Chapter 11

Testing Nanomaterial Toxicity in Unicellular Eukaryotic Algae and Fish Cell Lines

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Abstract

Nanoecotoxicology as a sub-discipline of ecotoxicology aims to identify and predict effects elicited on ecosystems by nano-sized materials (NM). Two key groups of model organisms in this context are algae and fish. In this chapter, we present considerations for testing NM with respect to their impact on unicellular algae and cell lines derived from various organs of fish.

Based on currently available literature on NM effects in unicellular algae and fish cell lines, and our own experience, we provide guidance on test design, including principle test considerations, materials, NM presentation to cells, exposure, bioavailability, and effect assessment. Assessment needs to be based on a meaningful choice of exposure scenario(s) related to the research question. As a first step, one needs to address whether effects of NMs are to be investigated under environmentally relevant or probable conditions, which may include processes such as agglomeration, or whether NM effects from mono-dispersed particles are of interest, which may require special steps to ensure stable NM suspension. Moreover, whether effects on cells are to be studied in the short- or long-term is important with regard to experimental design. Preparation of NM suspensions, which can be done in aqueous media different from the exposure medium, is addressed with regard to energy input, sterility (as required for algae and fish cell exposure) and particle purity.

Specified for the two model systems, algae and fish cell lines, availability and choice of culture media are presented and discussed with regard to impact on NM behavior. Light, temperature, and agitation, which are variables during exposure, are discussed. We further provide guidance on the characterization of the NM in the chosen aqueous exposure media regarding size, zeta potential and electrophoretic mobility. The state of NM in exposure media is decisive for their bioavailability and therefore for potential particle effects. Therefore, we present ways of deriving a mass balance and quantitative/qualitative information on the uptake and distribution of NM in cells.

As NM have a high surface-to-volume ratio and possess specific physical-chemical properties, which make them prone to interfere with various compounds and certain types of toxicity tests, potential interferences and appropriate controls are introduced. Furthermore, different types of dose metrics, which is still a strongly debated issue in nanotoxicology, are highlighted. We also consider laboratory safety regarding NM handling and disposal.

Key words Ecotoxicology, Algae, Fish cells, Nanomaterial properties, Nanoparticle characterization, Test design, Exposure scenario, Toxicity, Uptake, Accumulation

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1 Introduction

Nanoecotoxicology has recently emerged as a subdiscipline of ecotoxicology and aims to identify and predict effects elicited on ecosystems by nano-sized materials. According to a recent definition by the EU, "nanomaterial" (NM) means a natural, incidental, or manufactured material containing particles in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm [1]. In the following, we refer to spherical NM as nanoparticles (NP). Agglomerates of NM are based on weak interactions, whereas chemical bonds between NM are formed in aggregates [2]. To achieve its aims, nanoecotoxicology needs to take into consideration the entry routes and fate of NM in the abiotic and biotic environment to define exposure. It moreover needs to identify those interactions of NM with biota that alter the proper function of cells comprising an organism, thus impacting populations, which in turn can lead to changes in community structure and function. For nanoecotoxicology concerned with the aquatic environment, key species of consideration are algae and fish. Algae are important primary producers in aquatic ecosystems and represent the group of photoautotrophic organisms. In contrast, fish are heterotrophs further up the food chain; with many conserved functions in vertebrates, they are also considered early indicators of impacts on water quality on higher vertebrates, including humans.

In this chapter, we present key considerations for testing NM with respect to their impact on algae and fish using cellular models, specifically, unicellular algae and cell lines derived from various organs of fish. Currently, algae are a paraphyletic group of eukaryotes that conduct photosynthesis in chloroplasts and that differ from plants in, a.o., reproduction and anatomy (e.g., ref. 3). They fall into different groups according to the origin and architecture of their chloroplasts including pigmentation. Cyanobacteria are not included in this definition. Here we focus on unicellular algae as opposed to colonial or filamentous algae. A selection of unicellular algae has become widely used model organisms. Algae form the base of the food web in aquatic ecosystems. In toxicity tests, algal species often prove to be more sensitive than other model organisms [4]. Algae can be isolated from their natural habitat and cultured at standardized conditions in the laboratory [5]. The challenge is to generate cultures of a single species that is not contaminated by bacteria or fungi (axenic). Some algae species cannot be kept axenically as they depend on symbiotic bacteria (e.g., in *Rhopalodia gibba*, a diatom [6]). If the culture conditions are appropriate, algae can be cultured indefinitely. Fish cell lines can be

derived from primary cultures of cells, tissues, or organs taken directly from organisms. If a primary culture can be divided into new culture vessels and successfully propagated, it becomes a cell line. A cell line may be propagated a limited number of times, in which case it is finite or, indefinitely, in which case it becomes an immortal or continuous (or permanent) fish cell line [7, 8]. Cell lines from a variety of different species of fish and from different tissues and organs have been established in this way. In contrast to mammalian cells, many fish cell lines arise spontaneously without any specific immortalization treatment, possibly owing to a high telomerase activity [7]. A wide range of both unicellular algae and fish cell lines are commercially available through dedicated repositories, such as the Culture Collection of Algae and Protozoa (CCAP [9, 10]) and the American Type Culture Collection for fish cell lines (ATCC, e.g., refs. [7, 11]), or they can be obtained from other research laboratories.

Owing to their small size and ease to produce them in rather large numbers, unicellular algae and fish cells are very useful to study a variety of NM in small-scale assays on integrative parameters, such as cell survival and reproduction, but as well on mechanisms of NM uptake and toxicity. Small scale refers to small flasks, holding a few milliliters of medium, down to multi-well plates with micro- to nanoliter volumes of exposure medium, making these tests amenable not only to high throughput screening but also to reduced material resources and, consequently, reduced waste. However, the small scale also bears problems, such as the high surface-to-volume ratio of test containers or little material for characterizing NM as they are present in the exposure chambers.

A summary of currently available publications on NM effect studies using unicellular algae and fish cell lines is presented in Tables 2 and 3. Based on these studies, and taking our own experience into consideration, we would like to provide guidance on test design in terms of principle test considerations, NM presentation to cells, exposure, bioavailability, and effect assessment.

2 Materials

Materials specifically recommended for working with NM in algae or fish cell culture environments are as follows:

2.1 Equipment

1. High-precision, antistatic scale to weigh NM provided as powder.
2. Ultrasonic bath or sonication cup to fit on a sonication tip for indirect sonication of NM dispersions (direct sonication may add impurities to your sample!).

