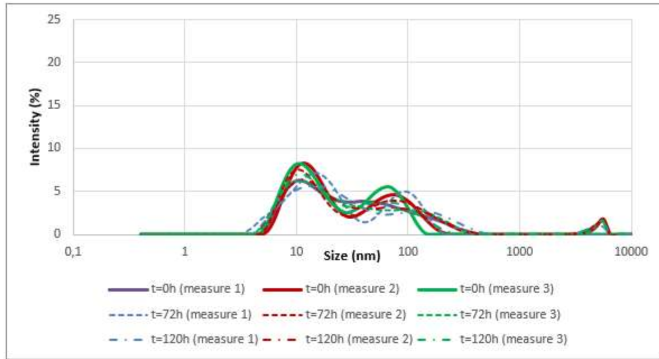

Figure 8. ZnO (Sigma-Aldrich) DLS dispersion measurements. Intensity (%) against size (d.nm) in DMEM/F-12 (10% FBS).

TiO_2 (JRC)

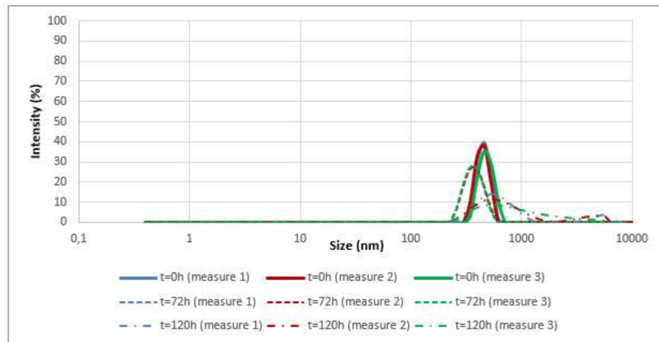

Figure 9. TiO_2 (JRC) DLS measurement. Intensity (%) against size (d.nm) in DMEM/F-12 (10% FBS).

RR2-particles

CuO (PlasmaChem)

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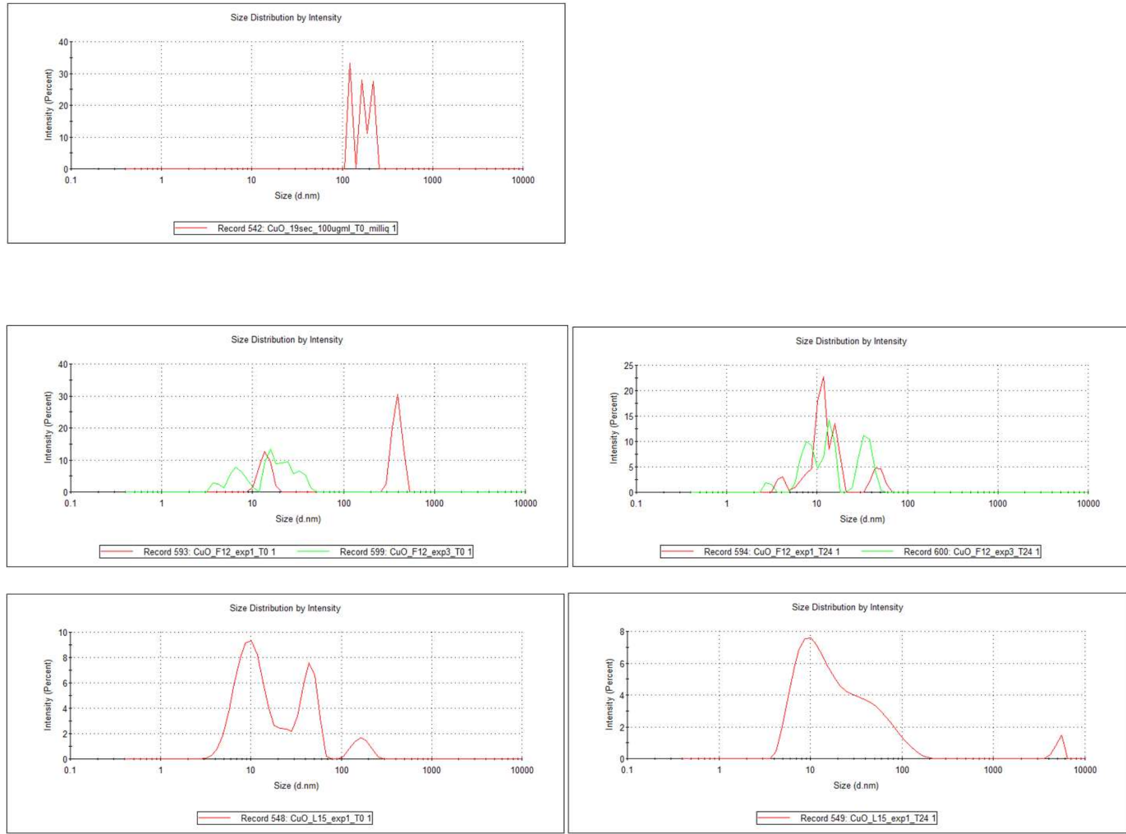
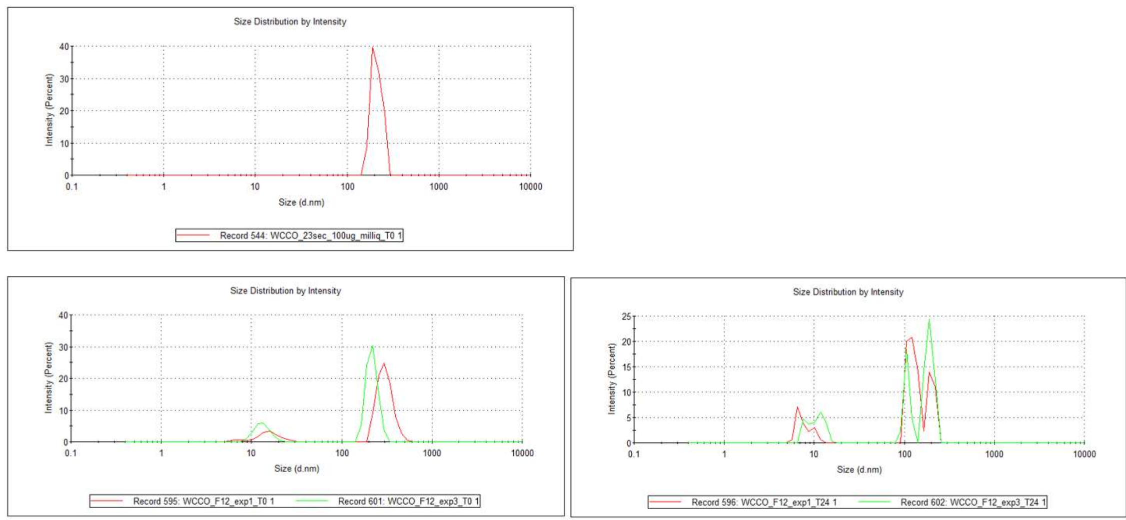


Figure 10. CuO (PlasmaChem) DLS measurements. Intensity (%) against Size (d.nm) for Stock dispersion (Top), in DMEM/F-12 (10% FBS) (middle row) at T0 (left) and T24 (right), and in L15 (10% FBS) medium (bottom) at T0 (left) and T24 (right).

WC-Co (NanoAmor)



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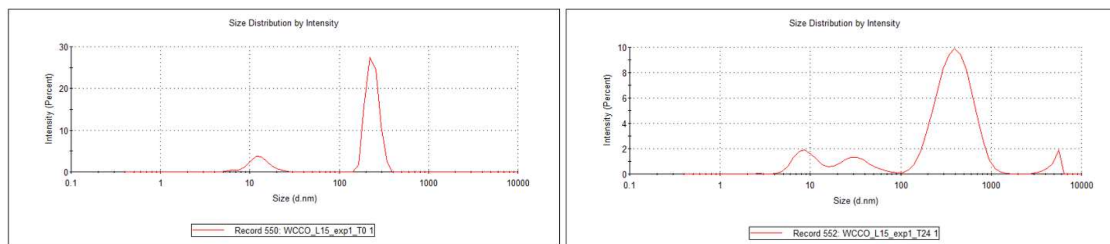

Figure 11. WC-Co (NanoAmor) DLS measurements. Intensity (%) against Size (d.nm) for Stock dispersion (Top), in DMEM/F-12 (10% FBS) (middle) at T0 (left) and T24 (right), and in L15 (10% FBS) medium (bottom) at T0 (left) and T24 (right).

MWCNT (AQUACYL 3 wt. % NC7000-AQ0303)

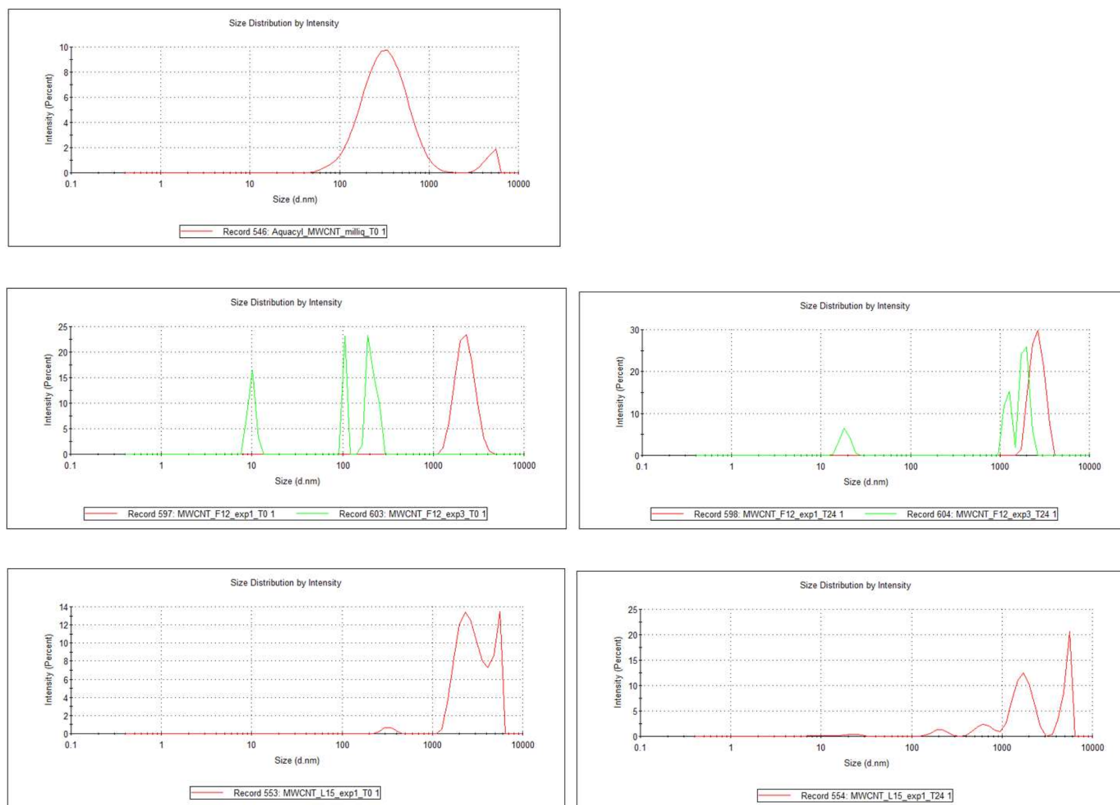

Figure 12. MWCNT (AQUACYL 3 wt. % NC7000-AQ0303) DLS measurements. Intensity (%) against Size (d.nm) for Stock dispersion (Top), in DMEM/F-12 (10% FBS) (middle) at T0 (left) and T24 (right) and in L15 (10% FBS) medium (bottom) at T0 (left) and T24 (right).

3.2 Cytotoxicity test (AlamarBlue and CFE assay with fish cells

For cytotoxicity the RTgill-WI cells (ATCC® CRL-2523™), epithelial adherent cells derived from Rainbow trout, were used. The cells are heteroploid and established from a 15-month old primary culture of apparently normal rainbow trout gill fragments. Prior to being deposited at the American Type Culture Collection (ATCC), the line was found to be contaminated with mycoplasma, and was cured by treatment with BM-Cycline and mycoplasma removal agent.

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The AB test as well as CFE assay were performed on RTgill-W1 cells with the ENMs selected in WP6. The data have been collected into the templates and are uploaded to RiskGONE's share platform (Teams).

Results from the AB assay show no cytotoxicity of any of tested ENM to RTgill-W1 cells. Neither ZnO nor TiO_2 ENMs show cytotoxicity to RTgill-W1 cells, as shown in Tables 3 and 4 below.

As RTgill-W1 cells have very low cloning efficiency it was not possible to evaluate the CFE experiments.

Table 3: Results of the AB test on RTgill-W1 cells exposed to Zinc Ozide (ZnO) ENMs (Zinc oxide (Sigma Aldrich 721077).

	Relative cell viability %	Relative cell viability %	Relative cell viability %	Relative cell viability %	Relative cell viability %
	Experiment 1	Experiment 2	Experiment 3	Average	SD
	100	100	100	100	0
1	109.43	89.84	88.65	95.97488232	11.66992618
2.5	108.9	88.53	91.11	96.17849339	11.08828932
5	113.16	86.21	90.27	96.54873935	14.527186
10	107.65	80.56	90.13	92.78427353	13.73737371
PC_mms	-1.58	-8.6	4.49	-1.89507572	6.552345929
KBrO	97.08	79.76	64.24	80.36205805	16.42659566
SC	96.51	84.32	58.73	79.85227386	19.28068727
INT_100	6.55	6.07	12.74	8.451341823	3.719406833

Table 4: Results of the AB test on RTgill-W1 cells exposed to JRC TiO_2 ENMs ERM00000063).

	Relative cell viability %	Relative cell viability %	Relative cell viability %	Relative cell viability %	Relative cell viability %
	Experiment 1	Experiment 2	Experiment 3	Average	SD
0	100	100	100	100	0
1	102.28	105.47	92.97	100.2376217	6.495992403
2.5	103.89	101.16	97	100.6846705	3.472493244
5	105.21	105.64	102.57	104.4748003	1.660739413
10	107.33	107.03	102.66	105.6742315	2.615048766
PC_mms				0.016635973	1.402815673
	1.61	-0.53	-1.03		
KBrO	85.21	79.02	72.55	78.92641041	6.330633615
SC	82.2	84.01	73.21	79.80501475	5.78621202
INT_100	1.32	30.01	6.78	12.70093178	15.23692404

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3.3 Comet assay with fish cells

The RTgill cells were also prepared for the comet assay. The protocol for used in WP5 was adopted for fish cells with small modification (see Appendix 2).

Unfortunately, RTgill cells appeared not to be suitable for the comet assay due to an extensive formation of micronuclei closely attached to the nucleus of the cells which made the comet scoring challenging (Figure 13). We, therefore, suggest testing another cell line for eco-toxicological safety assessment *in vitro*.

