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Applicability of nanomaterial-specific guidelines within long-term *Daphnia magna* toxicity assays: A case study on multigenerational effects of $nTiO_2$ and $nCeO_2$ exposure in the presence of artificial daylight

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ABSTRACT

In recent years, various ecotoxicological test guidelines and (technical) guidance documents have been evaluated and updated with regard to their applicability to nanomaterials (NMs). Several of these have currently reached official regulatory status. Ensuring their harmonized implementation with previously recognized methods for ecotoxicity testing of chemicals is a crucial next step towards effective and efficient regulation of NMs. In the present study, we evaluated the feasibility of assessing multigenerational effects in the first generation of offspring derived from exposed *Daphnia magna* whilst maintaining test conditions in accordance with regulatory test guidelines and guidance documents for NMs. To do so, we integrated the recommendations for ecotoxicological testing of NMs as defined in OECD Guidance Document 317 into an extended long-term *D. magna* reproduction test method (OECD Test Guideline 211) and assessed effects of two poorly soluble NMs ($nTiO_2$ and $nCeO_2$). Our results show adverse effects on life-history parameters of *D. magna* exposed to the selected nanomaterials within the range of reported environmental concentrations. We argue that conforming to OECD test guidelines and accompanying guidance for nanomaterials is feasible when performing *D. magna* reproduction tests and can minimize unnecessary duplication of similar experiments, even when extensions to the standardized test setup are added.

1. Introduction

Environmental risk assessment (ERA) of chemicals and substances relies on harmonized Test Guidelines (TGs) and Guidance Documents (GDs) developed by organizations such as the International Organization for Standardization (ISO) and the Organisation for Economic Co-operation and Development (OECD). In particular for the OECD the use of such test guidelines is linked to a mutual acceptance of data (MAD), i.e. the legally binding instrument which states that all OECD member countries must accept study data for regulatory safety assessment if the study has been performed under good laboratory practice (GLP) and in accordance with an OECD Test Guideline (OECD, 2020b). In this way, unnecessary repetitions of regulatory ecotoxicological tests are avoided, thereby minimizing the costs and number of test animals needed for risk assessment purposes. The *Daphnia magna* reproduction test (OECD TG 211, OECD 2012) constitutes a prominent example of a TG and is used to determine the likelihood of population-level effects upon exposure to contaminants by measuring impairment of reproduction-related endpoints over a 21 day period. The use of the *D. magna* reproduction test for effect assessment of soluble chemicals has been well established within the scientific and regulatory communities for decades (initial version of this TG was published in 1984), and plays a decisive role in international regulatory frameworks and legislation. These include the REACH regulation (Registration, Evaluation, Authorization and restriction of Chemicals, EC No. 1907/2006), the Biocidal Products Regulation (BPR, EC No. 528/2012) and the revised regulation for plant protection products (Regulation EC No. 1107/2009) in Europe, and the TSCA (Toxic Substances Control Act) in the United States of America (USA).

In the last decade, nanomaterials (i.e. NMs, materials with particle sizes between one and 100 nanometers) have become an integral part of society, economy and major industries. In order to mitigate potential adverse environmental effects resulting from (inadvertent) NM emissions, the OECD has initiated (re-)evaluations of currently in place

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regulatory documents used in ERA (OECD 2021). The goal of these (re) evaluations was to determine to what extent and how TGs and GDs which have initially been developed for (soluble) chemical stressors need to be adapted to ensure their applicability to (poorly- and highly soluble) NMs (OECD 2021). To this end, the overall consensus within the regulatory and scientific community which has emerged from multiple extensive national and international research projects (see listed projects in OECD 2020a) on NM safety states that obtaining understanding of which characteristics of NMs contribute to harmful effects is of high importance, and that in order to optimize ecotoxicological tests to this extend, primary emphasis needs to be placed on (1) establishing suitable exposure conditions, (2) ensuring sufficient characterization of NM properties and (3) selecting relevant endpoints (OECD 2020[a], OECD 2019; Rasmussen et al., 2019, Savolainen et al. 2013).

In order to establish suitable exposure conditions in toxicity tests, reproducible preparation of exposure media and obtaining a highly dispersed state of the tested NM by minimizing aggregation and sedimentation have been reported as key factors (Kaur et al., 2017; OECD 2017; Hotze et al., 2010). To further minimize inconsistencies and ensure transferability of findings between studies, standardized operating procedures (SOPs) specifying required characterization efforts have been proposed by Kaur et al. (2017). These considerations have been acknowledged by OECD and are incorporated in the recently published OECD GD on aquatic and sediment toxicological testing of NMs (OECD GD 317, OECD 2021), which specifically outlines the practical aspects and requirements for carrying out valid ecotoxicological tests with NMs. Although not considered within the scope of OECD GD 317, the selection of relevant endpoints with regard to NM toxicity has been amply discussed within the scientific literature (e.g. Liu et al., 2021; Ellis et al., 2020). In this regard, it has been noted that the persistence of poorly dissolving NMs in the environment is inherently high, and that uptake by organisms and resulting effects may manifest relatively slowly in comparison to soluble chemicals (Scott-Fordsmand et al., 2017). In line with this, Valsami-Jones and Lynch (2015) have argued that acute impacts resulting from exposure to realistic concentrations of NMs are rarely observed in ecotoxicological tests, underscoring the need for consideration of long-term exposures when assessing NM ecotoxicity.