Table 1
Possible interferences of NM with cell-based toxicity tests and adequate controls

Interference	Control
Quenching of fluorescence emitted from test reaction product	Measure known concentrations of NM and reaction product in combination
Quenching of fluorescence emitted from organism (e.g., algal autofluorescence)	Difficult to control as change in fluorescence is the end point; wash organisms to ± remove NM
Light absorption at the wavelength used in a fluorescence or light absorbance measurement	Measure light absorption spectrum of NM at known concentrations
Autofluorescence (in the respective wavelength required for detection in the assay)	Measure fluorescence of NM suspension
Conversion of assay substrates, e.g., by catalysis or ROS formation	Incubate NM with assays substrates and measure end point taking into account possible optical interference and sorption (see above)
Binding of assay compounds	Difficult to control (separate them from cells and prevent necessary interactions)
Interference with an enzyme (inhibition, enhancement) and/or adsorption of assay substrates or products	Incubate known concentrations of enzyme, substrate, and NM and measure end point, taking into account possible optical interference and sorption (see above)
Interaction with RNA/DNA, PCR mixes	Treat known concentrations of RNA/DNA with NM dispersions and perform PCRs in the presence of NM to check for interference/destruction
Shading	Use external shading
pH is influenced by NM–e.g., change in fluorescence of fluorophores	Check pH in the presence of NM
Nutrient or growth factor depuration (NM adsorb compounds from the media)	Analyze compounds associated with NM

3. Stir plates with exact (digital) displays for reproducible stirring of NM dispersions.
4. Room or incubator with controlled light and temperature conditions for the generation of dispersions and exposure of test organisms.

2.2 Reagents and Supplies

Please refer to specific literature on NM characterization (e.g., ref. 12) and on algae (e.g., ref. 5) and fish cell culture (e.g., ref. 13, 14), as well as the small-scale assays cited in Tables 1 and 2, for details on specific infrastructure, instruments, consumables, and chemicals. Be sure to protect yourself and your colleagues from exposure to NM (*see* **Note 1**).

Table 2
Reports on toxicity tests of NM in algae

Type of NM	Test organism	Division	NM dispersions	NM characterization	NM concentration (mg/L)	Temperature (°C)	Light (μEm²/s)	Light–dark cycle	Agitation	Exposure time (h)	End points 1 OECD 201 2 ISO 8692 3 DIN38412-33 4 US EPA	Effects expressed per…	Reference
Ag NP	*C. reinhardtii*	Chl		1, 2, 3, 4	10–10^5 nM	25	267	x	x	1–5	A	Total mass, $[Ag^+]$	[37]
Ag NP, 0.3 % PVP	*T. weissflogii*	Het	Fulvic acid, 1 week in Teflon, filtration	1, 5, 6	2.12×10^{-10} to 1.03×10^{-7}	20	170	x		48	A, B, C, E, EPS	$[Ag^+]$	[54]
Al_2O_3 NP	*P. subcapitata*	Chl	10 % (w/w) in H_2O, dilution in medium	7, 5	100	20	100		x	4.5	A	Total mass	[20]
Au NP	*S. subspicatus*	Chl			NPs/cell			x	x	24	B, D, F	Total mass	[55]
C60	*P. subcapitata*	Chl	Stirring (stock, 60 d; dilutions, 5 d)	1, 8	6–10	20	80–90				B, ISO 8692	Total mass	[56]
C60	*P. subcapitata*	Chl	Stirring in H_2O, dilution in medium	7, 5, 8	100	20	100		x	4.5	A	Total mass	[20]
CdTe QD	*P. tricornutum*	Het	Sonication, resuspension	2, 4, 8	0.1–0.3	20	3,000 Lux		x	24–144	B, E	Total mass	[48]
CeO_2 NP	*Anabaena* CPB4337	Cy	Sonication, dilution, stirring	1, 2, 4, 6, 9	0–100	28	65			72+96	D, F, G, ATP	Total mass	[57]
CeO_2 NP	*P. subcapitata*	Chl	Sonication, dilution, stirring	1, 2, 4, 6, 9	0–100	22	100			72+96	B	Total mass	[57]

(continued)

Table 2
(continued)

Type of NM	Test organism	Division	NM dispersions	NM characterization	NM concentration (mg/L)	Temperature (°C)	Light (μEm²/s)	Light–dark cycle	Agitation	Exposure time (h)	End points 1 OECD 201 2 ISO 8692 3 DIN38412-33 4 US EPA	Effects expressed per…	Reference
CeO_2 NP	*P. subcapitata*	Chl	Sonication, shaking, filtration	1, 3, 4, 5, 10[a]	0–100	24	70			72	B, membrane	Total mass	[40]
CeO_2 NP	*P. subcapitata*	Chl		1, 2, 11	0–32	25	70			72	B, 1	Total mass, BET	[58]
CeO_2 NP	*P. subcapitata*	Chl	10 % (w/w) in H_2O, dilution in medium	7, 5	100	20	100		x	4.5	A	Total mass	[20]
$CuZnFe_4O_4$ NP	*P. subcapitata*	Chl	Rotation, filtration		0.1–100						B	Total mass	[59]
CuO NP	*P. subcapitata*	Chl	Sonication		0 to ~6.5	24					B, F, 1	Total mass	[60]
CuO-PS NP	*C. reinhardtii*	Chl		1, 4	0–20	23	200			6	A, F, G, ROS	Total mass	[61]
Er_2O_3 NP	*P. subcapitata*	Chl	Rotation, filtration		0.1–100						B	Total mass	[59]
g-Al_2O_3 NP	*P. subcapitata*	Chl	Sonication	2, 3, 6	10–200					24	B	Total mass	[62]
Ho_2O_3 NP	*P. subcapitata*	Chl	Rotation, filtration		0.1–100						B	Total mass	[59]

In_2O_3 NP	*P. subcapitata*	Chl	Rotation, filtration		0.1–100					B	Total mass	[59]
MWCNT	*D. tertiolecta*	Chl	Stock: filtered, washed, autoclaved		0–10	19	120	x	24	A, B, D, GSH	Total mass	[63]
MWCNT	*P. subcapitata*	Chl	Sonication in presence of NOM	2	0.1–100	24	80 ± 5	x	96	A, B, 1	Total mass	[43]
MWCNT	*C. vulgaris*	Chl	Sonication in presence of NOM	2	0.1–100	24	80 ± 5	x	96	A, B, 1	Total mass	[43]
$Ni_{0.5}Zn_{0.5}Fe_2O_4$ NP	*P. subcapitata*	Chl	Rotation, filtration		0.1–100					B	Total mass	[59]
NIPAM/BAM NP	*P. subcapitata*	Chl	Dispersed on ice after freeze-drying	1, 2, 3, 6	25–1,000	20	10^4 lx	x	72	B, 1	Total mass	[64]
PMMA NP	*P. subcapitata*	Chl	10 % (w/w) in H_2O, dilution in medium	7, 5	100	20	100	x	4.5	A	Total mass	[20]
PNIPAM NP	*P. subcapitata*	Chl	Dispersed on ice after freeze-drying	1, 2, 3, 6	25–1,000	20	10^4 lx	x	72	B, 1	Total mass	[64]
QD	*C. reinhardtii*	Chl			0–100	25	55	x	24 + 120	B, C, ox. stress	Total mass	[65]
QD	*P. subcapitata*	Chl			0–50 ppbm				96	B, F, G, 4	Total mass	[21]
QD	*Chlamydomonas sp.*	Chl		1, 2	0.05–5 ppm	RT			2	D, G, [CO_2]	Total mass	[22]