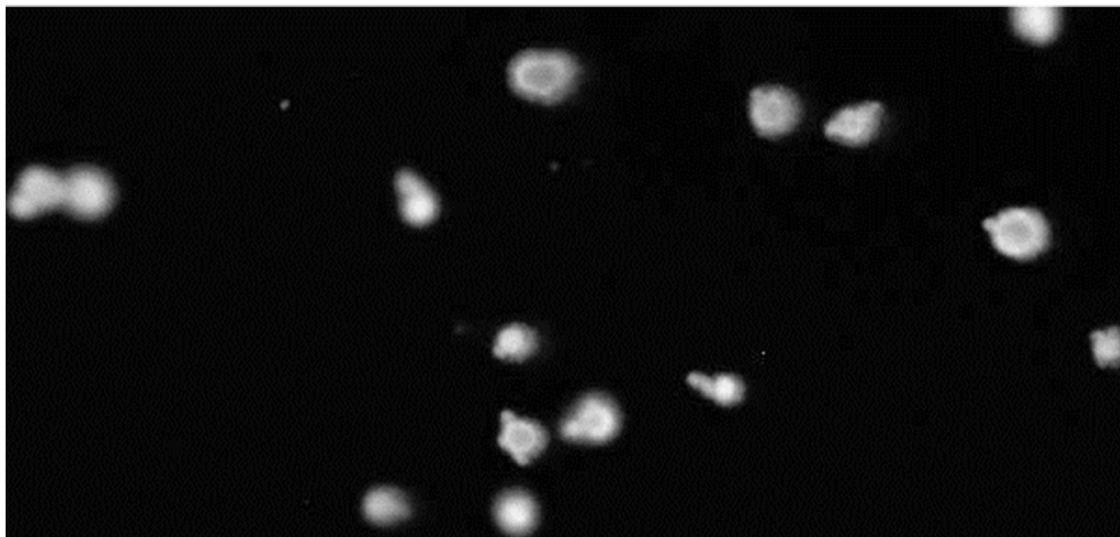

Figure 13. RTgill-W1 cells in Comet Assay, showing micronuclei formation suggesting that this assay is not suitable with this cell line.

However, successful application of miniaturized version of the Comet assay was applied for Zebrafish embryo cells (ZF4 cells) as shown in Figure 14. Three individual replicates were used to calculate the mean intensity of the scored tail percentage (%) using comet IV assay macro software by Instem solutions, https://www.instem.com/solutions/genetic-toxicology/comet-assay.php. Table 4 shows summary data for three sizes of AgNPs exposed to the ZF4 cells at 3 different concentrations. Confocal reflectance microscopy (Figure 15) confirmed the localization of the ENMs in the ZF4 cells and was used to quantify the Ag content (Table 5). Further work to confirm the suitability of the ZF4 cell line for use in ENM toxicity testing is reported in a series of publications - Quevedo et al., 2021a, 2021b, 2021c.

Table 4: Summary of the DNA percentage strand breaks (%). Results represent the mean DNA tail percentage of three individual replicates, obtained by scoring 50 comets per replicate, for a total of 150 comets per treatment. Comets were analysed and scored using IV comet macro software. H_2O_2 was used as the positive control.

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AgNPs					
AgNPs Size (nm)	**Naive**	**H_2O_2 200µM**	**2.5 AgNPs**	**5 AgNPs**	**10 AgNPs**
10nm	1.88 ± 1.45	46.81 ± 6.02	7.55 ± 2.61	8.70 ± 4.52	11.45 ± 3.53
30nm	1.88 ± 1.45	46.81 ± 6.02	8.32 ± 6.03	8.88 ± 9.69	10.46 ± 5.02
100nm	1.88 ± 1.45	46.81 ± 6.02	6.36 ± 0.86	6.62 ± 2.63	10.13 ± 4.56
$AgNO_3$					
$AgNO_3$	**Naive**	**H_2O_2 200µM**	**1 $AgNO_3$ (µg/mL)**	**1.5 $AgNO_3$ (µg/mL)**	**2 $AgNO_3$ (µg/mL)**
	1.88 ± 1.45	46.81 ± 6.02	79.48 ± 2.67	82.67 ± 1.46	82.90 ± 0.91

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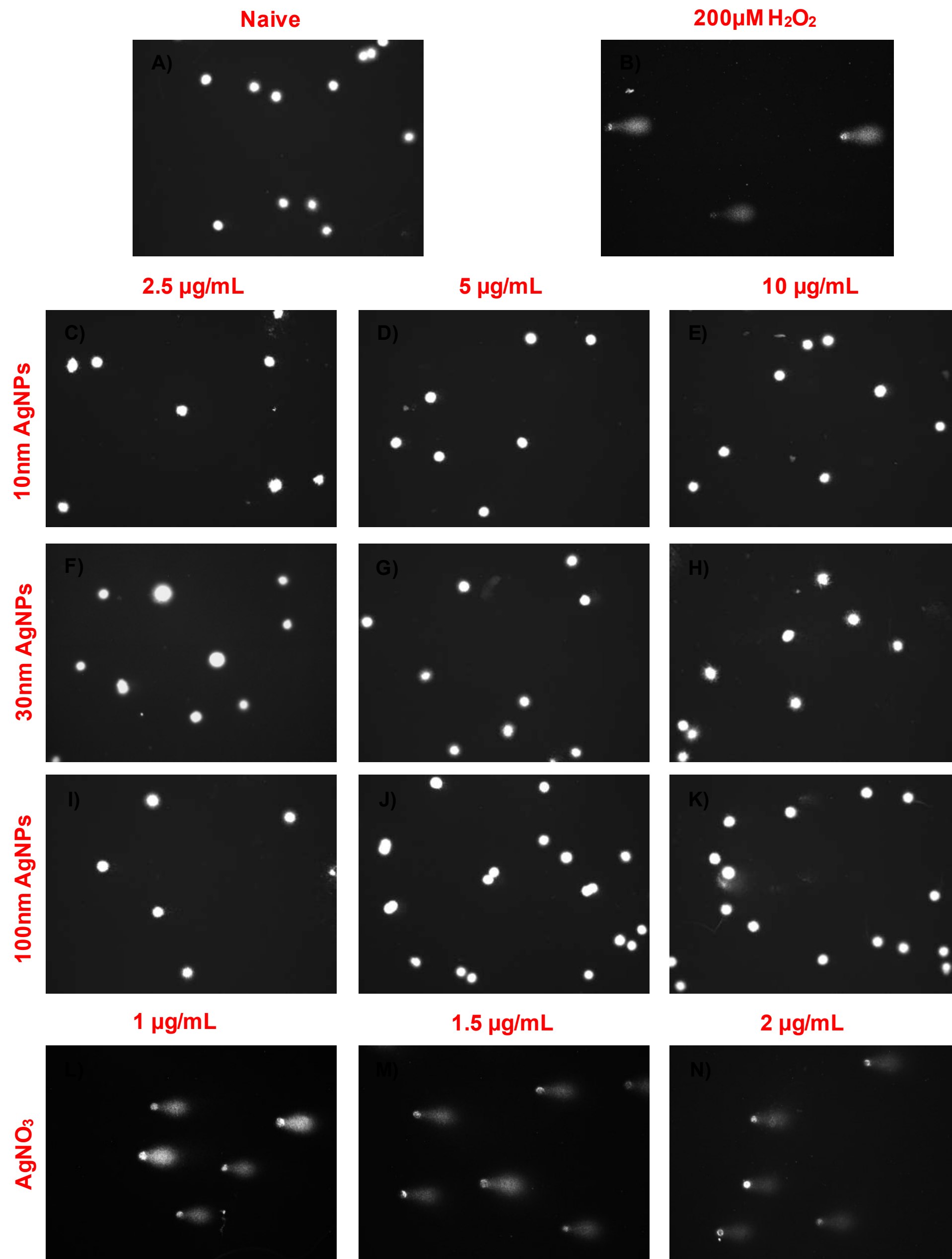

Figure 14. Images of the acquired comets from ZF4 cells exposed to the 3 sizes and 3 concentrations of AgNPs as indicated in the figure labels. Three individual replicates were used to calculate the mean intensity of the scored tail percentage (%) using comet IV assay macro software. The images here represent only one of the three replicates for each treatment.

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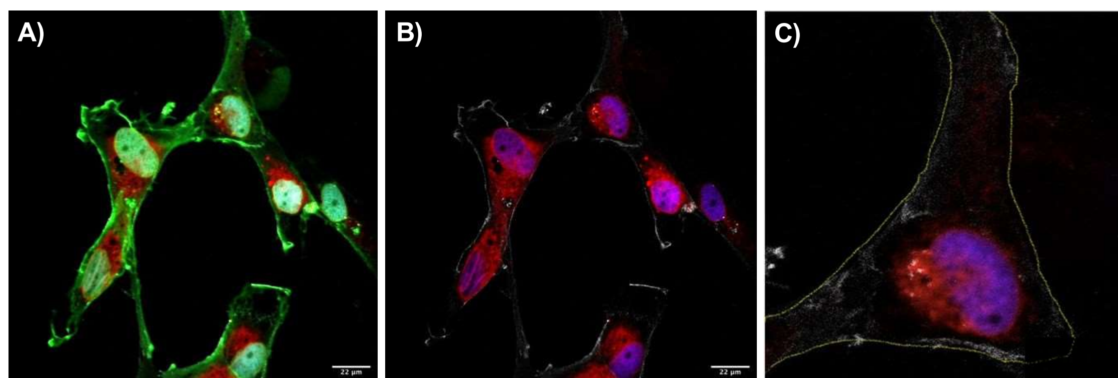

Figure 15. Example of the manual analysis of the AgNP reflectance intensity following exposure of the ZF4 cells to 10 nm AgNPs for 2 hours at 2.5 µg/mL. A) Composite image of a group of cells. Green shows the cell membrane, blue/light green the nucleus, red are the lysosomes and white are the NPs. Due to interference between the reflectance channel and the dye for cell membrane (A), the green channel was removed to visualize better the presence of the NPs for images B and C. B) Reflected intensity of the AgNPs – comparison with A indicates that the NPs are broadly associated with the cellular membranes. C) Zoomed image of a cell in figure B to show the manual drawing of the region of interest (ROI) by FIJI. The scale bar for all the images is 22 µm. Images were taken with a NIKON A1R 808 series microscope at 60X objective. Reproduced from Quevedo et al., 2021b.

Table 5: Calculation of mass concentration and NPs/mL. The mass concentration (10 µg/mL) was calculated to obtain the number of particles per millilitre (NPs/mL). Reproduced from Quevedo et al., 2021b.

AgNPs size	NPs/mL
10 nm	1.76E+12
30 nm	5.85E+10
100 nm	1.67E+09

The fact that the Comet assay is only applicable in some fish cells is an important finding, and more work is underway to determine whether this was a consequence of the specific ENMs tested or a general feature of the specific cell type / fish type. A more thorough review publication on the topic is planned.

3.4 xCELLigence measurements

Cell density screening of ZF4-cells

A cell density screening was performed on RTgutGC and ZF4-cells to select the optimal density for the toxicity testing and based on the results, a RTgutGC density of 10000 cells/well and a ZF4-cell density of 8000 cells/well were selected.

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Viability and proliferation of cells exposed to ENMs used in RR1 and RR2

For the toxicity screening, the following cell densities were used: and 10000 RTgutGC cells/well. The effects of RR1 ENMs were tested on RTgutGC (10000 cells/well). The effects of RR2 ENMs were tested on both RTgutGC (10000 cells/well) and ZF4 cells (8000 cells/well). The CI plots obtained by impedance sensing with the xCELLIgence system are presented bellow (Figs. 16 to 20). The overview of the results and the statistical analysis (One-way ANOVA and Tukey, p< 0.05) are presented in Figs. 21 to 23.

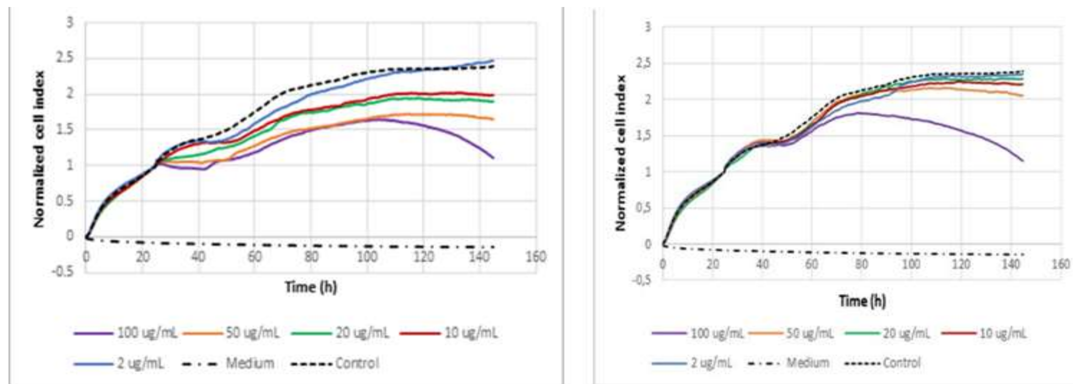

Figure 16. Normalized cell index (CI) [a.u.] plotted against Time [hr] for RTgutGC exposed to TiO_2 (JRC) (left) and ZnO (Sigma) (right). The cells were exposed to ENMs 24h after seeding.

CuO (PlasmaChem)

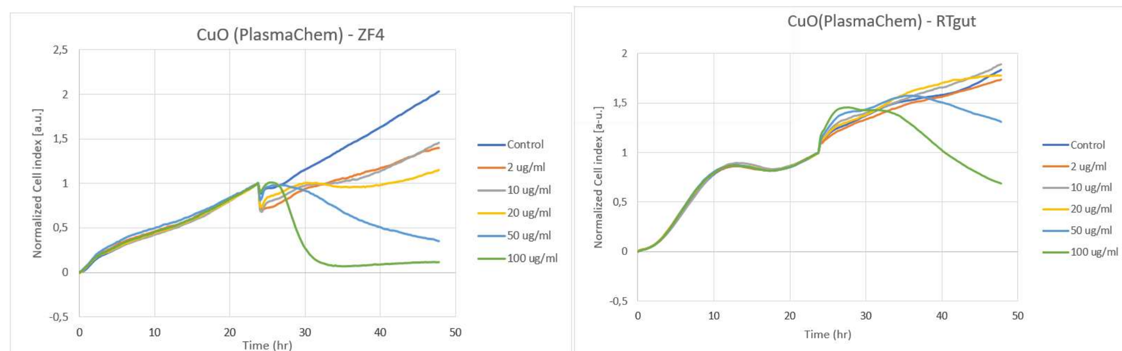

Figure 17. Normalized cell index (CI) [a.u.] plotted against Time [hr] for ZF4 in DMEM/F-12 (left) and RTgutGC in L15 medium (right) exposed to CuO (PlasmaChem). The cells were exposed to ENMs 24h after seeding.