Several specific challenges arise when performing long-term exposure studies with NMs. Firstly, organisms need to be fed during the course of the experiment. In the case of aquatic exposure, this will inevitably be accompanied by introducing organic matter into the exposure medium which is likely to impact NM fate due to the formation of an ecocorona (i.e. biomolecules bound to the particle surface) resulting in altered aggregation dynamics. Secondly, effective exposure concentrations are likely to be highly variable over time as aggregation, settlement, and dissolution (in the case of highly soluble NMs) processes often cannot be prevented without deteriorating the quality of the exposure medium. Currently, standardized test setups which are able to maintain the long-term exposure conditions of the NMs whilst maintaining suitable conditions for the test organism of interest are limited to a single example for algae (see Skjolding et al., 2020).

Long-term exposure studies also do not, by default, account for all relevant processes that may affect the longevity of natural populations. Importantly, effects that manifest in the offspring of exposed individuals (i.e. multigenerational effects) are currently not accounted for in most TGs, including the *D. magna* reproduction test, despite the fact that continuity in reproductive health of offspring over multiple generations drives population longevity. A primary obstacle here is the increase in resources, time and sampling effort which is often associated with extending a test setup to include assessment of multigenerational effects (Castro et al., 2018). To this end, Castro et al. (2018) proposed and validated a modification to the *D. magna* reproduction test which allows for the assessment of multigenerational effects resulting from exposure to conventional (soluble) chemicals without extending the timeframe or sampling requirements of the standard test significantly, by including an additional assessment of the reproductive performance of the first generation of offspring derived from the exposed parental generation.

In recent years, various studies have applied adapted versions of the *D. magna* reproduction test in order to assess multigenerational exposure and effects of NMs. Bundschuh et al. (2012) for example demonstrated increased sensitivity towards titanium dioxide nanoparticles ($nTiO_2$) in short-term immobilization tests with *D. magna* offspring that were derived from parents reared in a $nTiO_2$ contaminated environment. A more elaborate setup with the same species and the same NM was applied by Jacobasch et al. (2014), who assessed mortality, growth and reproduction over six generations during continued exposure. In line with the findings of Bundschuh et al. (2012), Jacobasch et al. (2014) found that the sensitivity to $nTiO_2$ for various endpoints generally increased over generations, even leading to full population collapse after 5 generations in nominal treatment concentrations >1.78 mg L^{-1}. More recently, Ellis et al. (2020) demonstrated that even when juveniles of *D. magna* derived from parents exposed to $nTiO_2$ and Ag NMs were reared in exposure-free conditions, adverse effects on growth and mortality were still present. In a subsequent study, the same authors elaborate on these findings by presenting extensive evidence that offspring of parental generations which had been exposed to $nTiO_2$ experienced alterations in expression levels of several genes involved in inflammatory and oxidative stress responses, and exhibited reduced reproduction rates (Ellis et al., 2020).

These studies provide valuable insights in potential long-term population level effects resulting from NM exposure and the underlying biological processes which govern such effects. For the purpose of implementation of multigenerational effect assessments in regulatory frameworks and TGs however, setups such as those applied in these studies may be considered undesirable due to their relatively large resource, time and sampling requirements.

In the present study, we aimed to explore the feasibility of incorporating an assessment of multigenerational effects of NMs in *D. magna* reproduction tests according to the criteria as stated for regulatory risk assessment. For the assessment of multigenerational effects, we applied an extension to the *D. magna* reproduction test as proposed by Castro et al. (2018), thereby minimizing the required time and sampling efforts to those of a standard *D. magna* reproduction test (OECD, 2012). To meet the criteria for regulatory risk assessment, we selected two poorly soluble NMs ($nTiO_2$ and $nCeO_2$, 0, 0.02, 0.2, and 2 mg L^{-1}) and followed the specific recommendations for ecotoxicological testing of NMs as defined in OECD GD 317 (OECD, 2021). $nTiO_2$ and $nCeO_2$ were selected, as these NMs are currently amongst the most produced and used NMs in the world, with applications ranging from (photo-catalytic) paints, coatings, cosmetics, sunscreens, to air- and wastewater treatment facilities and catalytic converters in the automotive industry (Peters et al., 2018). Since part of the mode of action of $nTiO_2$ and $nCeO_2$ has been hypothesized to be derived from their ability to form reactive oxygen species (ROS) in the presence of UVA radiation, we additionally applied full-spectrum artificial daylight at every exposure concentration following a full factorial design in order to assess potential effects of ROS induced toxicity (Coral et al., 2021; Farner et al., 2019).