(continued)

Table 2
(continued)

Type of NM	Test organism	Division	NM dispersions	NM characterization	NM concentration (mg/L)	Temperature (°C)	Light (μEm²/s)	Light–dark cycle	Agitation	Exposure time (h)	End points 1 OECD 201 2 ISO 8692 3 DIN38412-33 4 US EPA	Effects expressed per…	Reference
Sm_2O_3 NP	*P. subcapitata*	Chl	Rotation, filtration		0.1–100						B	Total mass	[59]
SiO_2 NP	*C. kessleri*	Chl			0–5 % w/v	25	3.4 W/m²	x	x	120	B, G, F	Total mass	[66]
SiO_2 NP	*P. subcapitata*	Chl	Stirring	1, 2, 4	0–460	25	70			72	B, D, 1	Total mass	[67]
$SrFe_{12}O_{19}$ NP	*P. subcapitata*	Chl	Rotation, filtration		0.1–100						B	Total mass	[59]
SWCNT	*P. subcapitata*	Chl	Stirring in H_2O, dilution in medium	7, 5	100	20	100		x	4.5	A	[C]	[20]
TiO_2 NP	*A. variabilis*	Cy	Sonication	2, 6	0–150	20				24	D, F, ROS	Total mass	[68]
TiO_2 NP	*D. subspicatus*	Chl	Sonication, ± light		12.5–50	20				72	G, 1, 2, 3	Total mass	[42]
TiO_2 NP	*D. tertiolecta*	Chl	Sonication, vortexing, dilution	2, 8	0–1	15	100–200	x		96	B	Total mass	[69]
TiO_2 NP	*I. galbana*	Chl	Sonication, vortexing, dilution	2, 8	0–1	15	100–200	x		96	B	Total mass	[69]
TiO_2 NP	*P. subcapitata*	Chl	Sonication	2, 3, 6	10–200					24	B	Total mass	[62]
TiO_2 NP	*P. subcapitata*	Chl	Sonication		0–120	24					B, F, 1	Total mass	[60]

TiO_2 NP	*P. subcapitata*	Chl	Stirring stock for at least 30 min	12		25	50–100	x	96	B, 4	Total mass	[70]
TiO_2 NP	*P. subcapitata*	Chl	Stirring, sonication, varying conditions	2, 3, 14	3.7–100	22	95	x		B, 1	Total mass	[27]
TiO_2 NP	*P. subcapitata*	Chl	10 % (w/w) in H_2O, dilution in medium	7, 5, 14	100	20	100	x	4.5	A	Total mass	[20]
TiO_2 NP	*P. subcapitata*	Chl	Rotation, filtration		0.1–100					B	Total mass	[59]
TiO_2 NP	*S. marinoi*	Het	Sonication, vortexing, dilution	2, 8	0–1	15	100–200	x	96	B	Total mass	[69]
TiO_2 NP	*T. pseudonana*	Het	Sonication, vortexing, dilution	2, 8	0–1	15	100–200	x	96	B	Total mass	[69]
TiO_2 (P25) NP	*A. carterae*	Dino	Directly mixed with algal culture	12, 13, 6, 8, 1	500	25	UV–Vis		72	B	Total mass	[71]
TiO_2 (P25) NP	*C. reinhardtii*	Chl		2, 14, 15	0–100	25	55	x	24 + 120	B, C, ox. stress	Total mass	[65]
TiO_2 (P25) NP	*P. subcapitata*	Chl		2, 6, 12, 15	0–100	23.8	9,500 lx	x	72	B, 1	Total mass	[72]
TiO_2 (P25) NP	*T. suecica*	Chl	Directly mixed with algal culture	1, 6, 8, 12, 13	500	25	UV–Vis		72	B	Total mass	[71]

(continued)

Table 2
(continued)

Type of NM	Test organism	Division	NM dispersions	NM characterization	NM concentration (mg/L)	Temperature (°C)	Light ($\mu Em^2/s$)	Light–dark cycle	Agitation	Exposure time (h)	End points 1 OECD 201 2 ISO 8692 3 DIN38412-33 4 US EPA	Effects expressed per…	Reference
TiO_2-Ag NP	*A. carterae*	Dino	Directly mixed with algal culture	1, 6, 8, 12, 13	500	25	UV–Vis			72	B	Total mass	[71]
TiO_2-Ag NP	*T. suecica*	Chl	Directly mixed with algal culture	1, 6, 8, 12, 13	500	25	UV–Vis			72	B	Total mass	[71]
$Y_3Fe_5O_{12}$ NP	*P. subcapitata*	Chl	Rotation, filtration		0.1–100						B	Total mass	[59]
ZnO NP	*C. gracilis*	Het	Sonication	1, 2, 4, 12	0–80	17	120	x			B	Total mass	[47]
ZnO NP	*D. tertiolecta*	Chl	Sonication, vortexing, dilution	1, 4, 8	0–1	15	100–200	x		96	B	Total mass	[69]
ZnO NP	*I. galbana*	Chl	Sonication, vortexing, dilution	1, 4, 8	0–1	15	100–200	x		96	B	Total mass	[69]
ZnO NP	*P. tricornutum*	Het	Sonication	1, 2, 4, 12	0–80	17	120	x			B	Total mass	[47]
ZnO NP	*P. subcapitata*	Chl	Sonication	–	0–0.5	24					B, F, 1	Total mass	[60]
ZnO NP	*P. subcapitata*	Chl	± Sonication, ± filtration	1, 2, 4	0.025–0.6					72	B	Total mass $[Zn^{2+}]$	[73]
ZnO NP	*S. marinoi*	Het	Sonication, vortexing, dilution	1, 4, 8	0–1	15	100–200	x		96	B	Total mass	[69]

ZnO NP	*T. pseudonana*	Het	Filtration	1, 2, 4	9.50×10^{-9} to 1.52×10^{-7} M Zn^{2+}	20	50	x			B, A, C	$[Zn^{2+}]$	[74]
ZnO NP	*T. pseudonana*	Het	Sonication, vortexing, dilution	1, 4, 8	0–1	15	100–200	x		96	B	Total mass	[69]
ZnO NP	*T. pseudonana*	Het	Sonication	1, 2, 4, 12	0–80	17	120	x			B, D, E	Total mass	[47]
ZrO_2 NP	*P. subcapitata*	Chl	10 % (w/w) in H_2O, dilution in medium	7, 5	100	20	100		x	4.5	A	Total mass	[20]