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WC-Co (NanoAmor)

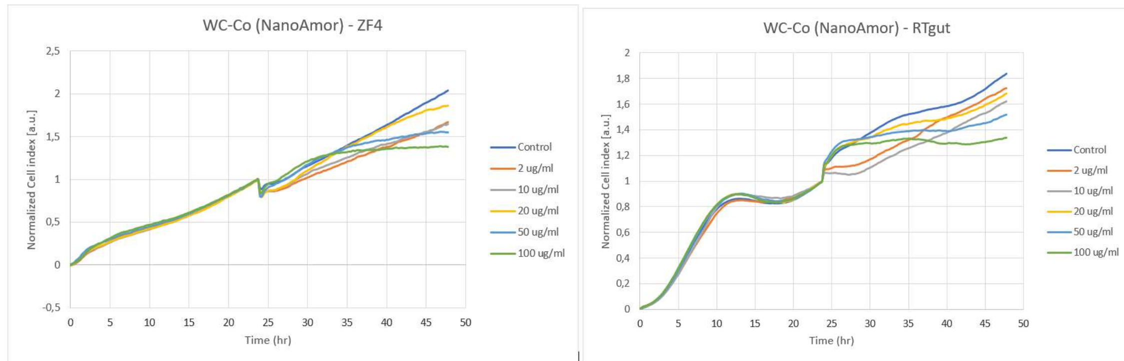

Figure18. Normalized cell index (CI) [a.u.] plotted against Time [hr] for ZF4 in DMEM/F-12 (left) and RTgutGC in L15 medium (right) exposed to WC-Co(NanoAmor). The cells were exposed to ENMs 24h after seeding.

MWCNT (AQUACYL 3 wt. % NC7000-AQ0303)

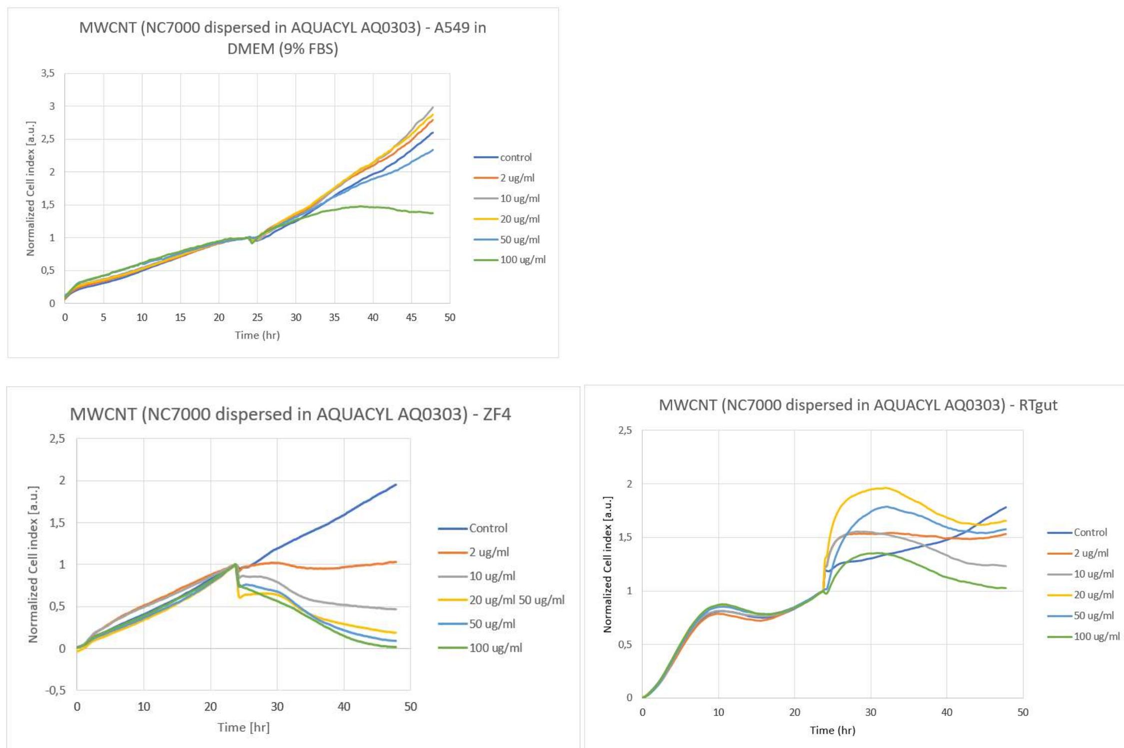

Figure 19. Normalized cell index (CI) [a.u.] plotted against Time [hr] for ZF4 in DMEM/F-12 (left), and RTgutGC in L15 medium (right) exposed to MWCNT (AQUACYL 3 wt. % NC7000-AQ0303). The cells were exposed to ENMs 24h after seeding.

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Dispersant (Nanocyl) – Proprietary Dispersant without MWCNT (AQUACYL 3 wt. % NC7000-AQ0303) particles.

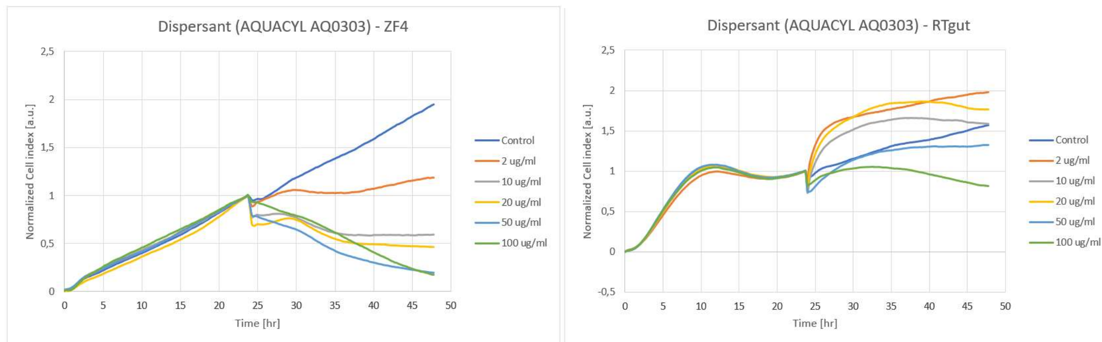

Figure 20. Normalized cell index (CI) [a.u.] plotted against Time [hr] for proprietary dispersant - ZF4 in DMEM/F-12 (left), and RTgutGC in L15 medium (right). The measurements were done only with the dispersant in the amounts used to achieve the exposure concentrations for MWCNT (AQUACYL 3 wt. % NC7000-AQ0303). The cells were exposed to dispersant 24h after seeding.

Fold-change vs control for particles in RR1 and RR2

RR1-Particles

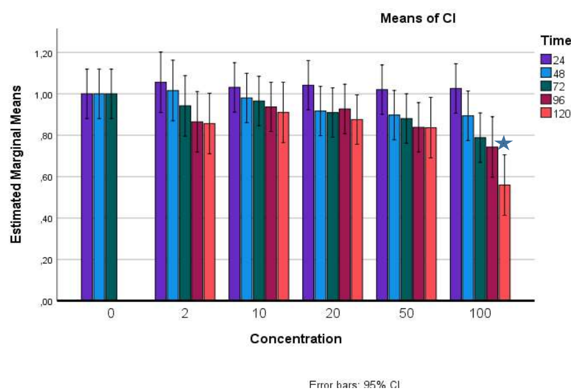

Figure 21. Fold change vs control over time for exposure of RTgutGC cells to TiO_2 (JRC). * Statistical significance in respect to control, $p<0.05$ (Two-way ANOVA, Tukey).

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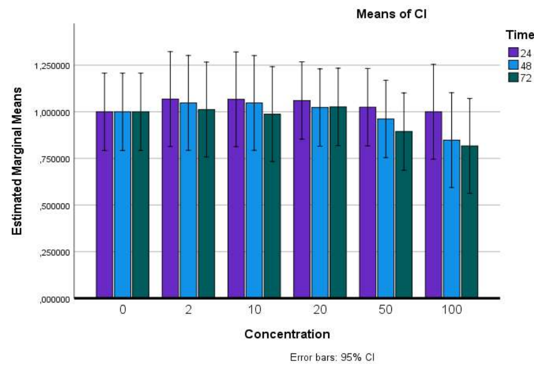

Figure 22. Fold change vs control over time for exposure of RTgutGC cells to ZnO (Sigma-Aldrich). * Statistical significance in respect to control, p<0.05 (Two-way ANOVA, Tukey).

RR2-Particles

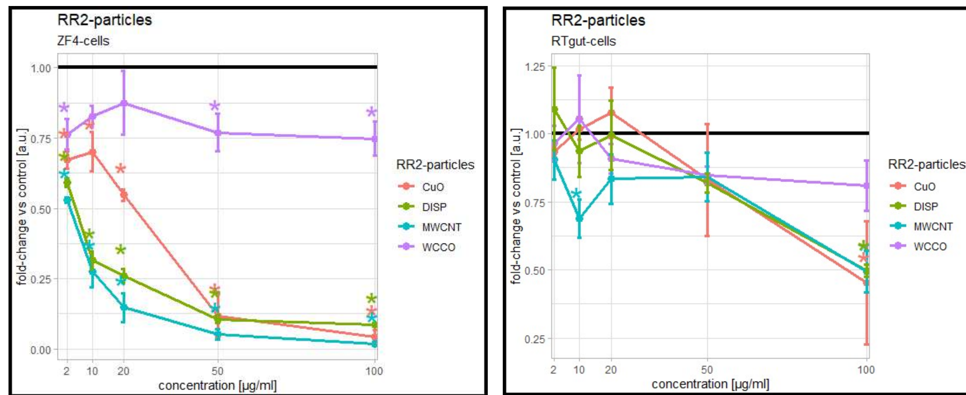

Figure 23. Fold-change vs control after exposure to RR2-particles: ZF4 cells (left) and RTgutGC (right) at 2, 10, 20, 50 and 100 ug/mL for 24h exposedto RR2 ENMs. Increased proliferation can be correlated to values above 1 and cell death to values below 1. * Statistical significance in respect to control, p<0.05 (Two-way ANOVA, Tukey).

3.5 Daphnia reproductive effects as a result of ENM exposure

3.5.1 Reproductive delays

Our experimental findings indicate that exposure to ENMs leads to delays in maturity and consequentially reproduction, resulting in delays to release of the first brood and subsequent broods of exposed daphnids. An extension to the test observation timeframe from the current 21 days to 28-30 days would enable a clearer picture to be established, by capturing the delayed broods that would be missed under the current timeframe. In addition, the induction of males is also a known toxicity response in *Daphnia,* and therefore it would be beneficial to capture the percentage of males, ephippia or fertilised eggs per brood during the test duration.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

3.5.2 Preconditioning of particles

The use of *Daphnia* conditioned medium to disperse the particles will allow a biomolecule corona to form prior to the exposure which will prevent membrane disruption as a result of exposure to unconditioned particles and therefore ensures that a more accurate effect concentration can be established. This can be by using medium from the running *Daphnia* cultures to exposure the particles, or could be additional *Daphnia* cultures that are age-matched to the life stage of the test organisms.

As shown in Figure 24, the toxicity of particles aged in the medium (even if salt-only medium) is much lower than that of freshly dispersed particles, and thus we recommend testing both freshly dispersed and medium-aged particles to see the range of potential effects.

Using four conditions (pristine and transformed/aged ENMs in salt-only medium versus environmentally relevant natural organic matter (NOM)-containing artificial water), we have demonstrated that even where toxicity is apparently reduced, some lasting effects persist in the subsequent generations affecting their apparent age and their reproductive success (Ellis et al, 2021). Sublethal effects from exposure to "pristine" TiO_2 ENMs in both the Daphnia culturing medium and the artificial river water representative were observed. Identical exposures of the aged TiO_2 ENMs in the artificial river water representative resulted in dramatically decreased effects on the *D. magna*. However, although the aged ENMs presented here appeared "safe" with fewer toxic consequences in the F0 generations, when the progeny were investigated (F1–3) as paired continuously exposed versus removed from the maternal exposure, there was still evidence of inherited dysfunction (Ellis et al, 2021).

3.5.3 Internalisation quantification

The internalised concentration of nanomaterials cannot be predicted using the octanol-water partition coefficients or equilibrium concentrations, and as such dose-response relationships can only be established experimentally. The method used to ascertain the internalised concentration will depend on the nanomaterial composition. Inductively Coupled Plasma Mass spectrometry (ICP-MS) is recommended for quantitative analysis, and we suggest doing this on organisms before and after 24 hours of depuration in clean medium, in order to distinguish particles in the gut lumen versus those embedded in the bush border or internalised by endothelial cells of the gut barrier. The number of timepoints at which to measure particles can be kept to a minimum (at the end of the experiment for example), matched to brood timings, or standardised (e.g., days 1, 3, 7, 14, 21 and 28) - the implications of these scenarios for experimental set-up and data re-use potential will be discussed. Visualization of the sub-cellular localisation of the nanomaterials, by Transmission Electron Microscopy (TEM) is also recommended and visualisation of any phenotypic or morphological changes to the daphnids following exposure to the nanomaterials, such as loss of tail length, presence of lipid deposits, changes to appendages or eyes etc. is also recommended, and such images have already been used to develop predictive models based on deep learning (Karatzas et al., 2020). Inclusion of mechanistic endpoints such as genotoxicity (e.g., Comet assay) is also recommended, with the goal of extracting maximum information from chronic studies.

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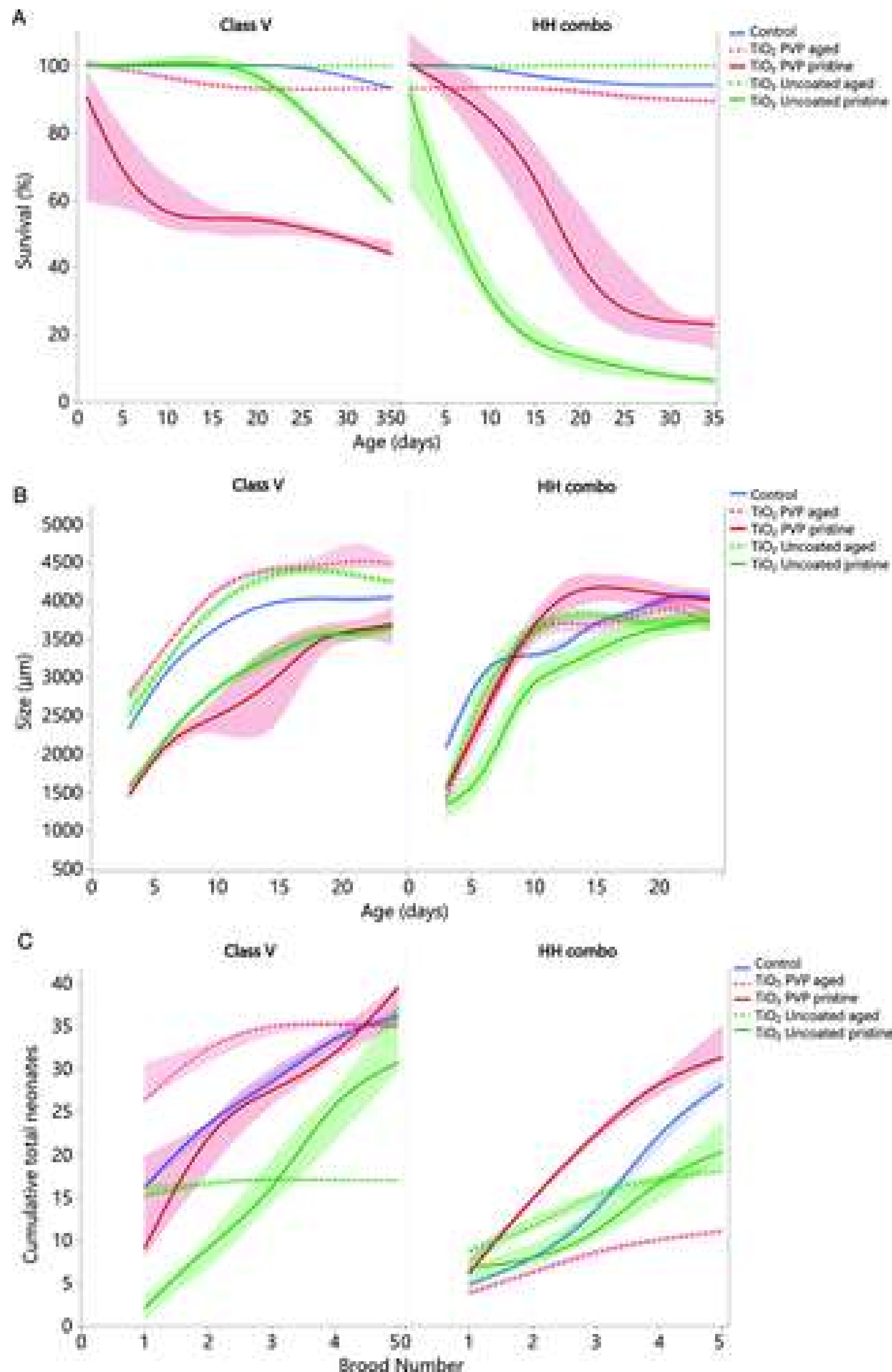

Figure 24: Aged TiO_2 ENMs are less toxic than the pristine ones in the F0 generation exposure: a) survival (%) versus age, b) the size versus age, and c) the cumulative total of the average neonates produced per daphnia for each brood. Each of the graphs (a–c) show the results for each medium condition and NM type (pristine vs aged) for each of the two coated TiO_2 ENMs. The data are expressed as the mean ± the standard deviation (represented by confidence bands). Reproduced from Ellis et al., 2021.