2. Materials and methods

The present study followed the criteria as specified in OECD GD 317 (OECD 2021), with exception of the use of probe-sonication and stabilizing agents for stock and exposure suspensions (see section 2.2). A summary of all criteria and their implementation in this study is provided in Supplementary information A (Table SI. 1 & SI 2.).

2.1. Primary characterization of the test materials

$nTiO_2$ (JRCNM01005a, European Commission – DG JRC, also provided by Degussa/Evonik as AEROXIDE P25®) and $nCeO_2$ (JRCNM212a, European Commission – DG JRC) with reported primary

particle sizes of 15–24 nm and 33 nm respectively were obtained as a dry powder from the repository for Representative Test Materials (RTMs) of the Joint Research Centre of the European Commission (JRC, Ispra, Italy). Both NMs were derived from a single production batch to enhance the comparability of test results between experiments and laboratories. The $nTiO_2$ used in the experiment is reported as consisting of a mixture of ~85% anatase: 15% rutile crystalline forms (JRC, 2014a) and the $nCeO_2$ is reported as consisting of 100% cubic cerionite (JRC, 2014b). Photocatalytic activity of the $nTiO_2$ used in this experiment is well documented and ROS formation has been observed after irradiation in water with natural and artificial sunlight (Ma et al., 2012). ROS generation of the $nCeO_2$ used in the experiment was previously compared to 100% anatase $nTiO_2$ (the most actively photocatalytic form of $nTiO_2$) and was found to be similar in terms of photocatalytic activity as demonstrated by quantification of triiodide formation in UV-irradiated deionized water using UV–visible spectroscopy (JRC, 2014b).

2.2. Preparation of stock- and exposure suspensions

Stock suspensions of both NMs were prepared freshly before every spike in 100-mL glass bottles by dispersing 100 mg L^{-1} $nTiO_2$ or $nCeO_2$ in Milli-Q water (Milipore Milli-Q reference A+ system, Waters-Millipore Corporation, Milford, MA, USA). Prior to use, stocks were ultra-sonicated for 10 min using a bath sonicator (Sonicor SC-50-22, Sonicor INC. NY, USA) which was calibrated as according to the NANoREG-ECOTOX Probe Sonication Calibration SOP (2015) to deliver 27 ± 0.2 Watt s^{-1} of acoustic energy. Bath sonication was applied to reduce chances of potential cross-contamination and no stabilizing agents (e.g. humic acid, organic matter) were used in order to ensure translatability between studies (OECD 2021). All exposure suspensions were prepared within 10 min after preparation of the stock suspension by pipetting the required volume of stock in Elendt M7 medium (Elendt, 1990).

2.3. Characterization of stock- and exposure suspensions

Particle size distributions (through dynamic light scattering, DLS) and zeta potential were determined in stock suspensions (at 0, 0.5, and 1h after preparation) and in Elendt M7 medium (at 0, 24 and 48 h after preparation using a Malvern Zetasizer Ultra (Malvern, Malvern, UK) thereby mimicking the test conditions.

Mass-based exposure concentrations and settling rates were verified in triplicate by inductively coupled plasma atomic emission spectrometry (ICP-OES) (Agilent Technologies, Santa Clara, CA, USA). To do so, 3 mL samples were collected from the center of the water column at 0, 1, 24 and 48h after preparation of exposure media as previously described. Prior to analysis, 3 mL of sample was digested in 0.3 mL of sulfuric acid (H_2SO_4 96%, Sigma-Aldrich - St. Louis, MI, USA), 0.3 mL of phosphoric acid (H_3PO_4 85%, Sigma-Aldrich - St. Louis, MI, USA) and 0.3 mL of HNO_3 (65% Sigma-Aldrich - St. Louis, MI, USA) followed by 1h of bath-sonication at 50 °C). Calibration curves were obtained by diluting a standard of titanium and cerium (Sigma-Aldrich - St. Louis, MI, USA) in Elendt M7 medium (concentration range 0.1–10.0 mg L^{-1}) and reproducing the same matrix as used for the sample digestion procedure.

Scanning electron microscopy (SEM) analyses of the particles used in this study have been described extensively (JRC 2014a; JRC 2014b) and were thus not conducted for the present study.