Chl (*Chlorophyceae*), Cy (*Cyanobacteria*), Dino (*Dinoflagellatae*), Het (*Heterokontophytae*)
A Photosystem II quantum yield
B Growth rate/inhibition/cell number
C Chlorophyll a content
D NM localization
E Bioaccumulation
F Morphology
G Fluorescence/luminescence
1 TEM
2 DLS
3 Electrophoretic mobility
4 Solubility/ICP-MS
5 EDS
6 BET
7 AFM
8 Spectrophotometry
9 TXRF
10 PEELS
11 XANES
12 XRD
13 XPS
14 Concentration
15 Visual observation
Empty cells not specified
[a]ROS formation, peroxidation of linoleic acid

3 Methods

This chapter guides through important elements in designing experiments to assess the toxicity of NMs in unicellular algae and fish cell lines. Inasmuch as the dominant route of exposure to NMs toward these organisms in the aquatic environment is via NM dispersions (aside from uptake in fish through food), we will focus this chapter on exposure via liquid media and NMs dispersed into it. A general design scheme is presented in Fig. 1. This scheme assumes a prior selection of cells. This selection needs to be decided upon according to the question at hand, availability of desired cell types, and other potential considerations as briefly summarized below.

3.1 Considerations for Selection of Cells

3.1.1 Unicellular Eukaryotic Algae

The three NM-specific considerations are the algal cell wall, algal class, and habitat. Algal cell walls are composed of silica in diatoms and of polysaccharides and/or glycoproteins in other groups. Consequently algal cell walls have different chemical and physical properties that will possibly influence the availability of NM at the cell membrane. The silica-based cell wall or frustule of diatoms has pores and slits of defined sizes which filter material from the surrounding medium. A study on two diatom species revealed a lower size limit of 40 nm in frustule pores, possibly limiting the diffusion of larger particles to the cell membrane [15]. Other classes may have additional routes of uptake for NM, such as dinoflagellates

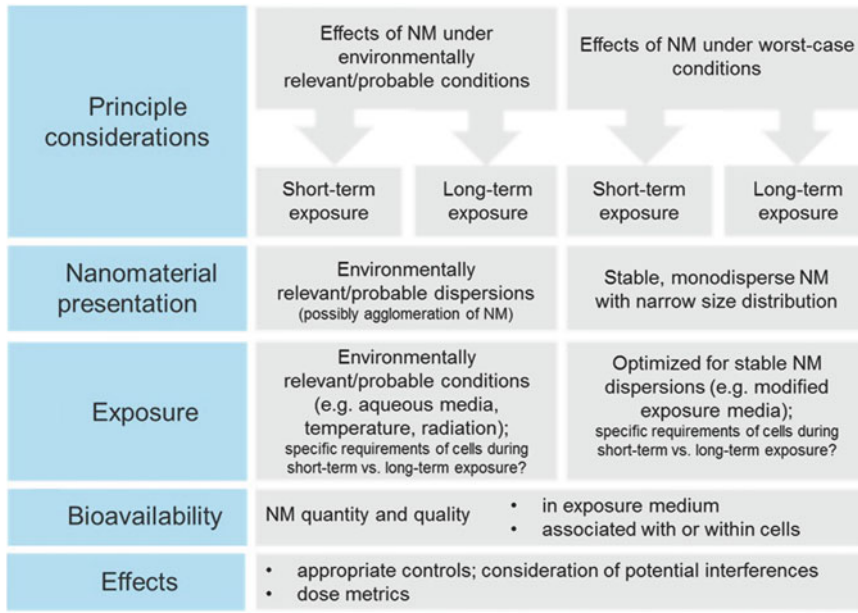

Fig. 1 Scheme for the design of toxicity tests of nanomaterials

that can ingest food particles (e.g., ref. 16). The original habitat and thus the required medium will influence the fate of NM during exposure. Marine algae, for example, require high salt medium as opposed to freshwater algae. The ionic strength strongly influences the stability of NM dispersions.

3.1.2 Fish Cell Lines

Fish cell lines are not as diverse in structure as algae cells, but the species and its associated habitat and the organ or tissue of origin are likewise important considerations. Inasmuch as fish cell lines generally thrive in temperatures closely mimicking the environment of their donor, cells from warm and cold water fish have very different temperature requirements. For example, cell lines from rainbow trout (*Oncorhynchus mykiss*) can be cultured between 4 and 25 °C, whereas cell lines from warm water fish, such as from zebra fish (*Danio rerio*), grow at temperatures also above 25 °C (23–34 °C). The organ or tissue of origin is important for consideration of specific target sites. For example, if one wants to mimic the exposure scenario at the gill, exposure media closely mimicking the fresh- or saltwater environment may be applied [11]. Moreover, in analogy to dinoflagellates, macrophage cells have additional routes of NM uptake, i.e., phagocytic routes, not of such relevance to other cell types.

3.2 Principle Considerations for Test Design

The assessment of NM toxicity needs to be based on a meaningful choice of exposure scenario(s) related to the research questions. The two possible "extreme" cases are sketched in Fig. 1. If the research focus is on the effects of NM under environmentally relevant or probable conditions, the use of natural water chemistry and low NM concentrations is implied. Due to the lack of measured data, different authors have estimated or modeled NM concentrations (such as TiO_2-, CeO_2-, Ag-based nanoparticles (NP)) which average in the ng/L range and below in surface waters, meaning several orders of magnitude below most reported test concentrations (Tables 2 and 3) [17–19]. To investigate NM effects in a "worst-case" exposure scenario, establishing a dose–response curve is necessary. However, the use of high NM concentrations may be limited by interference with toxicity assays (*see* Subheading 3.6.1), whereas low concentrations of NM are difficult to quantify and characterize (*see* Subheading 3.5).

Furthermore, the exposure duration needs to be considered (Fig. 1). In the literature, exposure is typically grouped in short-term (hours to days) and long-term (days to weeks) approaches. As the life cycle of NM in the environment has not been studied yet, we do not have any data to base our selection on. From other anthropogenic inputs into surface waters, we know that there may be pulsed and long-term/chronic exposure. Both may thus apply to NM, too. Reported exposure times in nanotoxicity studies range from 4.5 h [20] to 5 d [21] in algae and from 3 to 72 h [22]