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2.5.4 Comparison in various test media representing the compositions of natural waters

As with many biological elements, there is a narrow concentration range between deficiency and toxicity which needs to be carefully considered. Testing medium needs to be suitable for the test species (i.e., algae and *Daphnia*) and a fully defined medium is preferred for the standardisation of the assessment, as opposed to borehole water. Limitations of key elements, such as calcium for the carapace development, have been shown to significantly impact the growth and development of *Daphnia* (Hessen, 2000).

The medium also has the potential to impact the test species or toxicant in question; for example previous research has highlighted the need to ensure that there are no salts in standardised test medium solutions that will react with potential metal toxicants and thus change the overall metal toxicity, leading to the modification of the OECD medium by removing the EDTA when testing toxicants containing metals, or using an alternative medium that contains no chelating agents (OECD202).

When comparing the laboratory testing media, it become apparent how deficient the ARW1 medium is in the macro elements required by *Daphnia* for healthy growth and maintenance (see Figure 25). HH COMBO, ARW5 and borehole water were more comparable, although borehole was significantly lower in sodium in comparison to the other media tested.

Additional results on impacts of the tested ENMs on D. magna are included in the Deliverable Report D2.4 on the initial consultation on the AOP for daphnia reproduction, and the most recent data is included in the final AOP deliverable to confirm the Key events identified.

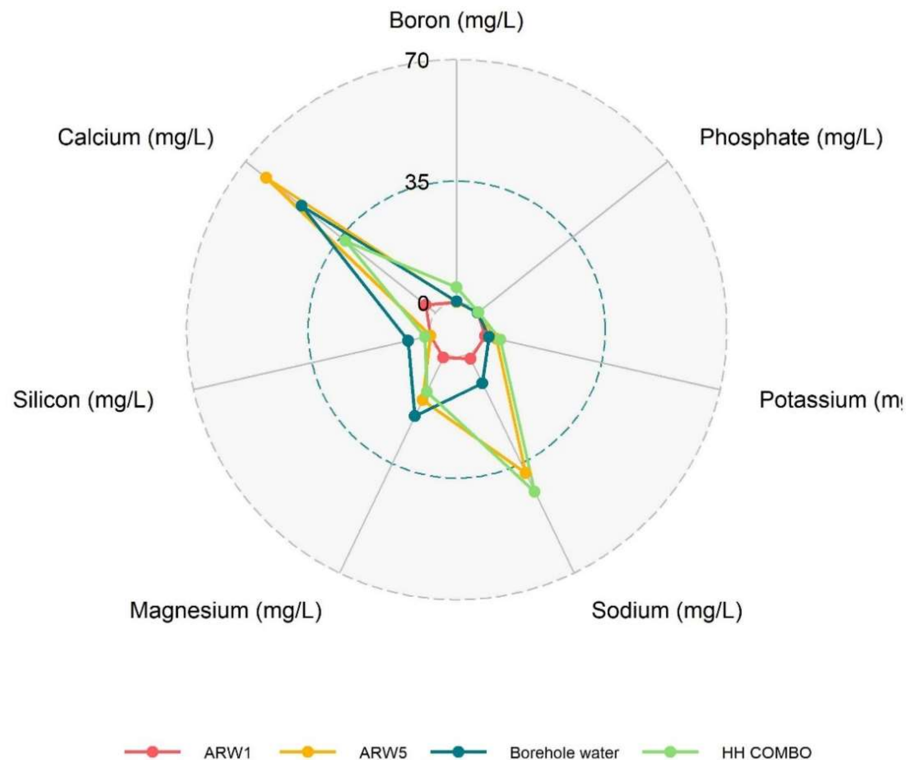

Figure 25. Radial diagram comparing the compositions of the 4 tested media in terms of their compositions of 7 macronutrients determined to be essential for *Daphnia* health and fecundity

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4. Conclusions and next steps

Cytotoxicity assessment

The SOP for RTgill-W1 cells cultivated *in vitro* has been developed and the cells have been used for testing ENMs. Two cytotoxicity methods (AB and CFE) have been adapted for testing ENMs in fish cells. While the AB assay appeared to be suitable for use with fish cells, the CFE could not be applied due to low cloning efficiency. Several other cell lines will be tested for cloning efficiency in the future.

Genotoxicity assessment

The Comet assay has been successfully adapted for eco-genotoxicity screening and the SOP both for fish cell culture was developed. While the comet assay appeared to be sensitive to detect DNA strand breaks in zebrafish embryo cells, the evaluation of DNA damage in RTgill epithelial cells originating from Rainbow trout was challenging due to multinuclear shape of the comets. The next step will be to compare several fish cell lines and to select the most suitable one for testing genotoxicity of ENMs in fish cells.

Similarly, the Comet protocol is currently being applied to whole daphnids to assess the role of genotoxicity in ENM-induced impairment of reproductive success.

Impedance based ecotoxicity assessment

In terms of the label-free impedance based cytotoxicity assessment, the ZF4 cells and RTgutGC reacted differently to different ENMs and ENM concentrations.

ZF4 cells appeared to be the most sensitive following exposure to ENMs. CuO (PlasmaChem), MWCNT (AQUACYL 3 wt.% NC7000-AQ0303) and the dispersant (without MWCNT AQUACYL 3 wt.% NC7000-AQ0303), for which a significant toxic effect was observed at all concentrations. For WC-Co (NanoAmor) a significant toxic effect was seen at the highest two concentrations.

RTgutGC cells were affected to a lesser extent, compared to ZF4 cells, as a significant toxic effect could be seen at 10 ug/mL and the highest concentration for CuO (PlasmaChem) and at the highest concentration for MWCNT (AQUACYL 3 wt.% NC7000-AQ0303) and dispersant (without MWCNT AQUACYL 3 wt.% NC7000-AQ0303), while for WC-Co (NanoAmor) no significant toxic effect could be seen at any of the concentrations for the RR2 ENMs.

The MWCNT (AQUACYL 3 wt.% NC7000-AQ0303) and dispersant (without MWCNT AQUACYL 3 wt.% NC7000-AQ0303), as well as CuO ZnO (Sigma-Aldrich) were more toxic than TiO_2 (JRC), ZnO (Sigma-Aldrich) showed some effect, and WC-Co (NanoAmor) at the concentrations and exposure times that were employed.

The fact that the zebrafish cells behaved differently to the rainbow trout cells in all the assays applied to date is also interesting and warrants further investigation as to the potential sources of these differences – either in terms of the underlying fish biology, or the limitations of the assays for the different cells in terms of the adherence, receptors for uptake of ENMs and so forth. This will be a key focus of the last period of the RiskGONE project.

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Impacts of ENM on daphnia reproductive success and population level effects

Based on the extensive work performed to date, a key outcome from RiskGONE will be the development of an SPSF on updating of the OECD Test No. 211: *Daphnia magna Reproduction Test* for use with engineered nanomaterials (ENMs) and other advanced materials.

The RiskGONE SPSF proposal being developed for the WPMN aims to address the following main issues:

1) incorporation of ENMs specific considerations that affect the relevance and reproducibility of the results;
2) inclusion of some 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).
3) extension of the assay to include 1-3 additional generations using a paired approach of parent only exposure versus continuously exposed to allow assessment of sensitisation versus adaption to low-level pollution.

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

This is a key aspect of the revision, as we have demonstrated important and significant epigenetic changes in daphnids (Ellis et al, 2021), as a function of exposure time throughout the parent daphnid lifetime (Ellis et al, 2021b), by assessing the reproductive fitness of continuously exposed versus parent-only exposed daphnids. Developed of a panel of genes for targeted assessment could be useful based on the identified epigenetic changes, or design of an eco-corona test to determine secreted proteins indicative of mode of action would be useful additional endpoints to support a tox21 and NAMs approach to regulation. At the very least, reproductive success in terms of the total number of living offspring produced per F1 and F2 daphnia 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 should be reported.

In the F1-F3 generations, as per the existing TG 211, the reported end-points would include: the daily counting of the offspring, the daily recording of the parent mortality, the 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. The reproductive output of the animals in each generation exposed to the test substance would be analysed, by comparing it with that of the control in order to determine the lowest observed effect concentration (LOEC) and hence the no observed effect concentration (NOEC), and by estimating the concentration that causes an x % reduction in reproductive output. Comparison of these values across generations and between the continuously exposed versus F0 generation only exposed, would provide important new insights into ENMs toxicity and potential population level effects that are currently missed. We note that very low effective concentrations (EC) of the ENMs would need to be tested to ensure survival into the subsequent generations, which was as low as EC3 for some of the TiO_2 ENMs tested in our previous studies (Ellis et al, 2021, 2021b).

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

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This project has received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement No 814425.

Appendix 1: SOP for high throughput CFE assay (highlighting adjustments for fish cell lines)

Reproduced from Rundén-Pran et al, 2022; https://doi.org/10.3389/ftox.2022.983316

Materials and equipment

Materials

Cells (adherent cell line), flasks 25 cm^2 or/and 75 cm^2, 12-well (or 6-well) plates, sterile plastic centrifuge tubes, microcentrifuge tubes, serological pipettes, pipettes and tips, cell culture medium (according to cell line) and additives (e.g., serum, Penicillin-Streptomycin), trypsin-EDTA, methylene blue (CAS number 122965-43-9), filtration paper, phosphate buffered saline (PBS), CO_2, distilled water, ethanol, Bürker chamber + Cover slips 22 × 22 mm/Cell counter slides, trypan blue stain 0.4%, ink pen or e-count pen.

Equipment needed

Laminar flow hood, light microscope, automated cell counter/Bürker chamber, pipettes, CO_2 incubator, refrigerator, water bath, vortex, autoclave.

Solutions

Preparation of methylene blue (1%): I g of methylene blue is dissolved in 100 ml of MilliQ water. Filter through filtration paper. It is not necessary to sterilize it. The solution can be kept at room temperature.

Methods

The CFE assay is performed on individual cells plated out in small inoculum (for fish cells i.e., -50-500 cells per well) on 12- (or 6) well plates at around 16 h (h), depending upon growth rate of the cells, before treatment. The cells should not divide after seeding before exposure. Then, cells are exposed to the test compound, positive and negative controls and cultured to allow for colony formation, generally for 5–12 days (d), depending on cell type and their doubling time. The colonies are stained and counted manually or by automated scoring.

Cell lines and preparation of culture

Human or other mammalian cells growing attached to the surface such as V79, A549 or HepG2 cells (El Yamani et al., 2017; Lee et al., 2022), are commonly used with the assay as they have high cloning efficiency. Any adherent cells growing with high cloning efficiency can be used including fish cells. However, so far fish cells are not commonly used for CFE. Thus, before applying fish cells to the CFE the ability of individual cells to form colonies (e.g. cloning efficiency) when seeded in low number (50-500 cells), must be tested. Cells in small inoculum are cultivated in complete culture medium and incubated in culture dishes or flasks in a cell incubator at 18-22° C and without addition of CO_{2-2} as described in the standard operating procedure (SOP) for cultivation of the fish cell line.

Seeding of cells for exposure

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RTgill-WI cells are cultured in Leibovitz's LIS medium supplemented with 9% or 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Additional reagents used are PBS-EDT A (0.2g EDTA(Nw1) per liter of PBS (0.48mM EDTA in PBS)} and Trypsin-EDT A (0.25%). The solution is stored at 4°C. All the reagents should be sterile and all the manipulations should be done in the laminar box to maintain the sterile conditions. The cells are cultivated at I 9°C, but temperatures between 18-22°C can be used. Medium and reagents should be kept in similar temperature range during cell handling. The cells grow attached to the surface (on Petri dishes, plates or flasks) in a regular incubator in the dark without CO2 in the atmosphere. When cells are approximately C95 % of confluence they need to be split and transferred to a new flask or dish:

1. Remove medium and rinse the cells with PBS-EDTA.
2. Remove PBS-EDTA and add 0.25% trypsin-EDTA to cover the whole surface (~1 ml in a 75 cm2 flask or petri dish) and incubate in the incubator at 18-22° C, for approximately 5 minutes. Remove excess trypsin. Observe in microscope until the cell round up and detach.
3. When the cells are rounded up (detaching), after approximately 3-5 minutes, add about 5-10 ml cultivation medium and properly re-suspend the cells immediately by pipetting. Take an aliquot of the cell suspension and calculate the number of cells with Countess or Burker chamber.
4 To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. A careful tapping on the flask is ok. Cells that are difficult to detach may be placed at 3 7°C to facilitate dispersal.

5. Transfer required number of cells into cultivation flask or Petri dish (e.g. 1-3 x 106 cells to 75 cm2 flask) with fresh medium. Spread cells well on the surface.

6. Grow the cells in a regular incubator in the dark without CO2 in the atmosphere at 18- 220C.

7. Check the cells in the microscope if they should be split. Cells are generally split or fed orrce a week, but this depends on the seeding density.

8. Flask should be labeled with the name of the cell line, the number of the passage, the date and the name (or initials) of the operator.