2.4. Test organisms

D. magna were obtained from an in-house culture at Leiden University which is maintained according to the conditions prescribed in OECD Test Guideline 211 (OECD, 2012). In short, cultures are maintained in a climate room at 22 ± 1 °C under a 16:8 h (light:dark) photoperiod and fed with a suspension of *Pseudokirchneriella subcapitata*. Prior to the experiment the sensitivity of the neonates of the culture was tested through a reference toxicity test using $K_2Cr_2O_7$ and the test result was confirmed as being within the recommended boundaries (24h-EC50 immobilization 0.96 ± 0.09 mg L^{-1}) as stated in the *Daphnia magna* Immobilization Test Guideline 202 (OECD 2004).

2.5. Experimental design

The experiment was initiated by introducing 12 neonates (<24h) in individual glass beakers containing 50 mL of Elendt M7 medium spiked with $nTiO_2$ or $nCeO_2$ at concentrations of 0, 0.02, 0.2, and 2 mg L^{-1} in the presence and absence of full-spectrum artificial daylight, following a full factorial design. Exposure concentrations were selected to be within the range of environmentally realistic concentrations of $nTiO_2$ as reported by Peters et al. (2018) on the low end, and effect concentrations of $nTiO_2$ as observed in previous multigenerational studies on $nTiO_2$ in *D. magna* on the high end (Jacobasch et al., 2014). $nCeO_2$ exposure concentrations were selecting accordingly, to ensure comparability between NMs. The exposure medium was refreshed every 48 h and aerated in order to achieve dissolved oxygen (DO) concentrations ≥3 mg L^{-1} prior to addition of the NMs. DO and pH were measured in triplicate per test concentration before and after every medium renewal. Offspring was counted and removed during every medium renewal. Feeding took place directly after every medium renewal at a rate of 0.1–0.2 mg C/Daphnid/day by addition of *P. subcapitata*.

UV radiation was applied using fluorescent tubes (Terra Exotica UV fluorescent tubes, Terra Exotica, Alfeld, Germany) that emit light at an intensity and spectrum that resembles natural lighting conditions, including 10% UVA and 2% UVB emission. Light intensity (in the UVA, UVB and UVC range) was verified (Supplementary information B) at the surface of the medium of the beakers in the experimental setup using a Flame-S photospectrometer (ser#FLMS02180, Ocean Insight, Duiven, the Netherlands) (SI Fig. 1). In addition, regular fluorescent tubes (Sylvania luxline plus, Osram Sylvania Inc., Massachusetts, USA) were used in the non-UV treatment and their spectral output was analyzed as described above. Controls without nanomaterial exposure but with full-spectrum artificial daylight were included to account for effects induced by UV radiation.

Multigenerational effects were assessed by following a modified version of the *Daphnia magna* reproduction test (OECD TG 211, OECD 2012) as proposed by Castro et al. (2018). In short, this modification consists of an additional test on top of the 21 day exposure period, in which individuals from the first brood of the F0 generation are reared in clean medium until release of their own first brood. The number of individuals in the first brood of the F1 is considered as a proxy for maternal investment in juvenile fitness, i.e. a multigenerational effect relating to reproductive abilities.

Mortality, time until maturity (defined as days elapsed before release of 1st brood), number of neonates in 1st brood, number of broods and total number of neonates produced over 21 days were recorded for the F0 generation throughout the 21 day exposure period at every medium replacement. Body size was measured in 5 individuals per treatment (at the end of the 21 day exposure period for the F0 generation, and in individuals from the first brood of the F1 generation) from the top of the head, through the eye, to the base of the apical spine from pictures taken with a Leica MZ16FA stereomicroscope equipped with a Leica DFC420C digital color camera (Leica, Wetzlar, Germany) using ImageJ image analyzer software. In addition, mortality, time until maturity and the number neonates in the first brood were recorded for the F1 generation.

2.6. Data analysis

All data was analyzed using R version 1.1.419 (R Core Team 2017). Effects of NM and UV treatments on the cumulative number of neonates produced over 21 days (F0) were assessed using Poisson distributed generalized linear models (GLMs, package:*stats*, function: *glm*) including all possible interactions between NM treatment, UV treatment and day.