Table 3
Reports on toxicity tests of NM in fish cell lines

Type of NM	Species	Cell type/cell line	Exposure condition/ scenario	NM characterization	NM concentration (mg/l)	Exposure time (h)	Temperature (°C)	Assay	End point	Effects expressed per…	Reference
Ag NP	Medaka (*Oryzias latipes*)	OLHNI2, adult fin tissue	DMEM w 20 % FBS	1,2,3,7,9,11	0.05–5 μg/ cm^2			A3.3, C2	A3, C	Total mass	[75]
Ag NP	Rainbow trout (*Oncorhynchus mykiss*)	Gill cells, RTgill-W1	L-15 with 10 % FBS	1,6		40, 48	37 (?)	A3.2, A3.4.	A, A3	Total mass	[76]
Ag nano-composite	Rainbow trout (*Oncorhynchus mykiss*)	Gill epithelial cells, RT-W1	Dispersion in distilled water, L-15 + 10 % FBS, alginic acid	1,2,4,7,10,11	0–25	6, 12, 24	24	A2.4, A3.1, B1.2	A3, B	Total mass	[77]
Au NP	Rainbow trout (*Oncorhynchus mykiss*)	Gill cells, RTgill-W1	L-15 with 10 % FBS	1,6		40, 48	37 (?)	A3.2, A3.4	A, A3	Total mass	[76]
Au NP polymer coated	Rainbow trout (*Oncorhynchus mykiss*)	Gill cells, RTgill-W1	L-15 with 10 % FBS	1,2,8,11	1–100	24	20	A1.3	A1, D	Total mass	[78]
CdO NP	Rainbow trout (*Oncorhynchus mykiss*)	Gill cells, RTgill-W1	L-15 with 10 % FBS	–		40, 48	37 (?)	A3.2, A3.4	A, A3	Total mass	[76]
CNT	Rainbow trout (*Oncorhynchus mykiss*)	Gill cells, RTgill-W1	L-15 with 10 % FBS	1,6		40, 48	37 (?)	A3.2, A3.4	A, A3	Total mass	[76]

NIPAM-NIPAM/BAM copolymers	Rainbow trout (*Oncorhynchus mykiss*)	Gonad cells (RTG-2)	Dispersed in Milli-Q, DMEM with 5 % FBS	2,3,5	25–1,000			A1.2, A2.2	A1, A2	Total mass	[79]
TiO_2 NP, anatase	Goldfish (*Carassius auratus*)	Skin cells (GFSk-S1)	Suspended in sterilized PBS, final dilution in growth media, w/o UVA	–	0.1–1,000	24		A2.2, B2.1, C1	A2, B, C	Total mass	[80]
TiO_2 (P25) NP	Rainbow trout (*Oncorhynchus mykiss*)	Gonad cells (RTG-2), passage 13–25	Suspension in dist. H_2O, sonication 12 h, exposure in PBS vs. MEM w/o UVA	1	0–50			A2.2, C1, C3	A2, C	Total mass	[81]
WC NP	Rainbow trout (*Oncorhynchus mykiss*)	Gill cells, RTgill-W1	Dispersion in distilled water, L-15 + 10 % FBS, L-15, L-15ex	1,2,3,4,5	0–30	3, 72	19	A1.2, A2.2, A2.3, D1	A1, A2, D	Total mass	[22]
WC-Co NP	Rainbow trout (*Oncorhynchus mykiss*)	Gill cells, RTgill-W1	Dispersion in distilled water, L-15 + 10 % FBS, L-15, L-15ex	1,2,3,4,5	0–33	3, 72	19	A1.2, A2.2, A2.3, D1	A1, A2, D	Total mass	[22]

(continued)

Table 3
(continued)

Type of NM	Species	Cell type/cell line	Exposure condition/ scenario	NM characterization	NM concentration (mg/l)	Exposure time (h)	Temperature (°C)	Assay	End point	Effects expressed per…	Reference
ZnO NP	Topminnow fish (*Poeciliopsis lucida*)	Hepatocellular carcinoma cell line, PLHC-1	Serum-free medium, 1 min vortexing, a-MEM with 5 % FBS	1,2,7	0.00078–0.1	24	30	A1.1, A2.1, A2.2, B1.1	A1, A2, B1	Total mass	[82]

End points
A Cytotoxicity/viability
A1 Metabolic activity
A2 Membrane integrity
A3 Cell number/protein content
B ROS
C Genotoxicity
D NM uptake and localization
Assays/tests
A1.1 MTT
A1.2 alamarBlue
A1.3 Resazurin
A2.1 LDH
A2.2 Neutral red
A2.3 CFDA, AM, fluorescein retention
A2.4 Propidium iodide
A3.1 Hoechst 33324
A3.2 Protein content (sulforhodamine B, Bradford)
A3.3 Colony-forming assay

A3.4 Impedance and resistance measurements
B1.1 DCF
B1.2 MitoSOX red
B2.1 Electron spin resonance
C1 COMET
C2 Chromosome aberration
C3 Micronucleus assay
D1 Microscopy
Characterization
1 TEM
2 DLS
3 Electrophoretic mobility/zeta potential
4 EDS
5 BET
6 AFM
7 ICP-MS
8 Spectrophotometry
9 UV–Vis spectroscopy
10 XRD
11 Concentration
Empty cells not specified

in fish cell lines. Recovery from NM-induced adverse effects was shown in cell culture and animal tests [23], but has not yet been investigated in ecotoxicological tests. Strikingly, many studies in algae are based on 72 h exposures, often conducted according to the standard procedures OECD 201 [24], ISO 8692 [25], or DIN 38412-33 [26] (Table 2). Using these standard protocols may imply an easy comparison to data on the toxicity of chemicals. However, these protocols may not be suitable to answer individual research questions on NM toxicity. We further discuss this point in Subheadings 3.4.1 and 3.6.1.

3.3 Nanomaterial Presentation

NMs are available as powders or dispersions depending on the production process and posttreatment. Both types need to be dispersed in liquid media for exposure. As described in Fig. 1, dispersions should be generated according to the research approach. The dispersion method can greatly influence the outcome of NM toxicity tests and should thus be carefully chosen [27]. Relevant parameters are dispersion medium chemistry, pH, energy input, time, temperature, and light (*see* **Notes 4**–**7**, **9**–**12**).

3.3.1 Generation of NM Dispersions

Stable, monodisperse NM suspensions with a narrow size distribution are needed to understand the specific effects of a defined size range of the NM under controlled conditions. Dispersion parameters need to be adapted to the respective NM. Depending on the NM surface properties (charged/uncharged, hydrophilic/hydrophobic), which are influenced by surface modifications and the exposure medium (*see* Subheading 3.4.1), more or less energy input during the dispersion process is required. Dispersion methods ranging from low energy input, like slow stirring or shaking, to high energy input, like vigorous stirring, shaking, or sonication, have been used (*see* Tables 2 and 3, *see* **Note 6**). The use of surfactants and temperature and/or pH different from exposure conditions may be required to achieve monodispersed NMs. Filtration or centrifugation may help to narrow the size distribution. Stock dispersions may be generated in a medium different from the exposure medium to ensure optimal dispersion (e.g., ref. 27).

Environmentally relevant or probable conditions may be contrary to the conditions needed to generate stable dispersions. Agglomeration (or possibly aggregation) of the NM may be the consequence (see, e.g., dispersion of Ag NPs in river water [28]). To design environmentally probable conditions, one should determine a relevant type of site in the environment and adapt the dispersion parameters mentioned above accordingly.