Seeding density examples:

- 25cm^2 flask: about 3 x 1 as cells in 5 ml medium
- 75cm^2 flask: Normal culture: 1-3 x10^6 cells in 10 ml medium. Seeding for weekend (Friday-Monday): 0.7 - 1.0 x10^6 cells in 15 ml medium

Preparation of cells for exposure and passaging

48-well plates should be added 5x10^4 cells/well and 96-well plates should be added 3x10^4 cells/well. The cells in the 48-wells form a confluent cell monolayer after 3 to 4 days in the dark at 18-22, whereas the cells in the 96-wells form a confluent cell monolayer after 2 to 3 days.

It is necessary to avoid any risk of infection and contamination. All work must be performed under sterile conditions in laminar flow hood. The temperature of the incubator must be between 18-22° C and without addition of CO_2. The cells are cultured in the dark.

Freezing of RTgill-WI cells:

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Use freezing medium made of 95% culture medium and 5% DMSO. The cells are frozen in 0.5 ml aliquots (e.g. 5 x 1 as cells/tube) at -80°C. Master stock is stored in liquid nitrogen, whereas working stocks can be stored at -80°C.

For the CFE experiment: Seed cells in 12-well plates in low inoculum at appr16 h before exposure. The time is selected depending on the generation time for the cells, as the cells should not divide between seeding and exposure to be able to expose individual cells.

The number of cells to be seeded per well is dependent on the plating efficiency and proliferation rate of the cell line applied. As these fish cells do not have high plating efficiency, seed 50-500 cells/well. See the suggested procedure below:

a. Prepare dilution of 1×10^5 cells/ml. Re-suspend well by pipetting and/or vortexing.
b. Prepare further 1×10^4 cells/ml ($10 \times$ dilution of 1×10^5 cells/ml) e.g., 0.1 ml of suspension of 1×10^5 cells/ml plus 0.9 ml of medium. Vortex.
c. Prepare further 1×10^3 cells/ml dilution e.g., 0.1 ml of suspension 1×10^4 cells/ml plus 0.9 ml of medium. Vortex.

Calculate the volume needed for all wells. It is recommended for more robust data to include six replicate exposure wells, three independent experiments. In case of shortage of test substances, the number of replicate wells can be reduced, but this will increase the margin of error. Place the cells in the incubator to settle before exposure to the test substance and controls. Remember to label the plate and the lid properly to avoid mix-up during the experiment.

Preparation of test ENM and controls

Prepare vials with 2x final concentrations of the test substance, diluted in cell culture media. Negative control is cells exposed to cell culture media only. A positive control should always be included to demonstrate responsiveness of the cells. This is especially important when non-cytotoxic results are obtained for the test substance. A good positive control would be e.g., chlorpromazine hydrochloride (50 µM) or staurosporine (200 nM). Concentrations to be applied should be tested for each cell line, as sensitivity will vary. A solvent control should also always be included. Test at least a concentration of the solvent used for the stock solution of the test substance equal to the solvent amount in the highest concentration of the test substance tested in the experiment. It is recommended to test also lower concentrations of the solvent and establish a concentration response curve.

Proper dispersion of the ENM is required. Dispersion protocol needs to be optimized for each ENM to be tested. The Nanogenotox protocol is a commonly applied dispersion protocol that works for many ENMs and purposes (Jensen et al., 2011). As toxicity of ENMs will depend upon physico-chemical properties, such as size, shape and surface coating, it is important always to perform physico-chemical characterization of the ENM to be tested - both pristine material and in the actual dispersion.

Exposure with ENMs and controls

At appr 16 h after seeding of the cells, they are ready to be exposed. You should use about the same time after seeding for all your experiments for consistency. Negative control, solvent control, positive control and at least three concentrations of the test substance should be applied. It is preferred to

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include more than three concentrations of the ENM tested for establishing a concentration response curve. It is recommended to include two sets of negative controls for increased robustness of the test method. For relatively non-cytotoxic compounds, it is important to test high enough concentration to be able to conclude about the effect. For standard chemicals, the maximum concentration for non-cytotoxic compounds should not be above 5 mg/ml, 5 ml/L, or 10 mM, whichever is the lowest. The concentration range should be selected regarding expected or demonstrated cytotoxicity, solubility in the test system, changes in pH or osmolarity. For ENMs, up to 100 µg/cm^2 should be used. This is equivalent to 380 µg/ml in 12-well plates (1 ml total volume) and 480 µg/ml in 6-well plates (2 ml total volume). However, for adherent cells, a dose metric per area is preferable. The highest concentration might be limited by agglomeration state of the ENM to be tested.

Solvents and ENMs suspension media with unknown effects should be also tested. A solvent control with the highest solvent concentration should be included in the assay. The stocks of test substances should then be prepared accordingly. The maximum solvent concentration depends on the type of solvent, but a general rule is that it should not exceed 5% for water, and 0.5% for solvents different from water or saline, (e.g. PBS and HBSS), such as methanol and DMSO.

A tip on how to choose concentrations: A linear range of concentrations (1, 2, 3, 4…) would normally be too tight, while a logarithmic range (1, 10, 100, 1000) is too much spread out. Steps of ~3-fold (e.g. 1, 3, 10, 30, 100) are often just right.

Expose the cells by adding 0.5 ml (or 1 ml for 6-well) of cell culture medium with diluted test substance (2x final concentration) or control solution, so that in total you will have 1 ml medium/well for 12-well format or 2 ml medium/well for 6-well format. Leave the cells in the incubator for colonies to form. For A549 cells, 9–12 days is sufficient. Exposure could also be stopped after 72 h by removal of medium, washing 3x in PBS and adding new medium (2 ml for 6-well plates and 1 ml for 12-well plates), however it will not be possible to wash out all the ENMs as they stick to the walls of the wells and to the cells (or are taken up). Thus, for ENMs it is recommended to use continuous exposure for the length of the experiment, which is until colonies clearly visible by eyes are formed. For longer exposure time points than what is mentioned above, the cell culture media could be replaced with new medium, with or without the test substance, based on the experimental setting.

Staining and counting of colonies

Colonies should be stained with 1% methylene blue. Add 20 µl methylene blue solution directly into the cell culture medium in each of the wells. Mix well by circular movements of the plate on the bench surface. Leave for minimum 30 min. The staining time can be increased if the staining is very weak. Pipette off all the medium with stain from all the wells. If needed, to reduce background staining, the plates can be rinsed carefully with water after staining but be careful not to wash off the colonies. Turn the plate upside down and leave on the bench to dry. Allow some air between the bench and the plate (e.g., place part of the lid under the edge of the plate).

Put the correct lid on each plate. Count the colonies from the bottom of the plate. Use an ink pen or a cell counter pen (e.g. e-count) to mark each counted colony to avoid double-counting. Only count colonies consisting of minimum 50 cells. Use a microscope to get familiar with selection of colonies sizes for counting. Create a template to note down the number of colonies for each well and each treatment group. Instead of manual counting, automatic counting equipment can be used (e.g., GelCount™ mammalian-cell colonies, spheroid and organoid counter, Oxford Optronix).

Calculation of relative colony forming efficiency

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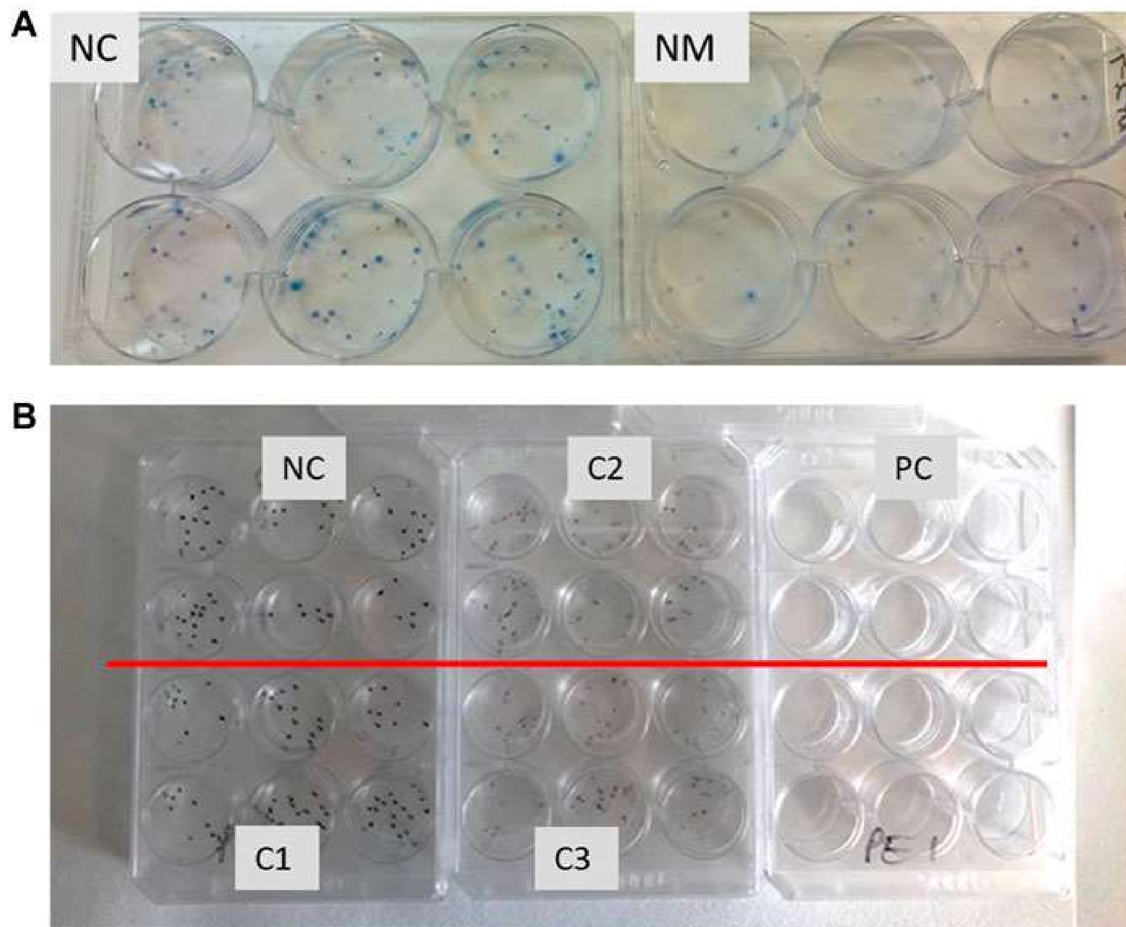
Each viable cell will form a colony (Figure A1). After counting the colonies, the CFE value is calculated as percentage based on the number of colonies formed relative to the number of inoculated cells, following the formula:



FIGUREA1. **(A)** Example of six well plates with cell colonies stained with methylene blue. A549 cells exposed to negative control (NC) and nanomaterial (ENM), showing cytotoxic effect. Six replicate wells were exposed for each sample. **(B)** Example of 12 well plates with cell colonies stained with methylene blue. Each independent sample (negative control NC, positive control PC and tested compound with concentrations C1-low, C2-middle, C3-highest) has six parallels.

CFE (%) = (colonies counted/cells inoculated) x 100.

The relative CFE (RCFE) is the ratio of viability ratio between treated cells and negative control cells. Calculate RCFE as the number of colonies in the exposed sample normalized against the negative control, by using the mean of the replicates for each treatment group:

RCFE (%) = (average number of colonies in treatment plate/average number of colonies in negative control) x 100.

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In addition to the number of colonies, a reduced colony size compared with control indicate a delay in the cell cycle. Thus, it is possible to distinguish between cytotoxic effects (reduction of the number of colonies formed) and cytostatic effects (reduction in colony size).

Interpretation of results

When results are analyzed, it is important to compare with historical control data. Historical control data need to be logged for each laboratory, cell line and test method, and should include data for negative and positive controls to map baseline level for the cell line, as well as responsiveness.

Acceptance criteria for the experiments to be considered valid:

1. Exposure to the positive control must result in significant reduction (50%) or complete cell death (no colonies in the wells)
2. The plating efficiency in negative control should be comparable to historical control data for the specific cell line.

Criteria for characterizing the tested compound as cytotoxic are:

1. Cell viability (RCFE) is reduced by at least 20% compared to negative control
2. A concentration-dependent reduction in cell viability
3. Reproducible effects in at least three independent experiments

A test substance, for which the results do not meet the above criteria, is considered non-cytotoxic under the experimental conditions.

Statistical analysis could be used as an aid in evaluating the test results for example by a parametric or non-parametric statistical test for multiple comparison, such as ANOVA or Kruskal–Wallis test. This can be performed by a statistical software. To compare effects between various substances it can also be valuable to calculate effect concentrations, such as EC_{50}-vaues or benchmark doses (e.g., EC_5). This can be performed by non-linear regression analysis, such as the four parameter Hill-equation.

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Appendix 2: RiskGONE SOP for genotoxicity assessment using the Comet Assay with fish cell lines

Preparation of fish cells

(i) Blood cells:

- Collect blood using a methods such as caudal puncture, which is easily applicable to specimens weighing >200 g.
- Alternatively, adopt more invasive methods such as caudal peduncle transection(e.g. Danio rerio), decapitation and sampling with heparinized capillary tubes in the cardiac region (recommended for very small fish and larval stage) or puncture on posterior cardinal vein or heart (most species)
- only 2 µL of blood are required
- When less than 2 µL of blood is available, to avoid obtaining insufficient cell number in the cell suspension, mix the sampled blood with <1 mL of ice-cold PBS (defined on a case-by-case basis).

(ii) Organs (liver, gills and gonads):

- Collect organs and place and rinse them immediately in ice-cold PBS, to remove blood cells
- Obtain a cell suspension by briefly homogenizing/mincing in PBS a small portion of the tissue into small pieces, using scissors, tweezers or scalpel. This can be followed by a soft mechanical dissociation (pipetting up and down) to further propmote cell dissociation

Additional digestion with trypsin can increase the cells' dispersion (10–15 min depending on the enzyme concentration and temperature of incubation). To get rid of larger tissue pieces, filter the cell suspension using a sterile mesh (usually with 50–100 µm pores). If necessary, centrifuge the cell suspension (5–10 min, 200g, 4 °C), discard the supernatant and resuspend the pellet in 1 mL of ice-cold PBS. Repeat the centrifugation/washing step (usually twice).

Protocol reproduced from El Yamani et al., 2022; https://doi.org/10.3389/ftox.2022.986318

Materials and equipment

2.1 Materials

2.1.1 Consumables and reagents

Cells (adherent or suspension cells), Flasks 25 cm^2 or/and 75 cm^2, Glass microscopic slides, Cover slips 22 mm × 22 mm or 22 mm × 60 mm, Sterile plastic centrifuge tubes 15 ml and 50, Pasteur pipettes 2, 5 and 10 ml, 96-well plates, Microcentrifuge tubes (1.5, 5 ml), Serological pipettes, Pipette tips.