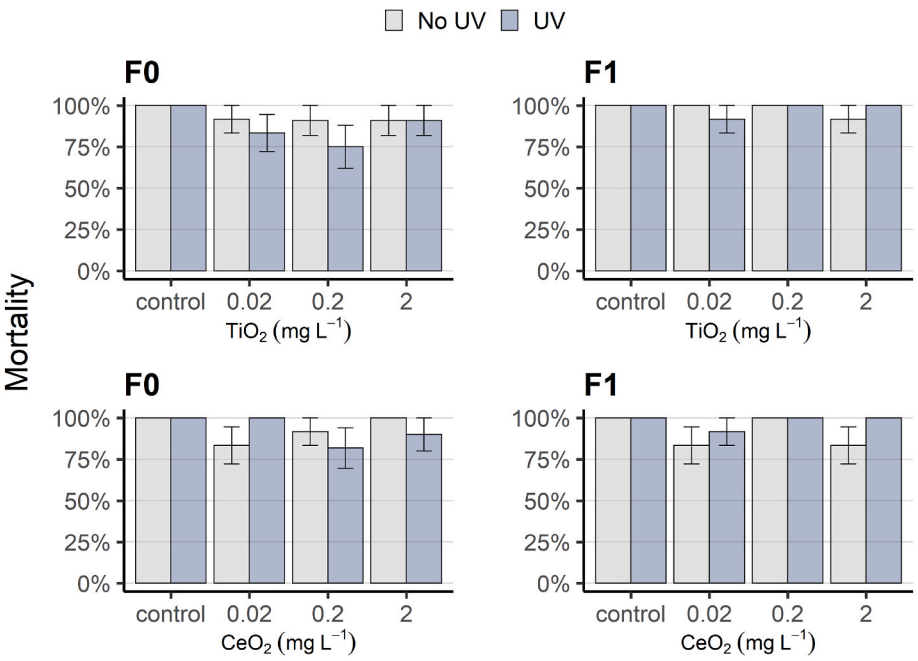

Fig. 1. Mean mortality of Daphnia magna during the 21 day exposure period of the F0 and until the first brood of the F1. Error bars represent standard errors. (Use of color requested for printing of figure). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Random intercepts were produced for each replicate to account for repeated measures over time. If models showed overdispersion, quasi-Poisson distributed GLMs were generated according to the same structure. Tukey adjusted estimated marginal means (EMMs, package: *emmeans*, function:*emmeans*) were calculated to assess on which specific days differences between controls and treatments were present.

Total number of neonates produced (F0 & F1), time until maturity (F0 & F1), number of neonates in first brood (F0 & F1), number of broods (F0) and body size (F0 & F1) were analyzed using two-way anovas (package:*stats*, function:*aov*) including interactions between NM treatment and UV treatment followed by Tukey pairwise comparisons. A subset of the data was generated prior to the analyses by removing all data points from individuals that died during the test. All data was checked for heteroskedasticity (Levenes test) and normal distribution (Shapiro Wilk test and visual inspection of histograms and QQ-plots) of the residuals prior to analysis. If assumptions for parametric tests were not met, the data was log10(x+1) or square-root transformed. In case assumptions were still not met, Kruskal-Wallis tests were performed followed by Bonferonni corrected Dunn's post-hoc tests.

To assess effects of NM and UV treatments on overall mortality (F0 & F1), binomial generalized linear models (GLMs, package:*stats*, function: *glm*) were generated including interactions between NM treatment and UV treatment. Tukey adjusted estimated marginal means (EMMs, package:*emmeans*, function:*emmeans*) were calculated to assess on which specific days differences between controls and treatments were present. Outcomes of statistical tests were considered statistically significant at $P < 0.05$.

3. Results

3.1. Mass-based concentrations and settling rates of $nTiO_2$ and $nCeO_2$ in exposure suspensions

Water column concentrations in the test setup initially showed only minor deviations from nominal test concentrations (Table 1). Relative deviations from nominal test concentrations were consistently larger at lower treatment concentrations, and over time, aggregation and subsequent sedimentation of particles consistently increased for both NMs at all except the lowest (0.02 mg L^{-1}) treatment concentrations (Tables 1 and SI Fig. 2). Time-dependent settling rates were most pronounced in the $nCeO_2$ treatment (SI Fig. 2). To aid in interpretation and ensure transferability of results, time weighted average (TWA) concentrations calculated over 48h medium renewal intervals are provided in Table 1.

3.2. Hydrodynamic diameter and stability of $nTiO_2$ and $nCeO_2$ in exposure suspensions

The hydrodynamic diameters (z-average) of $nTiO_2$ and $nCeO_2$ in the suspensions differed between exposure concentrations and generally increased over time (Table 1). Zeta-potential measurements ($nCeO_2$: -3.04 ± 1.16 mV; $nTiO_2$ -5.09 ± 0.58 mV) and polydispersity indexes (Table 1) further indicated that both NMs underwent rapid aggregation over the exposure period.

3.3. Validity criteria of the experiment

The experiment met all performance criteria as set in OECD TG 211 (OECD 2012): mortality of the parent *D. magna* in controls of the F0 remained <20% and the mean number of living offspring produced per parent at the end of the 21 day exposure period was >60. All endpoints showed comparable trends in controls reared in absence and presence of UV radiation.