3.3.2 Sterility

Contamination of the NMs by microorganisms or their toxins may affect the test organisms and thus confound toxicity test results [15]. Verifying the sterility of NM for a nonspecialized laboratory is challenging. A simple but superficial approach is to test NM for growth of nonspecialized bacteria in standard media

(e.g., lysogeny broth (LB)). If available, NM can be tested aerobically and anaerobically for sterility, applying fluid and solid media under long-term cultivation [15, 23]. The presence of common bacterial toxins (e.g., endotoxins) can be ruled out using available standard kits. Be sure to exclude interferences of the NM with the assay (*see* Subheading 3.6.1). Sterility of NM powders or suspensions can be achieved by autoclaving [22, 29], γ-irradiation [30], or possibly filtration. However, one has to verify that these processes do not change NM properties or concentration. We have previously used X-ray photoelectron spectroscopy (XPS) to verify that γ-irradiation did not change the surface properties of diverse NM [30].

3.3.3 Purity

Impurities originating from the production process (or side products formed during preparation of suspensions, e.g., by solvents) may result in measurable toxicity that is independent of actual NM effects and may confound the results [31]. The chemical purity of the NM should thus be determined. If impurities exist, washing steps (if possible) or appropriate controls in the toxicity tests (e.g., using the detected contaminants alone) are recommended.

3.4 Exposure

Depending on the research approach (Fig. 1), exposure conditions need to be adapted either to correspond as closely as possible to environmental conditions or to guarantee maintenance of stable, monodisperse NM suspensions with a narrow size distribution. The following sections describe the influence of different parameters on NM dispersions. They serve as basis to design the exposure conditions according to the desired scenario.

3.4.1 Exposure Media

Both algae and fish cells need growth media containing essential nutrients in order to ensure optimal growth and fitness. The constituents of these media will influence NM properties and thus their effects on the test organisms. If dispersion properties are to be controlled, e.g., to generate a stable monodisperse suspension or to minimize dissolution of metallic NM or loss of surface modifiers, the medium may have to be adapted. We recommend to model the speciation of media components including NM (e.g., with Visual MINTEQ, available from KTH, Department of Land and Water Resources Engineering). This helps to understand the fate of NM in the medium and allows optimizing the medium composition if necessary.

Typical liquid minimal algae media contain essential metals (sodium, potassium, calcium, magnesium), chloride, a source of nitrogen (nitrate or ammonium) and inorganic carbon (carbonate), trace metals (copper, zinc, cobalt, manganese, molybdenum, iron, possibly boron), and phosphate. Some algae require an organic carbon source (e.g., citrate), vitamins, or silicon (diatoms). A pH buffer may be necessary and a chelator (e.g., EDTA) to make the metals more bioavailable. Generally, bivalent cations such as

calcium and magnesium tend to increase the agglomeration of NM; phosphate and carbonate may stabilize NM depending on their surface charge and modification. Ionic strength, pH, and organic compounds will also influence the stability of NM dispersions (TiO_2 [30, 32], CeO_2 NPs [30, 33], polyethylene glycol (PEG)-coated quantum dots (QDs) (CdSe/ZnS) [34], Ag [28]). Media properties may also change the solubility of NM and possible contaminants which in turn may influence the biological effects. The coating of, e.g., Ag NPs influences their dissolution in different media [35]. Metal-binding compounds such as EDTA or citrate may change the bioavailability of metal ions dissolved from NM. NaCl has been shown to enhance dissolution of Ag NPs [36]. Thus, a medium without chloride has been used to expose *C. reinhardtii* to Ag NPs [28, 37].

As mentioned above, many studies on NM effects on algae have been conducted according to standard test guidelines. This implies the use of standardized culture media. As described in this section, certain components found in standard media influence the dispersibility of NMs or may affect dissolution of NMs. The application of standard protocols should thus be carefully evaluated with respect to the research questions.

For the cultivation of fish cells, different commercially available standard media are in use, e.g., Leibovitz-15 (L-15), minimal essential medium (MEM), or medium 199 (M199). These media contain amino acids, vitamins, inorganic salts, and sugars essential for the growth of heterotrophic cells and differ only slightly in their formulations. Other than mammalian cells, fish cells are usually maintained in CO_2-free systems, hence the media does not contain sodium bicarbonate as a buffer, but is buffered by the use of Hank's salts, which buffer at atmospheric CO_2 concentrations. Additionally, a higher amino acid content, and complement of galactose is used (e.g., L-15 medium). To allow cell growth and division, the addition of serum is generally needed and the most frequently used is fetal bovine serum (FBS). Serum contains essential growth factors and proteins (e.g., albumin) but may influence NM behavior and hence the toxicity assessment. For example, we and others have found the addition of serum to stabilize NP in the media, hence preventing NP agglomeration [22, 38]. For short-term assays not requiring cell division, serum is not essential and even very simple buffers may be applicable, such as L-15ex [22, 39]. The absence or presence of serum leads to very different particle states and may modulate the particle toxicity [22]. In media without serum, NP may agglomerate due to the high salt content. Depending on tissue of origin, fish cells are capable to grow under hypo- and hyperosmotic conditions (e.g., ref. 11); hence, media with different salt contents can be designed in order to control NM states. The addition of antibiotics to prevent growth of bacteria in the media is common. To date, there are no reports on interference of

antibiotics with NM; however, during exposures, the use of antibiotics is generally not needed and thus should be avoided.

3.4.2 Choice of Light, Temperature, and Agitation

Photoactive NMs such as TiO_2- or CeO_2-based NP generate reactive oxygen species (ROS) in the presence of UV and even visible light [40]. They may thus change exposure conditions by catalyzing the oxidation of media components (indirect damage due to change of nutrients). The light-induced generation of ROS may also result into phototoxicity of NM [41]. Photoreduction/oxidation of NM or ions in the medium may change NM dissolution, surface properties, and stability. The choice of light/dark conditions during exposure is therefore an important factor. Light conditions may be influenced locally due to NM shading the cells. This is especially relevant in studies based on photosynthetic organism [42, 43] for carbon nanotube-exposed algae, although only extremely high exposure concentrations resulted in this effect.

In previous studies, algae have been exposed under continuous light and light–dark cycles (Table 2). Continuous light produces a more or less homogenous culture and constant conditions, whereas light–dark cycles generate synchronized cultures similar to synchronization by the natural day–night cycle. Fish cells are usually cultivated in the dark and are only exposed to light during work steps performed under the sterile bench (splitting, exposure procedure, toxicity assays). If working with photoactive NM and if desired by the test design, light exposure during these steps can be minimized. On the other hand, it is reasonable to consider exposure under controlled light conditions for cell cultures that represent fish organs naturally exposed to light, such as gills, skin, or eyes [39, 44, 45].

In a non-agitated exposure system, stable-dispersed NMs move by Brownian motion which is dependent on, i.e., the temperature of the system. Consequently, the temperature influences NM behavior (e.g., speed, agglomeration, and contact with surfaces) and possibly interaction with organisms. Fish cells are typically cultured at 15–34 °C, depending on the species from which fish cells are derived (*see* Subheading 3.1.2), while temperatures reported for algae cultures range from 15 to 26 °C.