Cell culture medium (according to cell line) and additives (serum, penicillin-streptomycin, etc.), trypsin-EDTA solution (CAS. 59429C, Sigma), phosphate buffered saline (PBS) (Thermo Fisher, 10010049), dimethyl sulfoxide (DMSO) (Sigma-Aldrich, cat. number D5879- CAS. 67-68-5), Trypan Blue stain (Thermo Fisher, cat number 15250), Agarose—Electrophoresis grade normal melting point (NMP) (Fluka, cat number 05066), Agarose Low melting point (LMP) (Sigma-Aldrich, cat number A9414), distilled water, ethanol, Triton X-100 (Sigma-Aldrich, cat number T8787), Bovine serum albumin (BSA) (Sigma-Aldrich, cat number A9418), $CaCl_2$ (Mw = 74.55), $MgCl_2$, H_2O_2, 30%; (Sigma-

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Aldrich, cat number 31642-M), NaOH, Na_2EDTA (CAS 6381-92-6 SIGMA), Tris base (CAS 77-86-1 CALBICHEM), NaCl (CAS 7647-14-5 SIGMA), $KrBO_3$ (CAS 7758-01-2), KCl (CAS 7447-40-7 Sigma), HEPES (CAS7365-45-9 Sigma), KOH (Mw = 56.11), methymethane sulphonate (MMS) (CAS. M4016_ Sigma Aldrich), Fpg, Endo III, SYBR® Gold (Thermo Fisher S11494) (or other stains such as DAPI (4′,6-diamidino-2-phenylindole).PI (propidium idiode).

2.1.2 Equipment and software

Laminar flow hood, light microscope, countess cell counter or Bürker chamber with cover glass, pipettes, automatic pipettes and multi channel pipette (optional), microwave oven, CO_2 incubator, centrifuge, water bath or heat block, fridge 4°C, Incubator 37°C, electrophoresis equipment with power supplier, fluorescent microscope (with CCD camera).

For scoring comets, the use of a computer-assisted image analysis system with commercially available software is recommended to give the most reproducible results. Examples of scoring softwares: Comet assay IV (Instem), Comet Analysis software (Trevigen), Lucia Comet Assay™ software (Laboratory Imaging), Metafer (MetaSystems), KOMET 6 (Andor Technology). Several free scoring programs are also available such as Casplab or CometScore. The visual scoring system is an alternative (Dusinska and Collins, 2008) and (Azqueta et al., 2023).

2.1.3 Preparation of slides and solutions

2.1.3.1 Pre-coating of microscopic glass slides

Ordinary grease-free microscopic glass slides are pre-coated with (0.5%) NMP agarose. To prepare 100 ml of agarose solution, weigh 0.5 g NMP agarose and dissolve in 99.5 ml distilled H_2O by heating in a microwave oven. Fill a suitable vessel (Coplin jar or a narrow beaker) with the hot NMP agarose solution and place it in a water bath or a heat block set at (55°C) for approximately 15 min before using it as described below step by step:

- Dip one clean microscope slide vertically in the solution of agarose by holding it from the frosted area.
- Drain off excess agarose by holding the slide vertically for some seconds, then wipe the back of the slide with a tissue and leave the slide horizontally on the bench to dry overnight.
- Mark the coated side with a pencil mark in one corner on the frosted end (e.g., top left) to identify the coated side.
- Dried pre-coated slides can be stacked together in slide boxes and stored at room temperature for several months.

Note. Commercially precoated slides are also available and can be purchased.

2.1.3.2 Preparation of low melting point agarose solution

The LMP agarose solution is made in PBS. The concentration can vary between 0.6 and 1% depending on the cell type and genome complexity. For instance, a lower percentage % of LMP agarose can be recommended when working with plants. For cultured cells, we recommend 0.8% LMP agarose. The agarose can be prepared in batches and stored at 4°C in a fridge. LMP agarose is dissolved in PBS by careful heating in a microwave oven; after about 10-15 s, shake the flask to ensure uniform heating; repeat until the fluid is clear and the agarose completely dissolved. Make small aliquots (e.g.,10 ml per bottle/falcon tube) and keep at 4°C.

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2.1.3.3 Lysis solution

The preparation of lysis takes several hours to dissolve all reagents and to adjust pH. The lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, and 0.01 M Tris-base) is therefore usually prepared ahead in distilled H_2O and kept at 4°C. Generally, all the ingredients are weighed and added before adding distilled H_2O. Triton X-100 at 1% is added to the lysis solution before use. The solution should be mixed properly using magnetic blender and kept at 4°C until use.

2.1.3.4 Enzyme reaction buffer for Fpg

The enzyme reaction buffer (0.04 M HEPES, 0.10 M KCl, 0.0005 M EDTA, 0.2 mg/ml BSA) is prepared in H_2O and the pH is adjusted to 8.0 using KOH (e.g., 8 M). The buffer is used to dilute the enzyme to the desired concentration. The buffer can be used for both Fpg and Endo III.

2.1.3.5 Electrophoresis solution

The electrophoresis solution (0.3 M NaOH, 0.001 M Na_2EDTA) is prepared in distilled H_2O and kept at 4°C.

2.1.3.6 TRIS-EDTA for dilution of SYBR®Gold

The TRIS-EDTA (TE) buffer (2.5 mM Tris-base, 4 mM Na_2EDTA) is prepared in distilled H_2O and pH adjusted to pH 7.5–7.8 (e.g., HCl). The buffer is also commercially available.

2.1.3.7 SYBR®Gold solution

To avoid repeated thawing and freezing, the commercially purchased SYBR®Gold stock can be aliquoted after first thawing (e.g., 50 µl in microtubes) and stored at −20°C. The SYBR®Gold may be further diluted in DMSO and stored at −20°C. On the day of staining of the slides, the stock dye is diluted 10 times in TE buffer. For the 12 mini-gels staining, a drop of diluted SYBR®Gold (20 µl) is placed on top of each gel. The slide is covered with coverslip 22 mm × 60 mm and placed in dark for 5–10 min before visualization under fluorescence microscope.

Be aware DNA dyes are carcinogenic and should be handled with care. Use gloves and collect the waste in a hazard-labelled container.

2.1.3.8 Enzyme preparation

The lesion specific enzymes used in combination with the comet assay are commercially available from different sources. The purchased enzymes are usually followed with instructions for their use. Here, we are describing the procedure for two lesion-specific enzymes used to detected oxidized bases, Fpg and Endo III. These enzymes are isolated from bacteria containing over-producing plasmids. Upon receipt, they should be dispensed into small aliquots (e.g., 5 µl) and stored at −80°C. The final dilution of the working solution varies from batch to batch. A titration of the enzyme is used to find the optimum dilution for comet experiments and is usually carried out by the supplier. The stock solution is diluted using the Fpg reaction buffer described above, with the addition of 10% glycerol; aliquots are stored at −80°C. For use in an experiment, the Fpg is thawed and further diluted with Fpg buffer (no glycerol is needed) following instructions from supplier. It is usually recommended to keep the aliquots all the time on ice until adding to the gels. If any of this working solution is left over, do not refreeze.

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3 Principle and procedure

The principle of the assay is that strand breaks release the supercoiling in DNA loops and allow the DNA to extend towards the anode under electrophoresis, forming comet-like structures; the proportion of DNA in the tail represents the frequency of DNA breaks. Cells that have been experimentally exposed to a NMs, accompanied by appropriate control cells, are mixed with LMP agarose and set as gels on a microscope slide (two large gels or 12 mini-gels) or on a GelBond film (up to 96 gels in a 12 × 8 array) or in more elaborated devices such as CometChip. The cells are lysed with high salt and detergent, leaving the DNA attached to the nuclear matrix as a so-called nucleoid. Digestion with lesion-specific endonuclease is an option at this stage. Electrophoresis follows, and the comets (typically 100 per sample) are quantitated using image analysis software or by visual scoring (Dusinska and Collins, 2008; Azqueta et al., 2023). Due to its high sensitivity and to ensure reproducibility and reduce variability in the results, it is recommended to perform comet assay experiments always in the same manner following a standardized approach and experimental design taking into consideration, amount of medium to be used per treatment, plate layout type, dispersion of ENMs and the series of controls (including agent control and reference standards) to be included (Dusinska et al., 2019). Moreover, historical data for negative and positive controls should be stored as they are key information for conclusion statement. Additionally, to negative control, capping agents' control, positive control and interference controls, at least 4 concentrations of the test substance should be included. The length of exposure to ENMs is also crucial to consider as it should be sufficient for damage to occur. The comet assay normally measures an acute response and thus for testing chemicals in vitro an exposure time from 5 min (e.g., H_2O_2) to 24 h is usually recommended. However, for ENMs testing we recommend at least three hours to ensure cellular uptake. An access to DNA could be dependent on dissolution of the nuclear membrane during mitosis. Partly soluble ENMs could exert their effects in shorter time. Generally, we advise both short (e.g., 3 h) and long (e.g., 24 h) exposure to be conducted within the same experiment. Three independent experiments (including at least two duplicates) are recommended. When preparing the slides with 12 mini-gels and to increase the robustness of the results, it is recommended to include also replicate gels and replica slides in each experiment.

3.1 Exposure

On the day of exposure, the cells seeded in duplicate are exposed to the selected concentrations of the ENM including positive(s), capping agents (s) and negative/vehicle controls and placed at incubator for the required time. Before the end of the exposure, the lysis solution is mixed with 1% Triton-X as described above. The final lysis solution is kept at 4°C until use. The LMP agarose is carefully heated in the microwave oven until completely melted and placed in a pre-warmed bath or thermoblock at 37°C until use.

3.2 Embeding cells in LMP agarose

At the end of exposure , cells are mixed with LMP agarose. In the 12-gel fomat, each gel of 5–10 µl contains between 200–500 cells which is appropriate for image analysis. The volume of the cell suspension added to agarose to make the slides should not reduce the percentage of agarose to less than 0.45%. The cell embeding should be done as soon as possible after cell treatment. From each treatment, 1- 2 gels are made on a pre-coated slide, preferably on 2 replicate slides.

3. 2. 1 Interference controls

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The slides for ENMs interference control are prepared in parallel and as described above with one exception, no incubation time is needed for the ENMs to be tested for interference. At the end of exposure, cells from negative and positive controls are kept to be used for the interference controls. The cells are mixed directly with the tested ENMs in a way to achieve the highest tested ENM concentration in the mixture. The mixture is then directly embedded in agarose as described above. Interference control slides are then placed into lysis solution and incubated into electrophoresis solution for DNA unwinding before electrophoresis.

3. 2 Immersion of slides in lysis solution

Lysis step is an important step and keeping constant lysis conditions will help avoid variability within experiments. Once prepared, the slides are immersed in cold lysis solution already prepared with 1% Triton-X and at 4°C and incubated for at least 1 h or overnight.

3.3 Unwinding in alkaline solution and electrophoresis

At the end of lysis incubation and enzyme incubation, the slides are then placed in the electrophoresis tank filled with cold alkaline electrophoresis solution, side by side, for the unwinding step. This step is also critical and the solution conditions, (e.g., pH, temperature), length of incubation and volume used should be kept constant. The slides (with gel drops) should be totally covered (0.5 cm of solution above). If there are gaps in the tank (few slides), it is recommended to fill the gaps on the platform with some empty slides to maintain the depth of solution over the platform. The period of incubation is usually 20 min at 4°C in dark. At the end of the unwinding step, the electrophoresis is conducted. Electrophoresis should be run at 4°C in a cold room or a fridge if possible. Within the hComet project, conducting electrophoresis at 1 V/cm for 20 min was recommended. The duration of electrophoresis is considered a critical variable and the electrophoresis time should be set to optimize the dynamic range. Longer electrophoresis times (e.g., 30 or 40 min to maximize sensitivity) usually lead to stronger positive responses with known mutagens. It may also lead to excessive migration in control samples.

3.4 Neutralisation and fixation

After electrophoresis, slides are washd twice in cold PBS for 5 min follwoed by dH2O for 5 min. The slide are left to dry horizontally at room temperature (normally overnight). Fixation using 70% ethanol for 15 min followed by absolute ethanol for another 15 min is recommended when using the 12-gel format. The slides are dried overnight and can be stored for months at room temperature as long as they are protected from light and dust.

3.5 Staining, image analysis and data collection

Before image analysis, the gels are stained with SYBR®Gold (0.1 µl/ml in TE buffer) or another specific dye such as DAPI (1 µg/ml DAPI solution in distilled H2O). Slides are analysed using fluorescence microscopy with a computer image analysis program, e.g., Comet assay IV (Perceptive instruments), Metafer (Metasystems) or by visual scoring. We generally analyse at least 50 comets per gel (2 gels per treatment group). Cells close to the edge of the gel are not scored so as to avoid any potential “edge effects”. It is recommended that every gel is scored “blind” to its treatment. The % DNA in tail is considered the most informative parameter (Dusinska et al., 2008). At the end of image analysis, the data are collected in the templates (available through the eNanomapper database, www.enanomapper.com),

4 Data analysis and statistics

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The use of adequate statistical system is recommended (e.g., Excel, GraphPad Prism, SPSS). In general, data from comet assay are processed as below:
• Calculate the median of the ±50 comets (% DNA in tail) per gel replicate.
• Calculate the mean of medians and standard deviation SD (for the replicate gels of the same concentration/sample within the same experiment). Then, calculate the mean value ±SD for all independent experiments (at least three independent experiments are recommended).
• Compare the DNA damage of the positive and negative controls,
• Compare the DNA damage of the tested ENMs with negative control. Consider differences between replicates, differences between controls and treated cells, correlations and concentration response relationship.
The choice of the statistical tests to be applied, parametric or non-parametric tests, depends on many factors such as size of the data, data distribution, number of repeats.

5 Test acceptance criteria

For an experiment to be considered valid, it needs to include:

- Valid positive control: the effect should be within the range of mean ±2× standard deviation of historical control data for the same cells.
- Valid negative control: the background level observed should be within the range of mean ±2× standard deviation of historical control data for the same cell line or cell type.
- Adequate number of cells and concentrations have been analysed.
- The criteria for selection of the highest concentration of the ENMs are met.
  For cell lline: Quality control of test system (mycoplasma test) is shown to be negative

6 Evaluation and interpretation of the results

In addition to fulfilment of the acceptance criteria, since in the case of NMs a concentration response is not always observed (due to agglomeration at higher concentrations), in the EU FP7 project NanoREG2 we developed modified criteria for positive, negative and equivocal genotoxicity response (El Yamani et al., 2022).

A compound is considered positive if there is:
A. a significant increase in strand breaks or oxidised DNA bases at two of the tested concentrations (<30% cytotoxicity) compared to negative controls OR
B. a significant increase in strand breaks or oxidised DNA bases at one of the tested concentrations compared to negative controls AND a concentration response relationship when evaluated with an appropriate trend test.

A compound is considered equivocal if there is a significant concentration response OR a statistically significant increase in strand breaks or oxidised DNA bases at one of the tested concentrations (<30% cytotoxicity) compared to negative controls.

A compound is considered negative if none of the above criteria are met; additionally, all results are inside the distribution of the historical negative controls.

To summarize, negative results indicate that, under the test conditions, the tested ENMs does not induce DNA damage in the cultured cells used. Positive results indicate that, under the test conditions, the ENM tested is potentially genotoxic in vitro. If the response is neither clearly negative nor positive, the test substance is considered equivocal and further testing is needed.