3.4. Mortality (F0 & F1)

No statistically significant differences in overall mortality rates in the F0 and F1 were observed for either NM treatment in absence or presence of UV radiation (see Fig. 1).

3.5. Cumulative number of neonates produced (F0)

The number of neonates produced per individual in the F0 decreased significantly in the 0.2 mg L^{-1} test concentration of the $nTiO_2$ treatment in the presence of UV radiation from the 9th day onwards (see Fig. 2 & Table 2). No statistically significant differences in the mean number of neonates produced were observed at any of the applied $nTiO_2$

Table 1
Time-dependent concentrations, hydrodynamic size and polydispersity index (PDI) measured within the 48h medium renewal intervals of $nTiO_2$ and $nCeO_2$ in samples collected from the center of the water column of test vessels.

Nominal conc. ($mg\ L^{-1}$)		Measured concentration ± SD ($mg\ L^{-1}$) n = 3				
		T = 0h	T = 1h	T = 24h	T = 48	TWA
$nTiO_2$	0.02	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
	0.2	0.17 ± 0.00	0.17 ± 0.01	0.15 ± 0.00	0.15 ± 0.01	0.15 ± 0.01
	2	1.81 ± 0.00	1.79 ± 0.03	1.47 ± 0.05	1.20 ± 0.08	1.35 ± 0.06
$nCeO_2$	0.02	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.03
	0.2	0.15 ± 0.02	0.13 ± 0.02	0.12 ± 0.02	0.09 ± 0.02	0.11 ± 0.02
	2	1.67 ± 0.07	1.69 ± 0.07	0.84 ± 0.06	0.27 ± 0.07	0.58 ± 0.07
PDI ± SD (nm) n = 2						
		T = 0h		T = 24h	T = 48h	
$nTiO_2$	0.02	1.83 ± 0.02		2.03 ± 0.03	1.81 ± 0.05	
	0.2	0.99 ± 0.02		1.82 ± 0.02	1.76 ± 0.26	
	2	0.56 ± 0.05		2.02 ± 0.02	2.03 ± 0.01	
$nCeO_2$	0.02	0.68 ± 0.04		1.76 ± 0.04	2.43 ± 0.03	
	0.2	0.33 ± 0.09		2.28 ± 0.09	2.24 ± 0.02	
	2	0.37 ± 0.19		0.19 ± 0.04	2.20 ± 0.15	
Hydrodynamic size ± SD (nm) n = 2						
		T = 0h		T = 24h	T = 48h	
$nTiO_2$	0.02	4304 ± 116		7298 ± 78	5393 ± 206	
	0.2	1353 ± 334		4459 ± 101	4206 ± 1220	
	2	521 ± 57		6608 ± 853	6224 ± 739	
$nCeO_2$	0.02	721 ± 282		4003 ± 377	9865 ± 15	
	0.2	317 ± 9		7075 ± 584	6494 ± 344	
	2	302 ± 18		3266 ± 475	5585 ± 539	

SD = standard deviation, TWA = Time weighted average.

concentrations in the absence of UV radiation. Interestingly, no statistically significant differences in the mean number of neonates produced were observed at the 2 mg L^{-1} test concentration of the $nTiO_2$ treatment in presence of UV radiation either. In the F0 of the $nCeO_2$ treatment, a statistically significant decrease in the number of neonates produced was observed only at the lowest (0.02 mg L^{-1}) test concentration in absence of UV radiation (see Fig. 2 & Table 2).

3.5.1. Total number of neonates produced (F0 & F1)

No statistically significant differences in the total number of neonates produced in the F0 and F1 were observed for either NM treatment

Table 2
Results obtained from quasi-Poisson distributed GLMs and post-hoc pairwise comparisons showing effects of 0, 0.02, 0.2 and 2 mg L^{-1} $nTiO_2$ and $nCeO_2$ treatments in presence and absence of UV radiation on the cumulative number of neonates produced in the test setup. Results before the first day of reproduction are summarized for all days together.