3.4.3 Agitation

Agitation (e.g., stirring, shaking, flowing) influences the stability of the NM dispersion and the way and frequency of organism–NM encounter. Fish cells grown in monolayers are cultivated in non-agitated systems; however, suspension cultures may need agitation. Algae are typically agitated by CO_2 flow-through, stirring, or shaking.

3.5 Bioavailability

To understand toxic effects of NM, it is important to characterize the NM in the exposure system with regard to size and charge as detailed as possible (Fig. 1). Furthermore, the distribution of

the NM in the exposure system in the different compartments, i.e., the mass balance including possible dissolution, is an essential information for the interpretation of results. The NM characterization techniques need to be applicable to relevant NM concentrations. An overview of characterization methods is provided below. For a more detailed review, please refer to the literature [12, 46].

3.5.1 Characterization of NM in Exposure Media

Size

The size, among other properties, will determine the behavior of NM in suspension and thus its interaction with organisms. The most commonly used techniques for size characterization in NM toxicology are scanning electron microscopy (SEM) or transmission electron microscopy (TEM) or dynamic light scattering (DLS). The use of nanoparticle tracking analysis (NTA) is increasing. While EM provides images of typically dry material in high vacuum, DLS and NTA measure the hydrodynamic diameter in liquids, and calculations are based on spherical representations of the NM. Details and limitations of the methods can be found in specific literature (*see* **Notes 2**, **3**, and **13**).

In general, designing environmentally relevant or probable exposure scenarios may be contrary to the optimal sample properties for these techniques. For example, natural additives, like humic or fulvic acid or dissolved organic matter (DOC), can influence the measurements. All three methods have their limitations regarding the detectable NM concentration range. As environmentally relevant concentrations may be very low (e.g., up to μg/L instead of mg to g/L), characterizing NM size at these concentrations may be impossible or unfeasible with these methods to date. Field-flow fractionation (FFF) coupled to elemental analysis is applicable to very low NM concentrations but entails the removal of the NM from the exposure medium. The same holds true for the detection of single particles by inductively coupled plasma mass spectrometry (ICP-MS). The surface plasmon resonance (SPR) of metallic NP, especially silver and gold, can be measured by UV–Vis spectrophotometry and provide size information as the SPR peak wavelength depends on the NP size. DLS is not applicable to very polydisperse or unstable suspensions which might, however, represent the NP state in the environment. Large and/or sticky NM/agglomerates that adhere to the surfaces of the NTA sample compartment may also make reliable measurements challenging. Agglomerated NM are a common issue depending on the culture media and dispersion method (*see* Subheadings 3.6.1 and 3.6.2).

In summary, the more monodisperse the sample is and within the optimal concentration range of the methods, the more reproducible and meaningful are the results. We usually employ DLS, NTA, and EM in combination to profit from the complementary information gathered.

Zeta Potential and Electrophoretic Mobility

The zeta potential of a particle system is a measure for its surface charge and is indicative of particle stability and interactions with components of the suspension (molecules, other particles, surfaces) and test organisms. It can be derived mathematically from the electrophoretic mobility of particles within an electric field. Most publications list the derived zeta potential, but providing electrophoretic mobility data helps to compare results independent of the assumptions made when calculating zeta potential. Like size measurements in liquids, the optimal conditions for electrophoretic mobility analysis may not be fulfilled by environmentally relevant NM dispersions. The electrophoretic mobility/zeta potential strongly depends on the pH of a solution; hence, information on the pH value of a solution/medium is required. Shifts in pH (e.g., by oxidation processes or due to cell metabolism) during an exposure may lead to the presence of different particle states in an exposure period; usually this is prevented by using a buffer system with suitable capacity.

3.5.2 Mass Balance and Bioaccumulation of NM

Dispersed NM will partition to different parts of the exposure system depending on its material properties, exposure conditions (solution chemistry, agitation, temperature, illumination), material properties and surface area of the containers used, and the properties and number of the test organisms. Possible redistribution processes from the dispersion of both NM and ions or surface modifiers dissolved from the NM are:

1. Sorption to abiotic and biotic surfaces
2. Uptake by test organisms
3. Sedimentation.

The amount of NM in these different compartments should be quantified to understand the distribution of NM during the exposure. Quantifying NM adsorbed to abiotic surfaces may be difficult to determine but can be deduced from the initial total concentration of NM and the amount present on/in the organisms, in suspension, and possibly in the sediment.

The possible dissolution, coating or surface modification (engineered or by media components), and speciation of a NM change the bioavailability of its components and may influence its effects on test organisms. Thus, these properties should be analyzed under exposure conditions to understand which factor actually elicited measured effects.

The chemical characterization of nonorganic carbon-based NM is typically done via ICP-MS, ICP-AES (atomic emission spectroscopy), or ICP-OES (optical emission spectrometry) following adapted acidic digestion of the NM. When working in culture media, be sure to use matrix-adapted calibration curves. Organic carbon-based NM can be assessed via, e.g., OCEC (organic carbon–elemental carbon) analyzers based on infrared spectrometry.

Dissolved ions may be separated by, e.g., ultrafiltration, diffusive gradients in thin films (DGT) devices, or dialysis prior to chemical analysis or detected by ion selective electrodes. Surface composition may be determined by XPS.

Metal analysis (e.g., by ICP-MS) of entire cells (or possibly subcellular fractions) following acidic digestion informs about bioaccumulation of the NM within cells but does not differentiate between dissolved and particulate material. At the moment, determining intracellular mass balance and speciation is difficult or impossible, depending on the NM and test organism. One reported approach was based on radioactive NPs to facilitate detection and assess bioavailability of ZnO NP in algae [47]. To differentiate between Ag NP and Ag^+ adsorbed to the surface of cells and intracellular silver, one study used several wash steps including cystein to bind Ag^+ [37].

3.5.3 Intracellular Localization of NM

For the interpretation of toxicity test results, information on the localization of NM in or on cells is helpful. The method for detection of NM associated with and in cells depends on the NM and the organism. A combination of different methods should be used to account for the limitations of individual techniques. Optically dense NMs can be visualized by light microscopy (LM); however, it does not provide proof of the chemical identity of the detected particles. As fish cells are transparent, this type of visualization is feasible. LM can be unusable for algae with a cell wall and/or an optically dense cytosol. Electron microscopy of fixed and dried samples provides high-resolution images and can be coupled to, e.g., energy-dispersive X-ray spectroscopy (EDX) to give evidence of the NM chemistry. Preparation artifacts may distort the localization of NMs in the sample. Alternatives such as environmental SEM/TEM which do not require vacuum in the specimen chamber and thus do not require a dry sample are currently being refined. Fluorescence or preferably confocal laser scanning microscopy (CLSM) are valuable to visualize fluorescent NMs [48]. Depending on the fluorescence wavelength emitted by the NMs, algal autofluorescence may interfere with NM detection. Metal-containing NMs exhibit size-dependent surface plasmon resonance when interacting with light and can thus be visualized by CLSM in reflection mode [49]. For ions released from metallic NM, metal specific fluorescent probes may be used to detect dissolved fractions of NM within cells. This approach is limited by the detection limit of the probes and cross-reactivity with other metals. One possibly feasible approach is coupling CLSM with Raman spectroscopy to resolve subcellular chemistry in the nanometer range.