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Appendix 3: RiskGONE SOP for impedance-based cytotoxicity assessment with fish cell lines

Impedance-based real time assessment of cell viability, attachment and proliferation

1. Materials and method

An xCelligence® system (ACEA Bioscience. Inc., San Diego, CA) was used, consisting of a real-time cell analysis (RTCA) station, single-use 96-well E-plates (SP) with gold-plated electrodes at the bottom of the wells, and a computer with dedicated software. The ENMs used in the Round Robin 1 (RR1) testing were: ZnO (Sigma-Aldrich), TiO_2 (JRC), and TiO_2 (Sigma-Aldrich) and in Round Robin 2 (RR2): CuO (PlasmaChem), WC-Co (NanoAmor), and MWCNT (Nanocyl, NC7000™, AQUACYL 3 wt.% NC7000-AQ0303) with proprietary dispersant (Table 1). The types of cells exposed to ENMs were rainbow trout intestinal epithelial cells (RTgutGC) from the Swiss Federal Institute of Aquatic Science and Technology (Eawag, Dübendorf) and Zebrafish embryonic fibroblasts (ZF4) provided by the University of Birmingham, UK (Prof. I. Lynch Laboratory). The experiments involving RTgutGC cells were performed at the Institute for Marine Research (IMR), Bergen, Norway (L. Softeland).

Material and methods

Nanomaterial stock dispersions

- Stock ENM-dispersion (5 mg/mLfor the LIST dispersion protocol, 2.56 mg/ml for the NANOGENOTOX dispersion protocol)
- Pipettes and pipette tips (sterile)
- Leibovitz L15 culture medium, no phenol red (Life technologies-Gibco) supplemented with 10% (v/v) FBS (GE Healthcare-HyClone™) and 1% (v/v) Penicillin-Streptomycin (P/S) (GE Healthcare-HyClone™) – used for for RTgutGC-cells.
- DMEM/F-12 (Dulbecco's Modified Eagle Medium) (Life technologies-Gibco) supplemented with 10% (v/v) FBS (GE Healthcare-HyClone™) and 1% (v/v) Penicillin-Streptomycin (P/S) (GE Healthcare-HyClone™) – used for for ZF4-cells.
- Falcon Tube sterile: 15 and 50 ml (Sarstedt AG & Co).
- Tube rotator SB3 (Bibby Scientific Limited)
- Scale weight
- Glass scintillation vial, cryogenic tubes
- Aluminium foil, antistatic spatula

Nanomaterial stock dispersions were made as specified in the NANOGENOTOX protocol for the WC-Co ENMs (NanoAmor) and as described in the "LIST" protocol for CuO (PlasmaChem), MWCNT (AQUACYL 3 wt. % NC7000-AQ0303), ZnO (Sigma-Aldrich), TiO_2 (JRC), and TiO_2 (Sigma-Aldrich). A 130-Watt probe sonicator (VCX130, Vibra-Cell, 130W, Sonic & Materials) with a 12.8 mm probe with replaceable tip was used at 22% of the maximal amplitude. After sonication, a 200 µg/mL ENM dispersion in complete cell culture medium was prepared by adding the calculated amount of stock dispersion to the medium and mixed using a tube rotator at 40 rpm for 2 minutes. Five concentrations were prepared: 100 µg/mL, 50 µg/mL, 20 µg/mL, 10 µg/mL, and 2 µg/mL (corresponding to 50, 25, 10, 5 and 1 µg/cm^2).

DLS measurements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

DLS measurements were done for ENM stock dispersions and ENM dispersions diluted in cell culture medium at the highest concentration (100 µg/mL) at the beginning of the exposure, i.e., time 0h (T0), and at the end of exposure, i.e., 24h (T24).

Cell culture

- Cell types:
  - Rainbow trout intestinal epithelial cell line (RTgutGC) (Eawag and IMR).
  - Zebrafish embryonic fibroblast cell line (ZF4) (University of Birmingham.
- Leibovitz L15 culture medium (L15), no phenol red (Life technologies-Gibco) supplemented with 10% (v/v) FBS (GE Healthcare-HyClone™) and 1% (v/v) Penicillin-Streptomycin (P/S) (GE Healthcare-HyClone™)- for RTgutGC-cells.
- DMEM/F-12 – Dulbecco's Modified Eagle Medium (Life technologies-Gibco) supplemented with 10% (v/v) FBS (GE Healthcare-HyClone™) and 1% (v/v) Penicillin-Streptomycin (P/S) (GE Healthcare-HyClone™)- for ZF4-cells.
- Nunc™Cell culture treated EasYFlask™: 75 cm^2 (Thermo Fisher Scientific).
- Falcon Tube sterile: 15 and 50 mL (Sarstedt AG & Co).
- Pipettes and pipette tips (sterile).
- Sterile water
- Phosphate-buffered Saline (1 x PBS) (Life technologies-Gibco).
- Trypsin-Versene (EDTA) (Lonza group AG).
- Trypan blue solution 0.4% (Invitrogen™, Molecular Probes™).
- Countess™ cell counter and cell counting chamber slides (Invitrogen™)

Impedance-based monitoring

- RTCA SP system with computer and provided software (ACEA Bioscience. Inc., San Diego, CA).
- 96-well E-plate (ACEA Bioscience. Inc., San Diego, CA).

RTgutGC-cells were cultured in complete L15 medium (10% FBS, 1% P/S), while ZF4-cells were cultured in complete DMEM/F12 (10% FBS, 1% P/S) in 75 cm^2 cell culture flasks with 15 mL cell culture medium. RTgutGC-cells were kept at 19°C with no CO_2 and ZF4-cells were kept at 28°C in 5% CO_2 atmosphere. For all cells, the culture medium was changed every 2-3 days and cells were passaged at 80% confluency. For experiments, cells were used if their viability was above 90%.

Impedance measurements were performed at 15 min intervals using the xCELLigence system. First, 100 µL of complete medium were added to each well, and the E-plate was kept at room temperature for 30 min (equilibration time). The plate was then transferred to the RTCA station inside the incubator, and the background impedance value for each well was measured. Next, the cells were seeded in a 96-well E-plate at a density of 10 000 cells/well for RTgutGC and 8 000 cells/well for ZF4 in a total volume of 200 µL/well. After 24h, 100 µL were removed from each well and 100 µL ENM dispersion in fresh complete medium were added. The exposure ENM concentrations were 2, 10, 20, 50 and 100 µg/mL corresponding to 1, 5, 10, 25 and 50 µg/cm^2 and the exposure duration was 120 h for exposure to RR1 ENMs and 24h for exposure to RR2 ENMs. Fresh complete medium was also added to unexposed cells and to the wells without cells. Cell-free control composed of complete medium containing the highest concentration of ENM (100 µg/mL) were used to identify possible ENM contribution to the impedance measurements. The experiments were run in triplicates and repeated three times.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

To monitor the effects of ENMs on cells, the Cell index (CI) was used, which is calculated by the xCELLigence software as follows*:* $(Zn - Zb)/15$, where *Zn and Zb* are the impedance values in the presence and absence of cells, respectively (http://www.aceabio.com/theory.aspx?cateid=281). In the absence of cells, the CI is zero, and as cells adhere and proliferate, the Cell Index increases. A CI decrease on the other hand indicates detachment of cells and cell death. The CI fold-change of exposed cells at the end of exposure vs. unexposed cells was used.

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Appendix 4: RiskGONE SOP for chronic (reproduction) and multigenerational toxicity to *Daphnia magna*

1. *Daphnia* culturing

1.1 Introduction and principles

As a model organism, *Daphnia* are cultured in a controlled environment to establish the baseline health of the organisms in order to effectively test for toxicity response (deviations from the baseline health observations) to a range of different substances and conditions. The *Daphnia* facility at the University of Birmingham has a primary culturing room to maintain the *Daphnia* cultures at 20°C (±1°C) and a 16:8 light: dark cycle. Bham 2 strain *Daphnia magna* were used for all exposures and experimental work.

1.2 Resources and reagents

- Aerated medium or borehole water
- Glass jars (1L with metal screw tops)
- *Chlorella vulgaris* algal feed* (refrigerated to 4°C)
- Ethanol spray
- Glass pipette
- Light box

*Algae culturing information can be found in Annex 1.

1.3 *Daphnia* medium preparation

Stock solutions of the main salts used for the various *Daphnia* media were prepared and stored in the main prep room in the *Daphnia* facility. Stocks indicated with (*) in the tables below (Table 1-3) are stored at 4°C in the fridge. Aliquots of these stocks were then added to Milli-Q water as required to make up the working concentration of the various media and left to aerate for a minimum of 2 hours but ideally overnight (12 hours).

Table 1. Medium composition and stock salt preparations for Artificial River Water Class 1 based on (1)

ARW1					
pH 7.3-7.7		Stock conc	1L	4L	
		M, g/mol	m, mg	m, mg	(in g)
Powder	Calcium sulphate dihydrate ($CaSO_4$)	172.17	1.722	6.888	0.0068
Powder	Calcium carbonate ($CaCO_3$)	100.09	2.002	8.008	0.008
		C, mol/L	V, µL	V, µL	
Aqueous	Calcium nitrate tetrahydrate ($Ca(NO_3)_2$)	1	5	20	
Aqueous	Magnesium nitrate hexahydrate ($Mg(NO_3)_2$)	1	48.9	195.6	
Aqueous	Sodium bicarbonate ($NaHCO_3$)	1	13.5	108	
Aqueous	Calcium chloride ($CaCl_2$)	1	7.5	30	
Aqueous	Potassium bicarbonate ($KHCO_3$)	1	11.6	46.4	
Aqueous	NOM*	1	1.84	7.36	

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Table 2. Medium composition and stock salt preparations for Artificial River Water Class 5 based on (1)

ARW5					
pH 7.8-8.2		Stock conc	1L	4L	
		M, g/mol	m, mg	m, mg	(in g)
Powder	Calcium sulphate dihydrate ($CaSO_4$)	172.17	155.02	620.08	0.6201
Powder	Calcium carbonate ($CaCO_3$)	100.09	8.007	32.028	0.032
Powder	Magnesium carbonate ($MgCO_3$)	121.41	45.517	182.068	0.182
		C, mol/L	V, µL	V, µL	
Aqueous	Calcium nitrate tetrahydrate ($Ca(NO_3)_2$)	1	165	660	
Aqueous	Sodium bicarbonate ($NaHCO_3$)	1	696.6	5572.8	
Aqueous	Calcium chloride ($CaCl_2$)	1	363.5	1454	
Aqueous	Potassium bicarbonate ($KHCO_3$)	1	85.9	343.6	
Aqueous	NOM*	1	4.6	18.4	

Table 3. Medium composition and stock salt preparation for HH COMBO based on (2)

HH COMBO				
pH 7.6-7.8		Stock conc	1L	4L
		C g/L	V, ml	v, ml
Aqueous	Calcium chloride dihydrate ($CaCl_2$)	110.28	1	4
Aqueous	Magnesium sulphate heptahydrate ($MgSO_4$)	113.5	1	4
Aqueous	Potassium phosphate dibasic (K_2HPO_4)	1.742	1	4
Aqueous	Sodium nitrate ($NaNO_3$)	17	1	4
Aqueous	Sodium metasilicate nonahydrate ($NaSiO_2$)	28.42	1	4
Aqueous	Boric acid (H_3BO_3)	24	1	4
Aqueous	Potassium chloride (KCl)	5.96	1	4
Aqueous	Sodium bicarbonate ($NaCO_3$)	63	1	4
Aqueous	Sodium selinate ($NaSeO_4$)*	40 µg/mL	50 µL	200 µL
Aqueous	VIM*	50 µg/mL	0.5	*2
Aqueous	Animate*	100 mgL	1	*4

1.4 Protocol

1) *Daphnia* were cultured in groups within their respective laboratory medium, typically 15 adults were maintained in 900 mL of medium (in 1L jars) with metal screw tops loosely fitted.
2) Medium was prepared following the standard procedures (see below, section 1.3 medium preparation) and allowed to aerate for a minimum of 2 hours, but typically overnight.

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3) The pH of the medium was checked and altered as required with either 0.1M NaOH or 1M HCl to be within the specified range and any final medium constituent (VIM and animate) were added.
4) Medium was then poured into fresh culturing beakers, and *Daphnia* were carefully transferred using a glass pipette. A lightbox was used to make the *Daphnia* more visible within the jars at this stage if required.
5) Once *Daphnia* were added to the new culturing jars, algal feed was added in the following ratio, 0.5 mg C for days 0-7 and 0.75 mg C for days 7 onwards (based on 15 *Daphnia*/900 mL).
6) Glassware was washed with 70% ethanol spray and hot water to prevent any bacterial growth.
7) To split running cultures, adult *Daphnia* were removed from the medium and neonates were then filtered carefully through a mesh to concentrate them within the medium for exposures, or alternatively a lightbox was used to view the *Daphnia* and neonates were selected and transferred to new culturing jars to start the new generation of running cultures.
   N.B Running cultures were established with neonates from the third brood of running cultures to maintain optimum genetic health of the organisms.

2. *Daphnia* exposures- acute

2.1 Introduction and principles

The acute exposure protocol follows the guidelines set out by the OECD 202 test for *Daphnia* (3). The principle of the test is that daphnids are exposed to a series of different concentrations of a toxicant and are exposure for 48 hours. Observations are made at the 24 and 48-hour intervals to assess for "immobilisation" within the *Daphnia* test, which is defined as a daphnid that is not moving/swimming after gently agitating the vessel for 15 seconds (disregarding any movement of their antennae). Immobilisation is often used as it is hard to visually determine *Daphnia* death without the use of a microscope, and this therefore speeds up the observations. As a result of the immobilisation being used as the end point for the test, results are reported as the effect concentration (EC_{50}) compared to lethal concentrations/dose (LC_{50}).

2.2 Resources and reagents

- Aerated medium or borehole water
- Glass pipette
- Light box
- Toxicants/stock solutions
- Test vessels

2.3 Protocol

1) Neonates were filtered from the running cultures and pooled from the different culture jars.
   Daphnia exposures were undertaken with broods 3-6 from the healthy running cultures. Cultures were maintained in the same medium that the exposure was conducted in to remove any confounding factors associated with the change in medium.
2) Neonates were then allocated to a test vessel from the pooled stock to ensure that there is no bias associated to the different culture jars that could confound the results.
3) *Daphnia* were grouped, typically 10 individuals per vessel for an acute test unless otherwise stated, with a minimum of 3 vessels per treatment, in fresh medium.
4) The toxicant was then added to the test vessel in the nominal concentration outlined in the study.
   N.B. As stated in the OECD 202 test protocol *Daphnia* were not fed during the duration of the test.

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5) The labelled test vessels were then pooled and stored within the CT laboratory for the duration of the test unless otherwise stated.
6) For observation of results, test vessels were randomly selected, and results were recorded to minimise operator bias and fatigue within observations.

2.4 Data capture, processing, and presentation

Total immobilisation during the test period was recorded for each of the test vessels. This data was then plotted for a dose response curve with a log transformation of the concentration (x axis) to establish the sigmoid response curve over the 50% effect concentration range. This allows a more accurate Effect Concentration (EC_{50}) to be calculated. EC_{50} values can then be compared to other toxicants to establish a relative ranking of exposure hazard.