Pairwise comparisons Tukey adjusted P-values<0.05 controls vs. treatments ($mg\ L^{-1}$)								
		No UV				UV		
NM	Day	0.02	0.2	2	0	0.02	0.2	2
$nTiO_2$	0–7	ns	ns	ns	ns	ns	ns	ns
	8	ns	ns	ns	ns	ns	ns	ns
	9	ns	ns	ns	ns	ns	**0.04**	ns
	10	ns	ns	ns	ns	ns	**0.06**	ns
	11	ns	ns	ns	ns	ns	**0.03**	ns
	12	ns	ns	ns	ns	ns	**0.03**	ns
	13	ns	ns	ns	ns	ns	**0.03**	ns
	14	ns	ns	ns	ns	ns	**0.005**	ns
	15	ns	ns	ns	ns	ns	**0.02**	ns
	16	ns	ns	ns	ns	ns	**0.02**	ns
	17	ns	ns	ns	ns	ns	**0.001**	ns
	18	ns	ns	ns	ns	ns	**0.001**	ns
	19	ns	ns	ns	ns	ns	**0.001**	ns
	20	ns	ns	ns	ns	ns	**0.007**	ns
	21	ns	ns	ns	ns	ns	**0.007**	ns
$nCeO_2$	0–7	ns	ns	ns	ns	ns	ns	ns
	8	ns	ns	ns	ns	ns	ns	ns
	9	ns	ns	ns	ns	ns	ns	ns
	10	ns	ns	ns	ns	ns	ns	ns
	11	ns	ns	ns	ns	ns	ns	ns
	12	**0.02**	ns	ns	ns	ns	ns	ns
	13	**0.02**	ns	ns	ns	ns	ns	ns
	14	0.09	ns	ns	ns	ns	ns	ns
	15	**0.009**	ns	ns	ns	ns	ns	ns
	16	**0.009**	ns	ns	ns	ns	ns	ns
	17	**0.009**	ns	ns	ns	ns	ns	ns
	18	**0.009**	ns	ns	ns	ns	ns	ns
	19	**0.007**	ns	ns	ns	ns	ns	ns
	20	**0.007**	ns	ns	ns	ns	ns	ns
	21	**0.001**	ns	ns	ns	ns	ns	ns

ns = not significant (P ≥ 0.05). P-values ≤ 0.05 are indicated in bold.

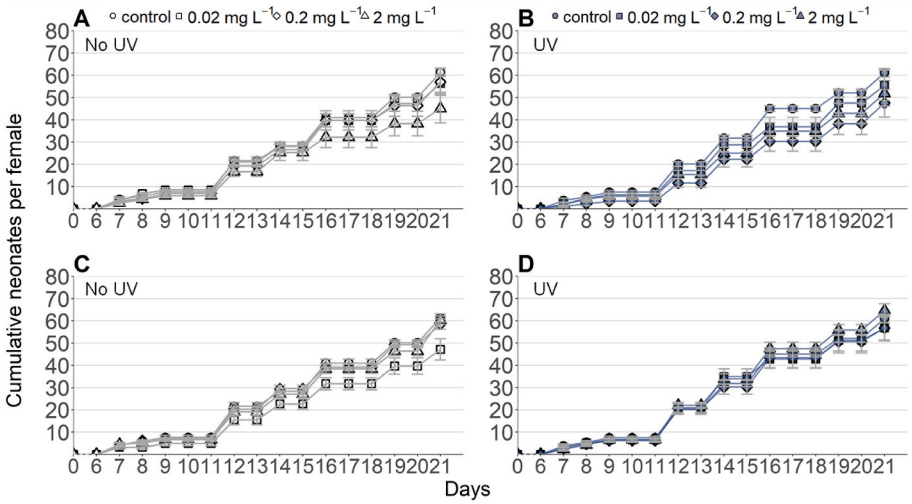

Fig. 2. Cumulative number of neonates of *Daphnia magna* produced in 0, 0.02, 0.2 and 2 mg L^{-1} $nTiO_2$ (**A** & **B**) and $nCeO_2$ (**C** & **D**) treatments in presence and absence of UV radiation during the 21 day exposure period in the F0. Error bars represent standard error of the mean (SEM). See Table 2 for Tukey adjusted P-values<0.05 per treatment in comparison to controls. (Use of color requested for printing of figure). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in the absence or presence of UV radiation (see SI Fig. 3 & Table 3).

3.6. Time until maturity (F0 & F1)

A clear dose-response relationship was observed for time until maturity (defined as time until release of first brood) in the F1 of the 0.2 mg L^{-1} ($p<.0001$) and 2 mg L^{-1} ($p<.0001$) $nTiO_2$ treatments, regardless of absence or presence of UV radiation. No differences in time until maturity were observed in the F0 of either NM treatment or the F1 of the $nCeO_2$ treatment (Fig. 3 & Table 3).

3.7. Number of neonates in 1st brood (F0 & F1)

No statistically significant differences in the number of neonates produced in the first brood of the F0 or F1 were observed for either NM treatment in the absence or presence of UV radiation (see SI Fig. 4 & Table 3).

3.8. Total number of broods (F0)

No statistically significant differences in the number of broods produced in the F0 were observed for either NM treatment in the absence or presence of UV radiation (see SI Fig. 5 & Table 3).