3.6 Effects

Algae and fish cells can be explored for NM effects in many different ways. One common approach is indicator dyes that can be assessed either by fluorescence, luminescence, or by absorbance;

a large variety of such indicator dyes is commercially available. Among them are indicator dyes to assess viability, ATP content, or formation of ROS (Fig. 1). Taking some limitations due to autofluorescent biomolecules, such as chlorophylls, into account, these indicator dyes can generally be applied to both algal and fish cells (*see* Tables 1 and 2 but as well as [13, 14] as examples for effect assessment in fish cells and [50, 51] for examples of effect assessment in unicellular algae). Also fluorescence-based are methods to quantify photosynthetic activity of NM-exposed algae, such as via pulse-amplitude modulated (PAM) fluorometry. PAM provides a measure of the relative activity of photosystem II reaction centers at a given time and information on which part of the electron transport chain is affected in response to a stressor. In principle, one can make use of the variety of effect measurements established also for exposure of cells to chemicals, including genotoxicity, gene and protein expression, and impact on enzyme activity (*see* **Notes 14–18** for critical points in choosing end points).

The key to successfully applying effect-focused assays is to include appropriate controls to take potential interferences by NMs into account. For example, carbon-based NMs have a high sorption capacity also for hydrophobic fluorescence indicator dyes, which may render some of these dyes impossible to use to measure NM cellular effects [52]. Another important aspect is how to express the effect assessment results in terms of exposure or dose. These two aspects, interferences/controls and dose metrics, are briefly discussed below.

3.6.1 Potential Interferences and Appropriate Controls

NMs have a high surface-to-volume ratio and possess specific physicochemical properties which make them prone to interfere with certain types of toxicity tests [30]. Possible interferences that need to be ruled out by appropriate cell-free controls are listed in Table 1 (also *see* **Note 8**). In certain cases it may be difficult to distinguish between the different types of interferences. Different surface modifications of NM may induce different interferences. Media components may also interact with the reaction substrate, product, or enzyme. The medium should thus be tested in the same way as NM dispersions.

With regard to the NMs, two types of controls should be considered: reference NMs to rank the test results and make them better comparable to other studies and positive controls to verify the functionality of the toxicity test. A repository of representative NMs has recently been established by the Institute for Health and Consumer Protection (IHCP) of the European Commission Joint Research Centre (EC JRC) [53]. However, round robin tests based on these materials have not yet been published. To distinguish between the effects of ions and particulate material of soluble NMs, appropriate characterization methods help to know the

extent of ion dissolution or impurities from the production process. Additionally, controls with ion chelators and metal salts are recommended. Surface modifications may also induce effects independent of the NMs. It may thus be reasonable to test surface modifiers independent of the NM.

3.6.2 Dose Metrics

Dose metrics is a widely debated issue in nanotoxicology. Possible bases to express measured effects are the total mass, the surface area of the dry material, or the number concentration. Furthermore, these can be expressed per cell surface, cell volume, cell number, or total exposure volume. The most common choice is total mass per total exposure volume, mostly given in mass NM/volume medium (*see* Tables 2 and 3). The total mass is the easiest to measure but does not account for size distribution. NM surface area and number have to be derived from other information (most often size distribution by intensity) which may be biased toward smaller or larger particles and also do not account for size distribution. Additionally, in case of NM agglomeration (or possibly aggregation) in the test media, both size and free surface area may change. Certain assumptions such as uniform shape have to be made. Cell number and exposure volume are easy to determine, while cell surface and volume have to be derived also in making certain assumptions. In summary, none of the combinations is a perfect solution. Using total mass per exposure volume and providing a thorough NM dispersion characterization and detailed description of exposure conditions is one approach to increase comparability of reported results because it also allows for recalculating different dose metrics. For soluble NMs it is recommended to use the dissolved fraction as well as total concentration as basis to compare toxicity of different treatments to see whether free ions can explain the measured toxicity and whether it is identical or different for different forms of the same material.

4 Notes

1. Laboratory safety: Be sure to protect yourself and your colleagues from exposure to NM. If possible, dedicate a specific area in the laboratory to handling NM. In particular, a dedicated hood should be used for powdered material. We additionally keep track of the NM handled in the nano-laboratory and the involved persons. Handling NM waste is an important issue and should be discussed with the personnel responsible for chemical waste.
2. Containers: Consider choosing your dispersion and exposure containers depending on the tested NM, e.g., using plastics to minimize metal adsorption during exposure.

3. Glass ware: Soak in diluted HNO_3 to minimize adsorption of metals to the surface during experiments; muffle in case you need to determine organic carbon.
4. Sonication: Be sure to use indirect sonication to avoid metal contamination by direct sonication.
5. NM may have to be cleaned every time before use (e.g., ref. 42).
6. NPs may change agglomeration state in the presence of cells [68].
7. Properties of purchased NM may vary between different lots. This may be a cause of variable responses in the toxicity tests.
8. Test results may prove to be irreproducible in independent experiments or show high standard deviations (e.g., ref. 56), which requires troubleshooting. One cause of this variability may be differences in NM dispersions or nonintuitive factors such as frequency of opening and closing of incubators during growth and exposure.
9. Due to the variation in NM preparation and of exposure conditions in available reports, results (*see* Table 1) may differ from reported values even if the same NM and same organism was used.
10. DLS: Discard all DLS data not meeting software quality criteria.
11. NTA: Carefully adjust the data processing settings to the sample analyzed.
12. The methods for NM characterization that are currently available are limited regarding very low (but likely environmentally relevant) concentrations of NM.
13. Most of the toxicity end points reported in the literature to date are descriptors of the general state of cell cultures (such as growth rate, membrane integrity, photosynthetic activity).
14. Most methods were adopted from the testing of chemicals.
15. Direct interaction with intra- and extracellular molecules (e.g., DNA, specific enzymes, and extracellular polymeric substances) has not been studied.
16. The influence of NMs on the cell cycle and biochemical pathways is still unexplored.
17. We are far from understanding the mechanisms of interaction between the considered biological models and NM.
18. To minimize interference with subsequent measurements, cells should be washed, taking into account the chemistry and charge of extracellular substances and of the NM (e.g., slightly acidic buffer to neutralize extracellular negative charges and thus weaken NM-cell interaction).

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