3 *Daphnia* exposures- chronic

3.1 Introduction and principles

Daphnia chronic toxicity response can be established using total reproduction and growth over a 21-day testing duration. The chronic toxicity exposures are based on the OECD 211 *Daphnia* chronic reproduction test (4). Observations are made over the test durations for time to first brood, time between broods, total neonates per brood (and over the whole period) and growth over time, often measured from the eye to the tail spine. These observations allow for sublethal toxicity to be observed in the *Daphnia* and the impact of the toxicant on the reproductive health of the *Daphnia* to be determined.

3.2 Resources and reagents

- Aerated medium or borehole water
- Glass pipette
- Light box
- Test vessels (50mL glass vials) and racks
- *Chlorella vulgaris* algal feed (refrigerated to 4°C)
- Nikon stereomicroscope with camera (or microscope with a camera fitting to enable images to be taken)
- Access to image analysis software (such as Image J- which is open access ImageJ (nih.gov)).

3.3 Protocol

1) Neonates were filtered from the running cultures and pooled from the different culturing jars. NB. *Daphnia* exposures were done with broods 3-6 from the running cultures. Cultures were maintained in the same medium that the exposure was conducted in to remove any confounding factors associated with the change in medium.
2) Neonates were then allocated to a test vessel from the pooled stock to ensure that there is no bias associated to the different culture jars that could compound the results.
3) *Daphnia* were maintained individually in the 50mL test vessels, with typically 12 replicates per treatment.
4) The toxicant was then added to the test vessel in the nominal concentration outlined in the study.
5) The labelled test vessels were then pooled and stored within the CT laboratory for the duration of the test.

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6) For observation of results, test vessels were randomly selected, and results were recorded. Typically, *Daphnia* were imaged on Day 0, 7, 14 and 21 during chronic tests and neonates were counted daily.
7) For imaging, *Daphnia* were removed from the test vessel and placed on a glass slide, excess medium was removed to limit the *Daphnia* movement to enable a clearer image to be taken. The slide was transferred to the microscope stage, focus adjusted, and image taken as quickly as possible to reduce the stress to the *Daphnia*. Once imaged, the *Daphnia* was returned to the test vessel. Light intensity and magnification were recorded on the respective observation sheets at the time of imaging. A scale bar was included with each photo to enable subsequent growth measurements.
8) Neonates were removed from the test vessel at time of observation. Care was taken to ensure minimal medium was removed.
9) Testing medium was replenished three times per week as outlines in steps 3 and 4 above.
10) Images were measured for total growth (centre of the eye to base of the tail spine) and total length of tail to allow for total growth to be calculated using Image J software.
11) At the end of the 21-day testing period *Daphnia* would be discarded or could be retained for subsequent lipid analysis.

3.4 Data capture, processing, and presentation

Daphnia observations were recorded in the data sheet outlined in Table 4 below. Daily observations were recorded, and this allows for subsequent analysis for total neonates or potential delays to broods etc.

In addition, measurements were taken for growth over time using image analysis software, such as Image J. This allowed the growth of the *Daphnia* to be measured by using the captured microscope images from the study. Typically, measurements were taken from the centre of the eye to the base of the tail spine (Figure 1) and then from the base to the tip of the tail for tail length. This measurement can then be replicated across all *Daphnia* measurements fairly consistently however, this measurement will use the carapace of the *Daphnia* and therefore will not take into consideration potential variability in mass. Calibration was based on the scale bar for the respective images.

4. *Daphnia* exposures- multigenerational

4.1 Introduction and principles

Following on from chronic toxicity, the impacts of toxicants on subsequent generations and the potential for the *Daphnia* to recover from exposures can be assessed in a multigenerational assay. This encompasses a series of chronic exposures undertaken synchronously, with the 3rd brood from each generation been used to established the next generation. The exposure generation is termed F0 and the subsequent generations are termed, F1, F2 etc. for the required number of generations (Figure 2). Chronic toxicity observations (OECD 211) end points are recorded for each generation (section 3.1), and can be supplemented with additional morphological end points, such as changes to lipid deposits or morphological defects such as growth of helmet spikes.

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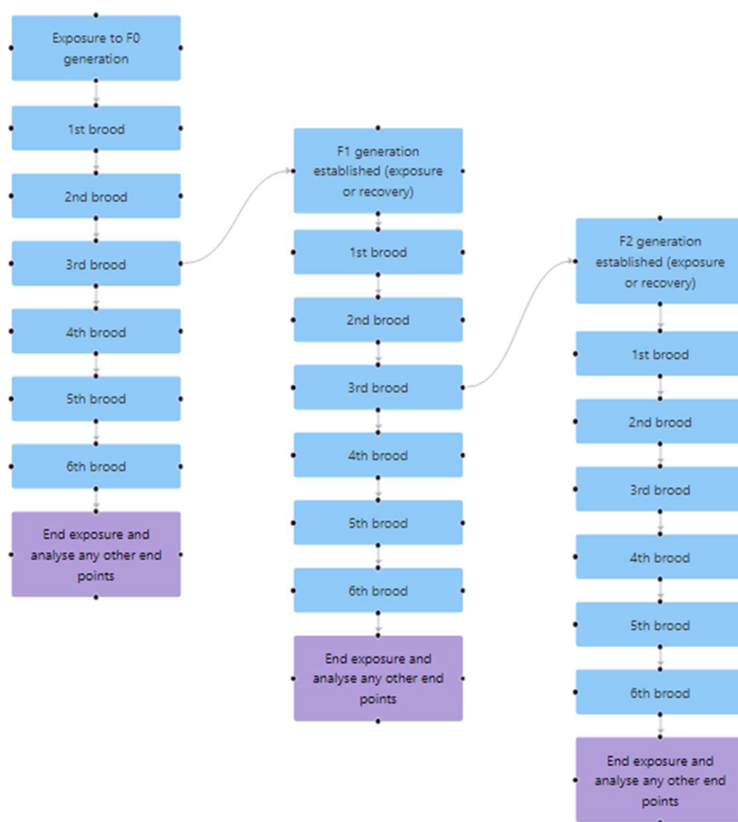

Figure 2. Schematic of the mutigenerational stepped approached to determine exposure recovery in subsequent generations.

4.2 Resources and reagents

- Aerated medium or borehole water
- Glass pipette
- Light box
- Test vessels (50mL glass vials) and racks
- *Chlorella vulgaris* algal feed (refrigerated to 4°C)
- Nikon stereomicroscope with camera (or microscope with a camera fitting to enable images to be taken)
- Access to image analysis software (such as Image J- which is open access [ImageJ (nih.gov)])

4.3 Protocol

12) Neonates were filtered from the running cultures and pooled from the different culturing jars. NB. *Daphnia* exposures (F0) were done with broods 3-6 from the running cultures. Cultures were maintained in the same medium that the exposure was conducted in to remove any confounding factors associated with the change in medium.
13) Neonates were then allocated to a test vessel from the pooled stock to ensure that there is no bias associated to the different culture jars that could compound the results.
14) *Daphnia* were maintained individually in the 50mL test vessels, with typically 12 replicates per treatment.

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15) The toxicant was then added to the test vessel in the nominal concentration outlined in the study.
16) The labelled test vessels were then pooled and stored within the CT laboratory for the duration of the test.
17) For observation of results, test vessels were randomly selected, and results were recorded. Typically, *Daphnia* were imaged on Day 0, 7, 14 and 21 during chronic tests and neonates were counted daily.
18) For imaging, *Daphnia* were removed from the test vessel and placed on a glass slide, excess medium was removed to limit the *Daphnia* movement to enable a clearer image to be taken. The slide was transferred to the microscope stage, focus adjusted, and image taken as quickly as possible to reduce the stress to the *Daphnia*. Once imaged, the *Daphnia* was returned to the test vessel. Light intensity and magnification were recorded on the respective observation sheets at the time of imaging. A scale bar was included with each photo to enable subsequent growth measurements.
19) Neonates were removed from the test vessel at time of observation. Care was taken to ensure minimal medium was removed.
20) Neonates from the 3rd brood of each generation were collected at the point of counting and pooled, before being allocated to test vessels for the next generation or either recovery or exposure (repeating steps 2-8).
21) Testing medium was replenished three times per week as outlines in steps 3 and 4 above.
22) Images were measured for total growth (centre of the eye to base of the tail spine) and total length of tail to allow for total growth to be calculated using Image J software.
23) At the end of the 21-day testing period *Daphnia* would be discarded or could be retained for subsequent lipid analysis.

4.4 Data capture, processing, and presentation

Daphnia observations were recorded in the data sheet outlined in Table 4. Daily observations were recorded, and this allows for subsequent analysis for total neonates or potential delays to broods etc for each generation.

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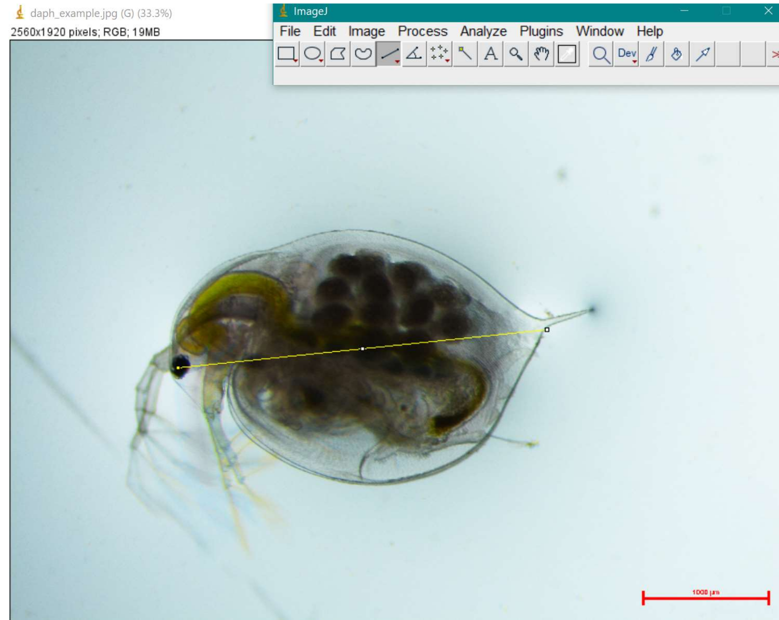

Figure 1. Example of image analysis in Image J measuring the growth of the daphnid. Red line depicts the scale bar and yellow line is the length being measured.

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Table 4. *Daphnia* chronic observations

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
Medium renewal (tick)																								
pH*																							new	
																							old	
O_2 (mg/l)*																							new	
																							old	
Temp (°C)*																							new	
																							old	
Food provided (tick)																								
No. live offspring**																								Total
Vessel 1																								
2																								
3																								
4																								
5																								
6																								
7																								
8																								
9																								
10																								
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12																								

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Experiment No: Date started: Clone: Medium: Type of food: Test Substance: Nominal conc:

Key references

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2. Kilham SS, Kreeger DA, Lynn SG, Goulden CE, Herrera L. COMBO: a defined freshwater culture medium for algae and zooplankton. 1998;13.
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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Annex 4.1: Daphnia facility algae culturing SOP

UNIVERSITY OF BIRMINGHAM

SCHOOL OF BIOSCIENCES, DAPHNIA FACILITY

STANDARD OPERATING PROCEDURE

SOP No.5 Version #2

ALGAL CULTURE

This project has received funding from the European Union's Horizon 2020
research and innovation programme under grant agreement No 814425.

The purpose of this Standard Operating Procedure (SOP) is to describe the culture of algae in the facility.

Culture of algae:

The algal species cultured will be *Chlorella vulgaris*. Other species may be cultured for specific experimental purposes.

The culture of algae will generally be performed once weekly with preparation and harvesting on Wednesdays. Alternatively, if there is greater demand for algae, then preparation and harvesting can be done on Monday and Thursday mornings. For holiday periods the regime will be altered accordingly. The number of flasks prepared will be dependent on demand in the laboratory.

Harvesting algae:

Make up new cultures by inoculating freshly autoclaved BBM medium in conical flasks from two or more good existing cultures by adding an aliquot of algae to each flask. The aliquot volume added to the fresh medium will normally be 20-25ml for 7 days incubation. Volumes for alternative incubation will be 100-110ml on Monday for a 3-day incubation period, and 75-80ml on Thursday for a 4-day incubation period. Other volumes may be used for other incubation periods. Decant the remaining algal culture into clean beakers and cover with cling-film to prevent aerial contamination.

Aerate the culture flasks vigorously using freshly autoclaved aeration tubes.

After use, place the used conical flasks in a sink and add ≈1 teaspoon of sodium bicarbonate. Fill with hot water then place the used aeration tubes in the flasks. Leave to soak for >1 hour.

Clean conical flasks and aeration tubes and leave to dry.

Centrifugation of algae:

Centrifuge the algae in 500ml centrifuge tubes using a Sorval Centrifuge (located in room S203 off S204) at 3500rpm for 15 minutes using the rotor SLA3000, min temp +4°C and max temp +9°C. The rotor can be found in the corner cupboard in room S204.

After centrifugation, decant off the supernatant. Re-suspend the concentrated algae in the minimum amount of modified standard Combo medium and then decant into a clean beaker. In order to ensure the maximum amount of algae is recovered, rinse the centrifuge tubes with a minimum amount of standard Combo so as not to over-dilute, then add this to the collection beaker.

Measure the optical density (OD) using a spectrophotometer. Measure the absorbance of a 1 in 10 dilution of the algal concentrate at 440nm using 1cm cuvettes. Dilute with standard Combo medium until the absorbance is approximately 0.80.

After adjustment to the correct OD, decant the algae into 1L Duran bottles and store in the fridge at +4°C.

Clean the centrifuge tubes and leave to dry.

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Preparation of fresh algal medium:

Prepare 10 litres of BBM medium (SOP No.3) in 10L aspirators or two 5L Duran bottles and dispense (approx. 1650ml) to an appropriate number of conical flasks and autoclave. The volume prepared will vary dependent on the demand for experimental procedures. Prepare and autoclave ready for Wednesday morning.

Approved by: Caroline Sewell **Signature**:

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Appendix 5: RiskGONE SOP for cytotoxicity assessment by AlamarBlue with fish cell lines

The Alamar Blue (AB) assay is widely used to investigate cytotoxicity, cell proliferation and cellular metabolic activity within different fields of toxicology. The use of the assay with ENMs entails specific aspects that are here addressed by drawing relevant considerations, discussing possible challenges such as interference of the ENMs with the test. The procedure of the AB assay applied for testing ENMs is described in detail and step-by-step, from the ENM preparation, the cell exposure, the inclusion of interference controls, to the analysis and interpretation of the results. Provided that the proper procedure is followed, and relevant controls are included, the AB assay is a reliable and high throughput test to evaluate the cytotoxicity/proliferation/metabolic response of cells exposed to ENMs. SOP is available at Longhin et al., 2022.

Key references:

Longhin, E.M., El Yamani, M., Rundén-Pran, E., Dusinska, M. The alamar blue assay in the context of safety testing of nanomaterials. Front. Toxicol., 2022, https://doi.org/10.3389/ftox.2022.981701

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

www.riskgone.eu | riskgone@nilu.no

Birmingham, 31 12 2022

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.