3.9. Body size (F0 & F1)

A statistically significant decrease in the body size of adults (F0) was observed in the 0.2 ($p < 0.0001$) and 2 mg L^{-1} ($p < 0.0001$) test concentrations of $nTiO_2$ relative to controls in the presence of UV radiation (Fig. 4 & Table 3). No statistically significant differences in body size of adults were observed in absence of UV radiation. Additionally, although overall models showed a minor effect of $nTiO_2$ exposure on the body size of neonates in the F1, subsequent post hoc tests showed no differences between any of the treatment concentrations and controls regardless of the presence of UV radiation (Fig. 4 & Table 3).

The mean body size of adults showed a statistically significant decrease relative to controls in the 0.02 ($p = 0.03$), 0.2 ($p = 0.005$) and 2 mg L^{-1} ($p = 0.01$) treatment concentrations of $nCeO_2$ in the presence of UV radiation, and in the 0.2 mg L^{-1} ($p < 0.0001$) treatment concentration in the absence of UV radiation (Fig. 4 & Table 3).

4. Discussion

4.1. Implementation of the (extended) Daphnia magna reproduction test for effect assessment of NMs

Effect assessment of NMs through the use of the *Daphnia magna* reproduction test poses specific issues with regard to exposure conditions which need to be accounted for in order to assure validity, reproducibility and translatability of results. In the present study, we observed >20% deviations from nominal test concentrations of both NMs tested, likely as a result of time-dependent aggregation and sedimentation of particles out of the water column. When encountering this, researchers are posed with a dilemma in which the decision needs to be made to either (1) enhance the stability of the exposure suspensions, (2) increase the frequency of medium renewal, or (3) accept a dynamic exposure scenario in which exposure concentrations and aggregation rates changes over time. In the current study, it was decided to accept a dynamic exposure scenario whist ensuring thorough characterization over the exposure period. It should be noted that enhancing the stability of exposure suspensions e.g. through the introduction of natural organic matter (NOM) may impair comparability with other studies, and that increasing the frequency of medium renewal may drastically increase the amount of NMs needed for testing. To this regard, specifically designed exposure chambers which aim to maintain NMs in suspension over the exposure period such as those proposed for *Danio rerio* (see Boyle et al., 2015) and algae (see Skjolding et al., 2020) may serve as a suitable option for *D. magna* toxicity tests as well.

Specific methods may be applied to achieve stable stock- and exposure suspensions of NMs. To this end, the (draft) dispersion protocol developed as part of the NanoReg project (2015) proposes that stock suspensions are prepared in ultra-pure water at a maximum NM concentration of 2.56 mg L^{-1}, after which stocks are subjected to ultra-sonication using a calibrated probe sonicator (Kaur et al., 2017). In case stable suspensions are not achieved by this procedure, the protocol suggests that a pre-wetting step may be applied and that stock

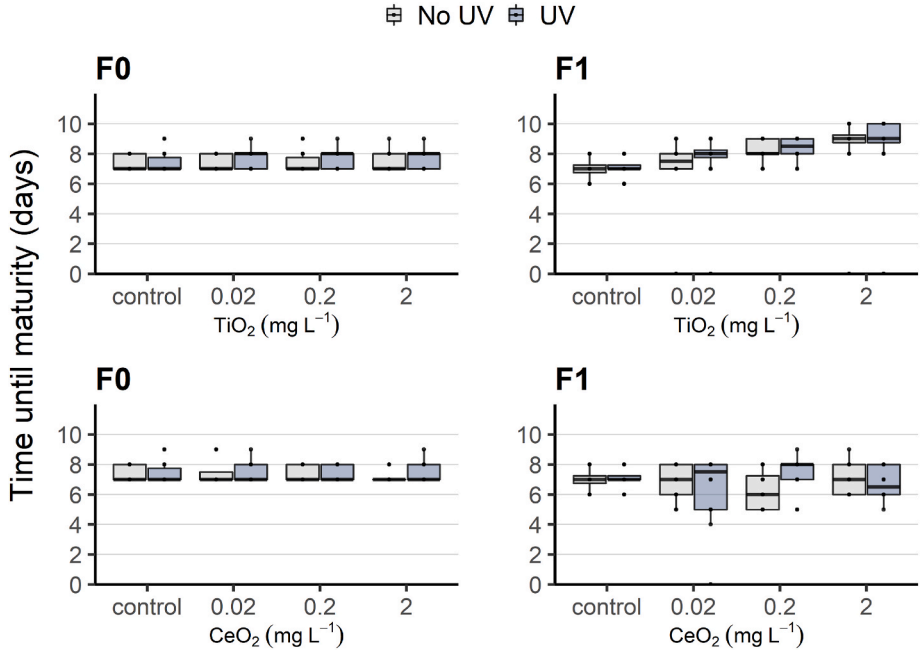

Fig. 3. Time until maturity (i.e. time until release of first brood) of Daphnia magna in the F0 and F1. (Use of color requested for printing of figure). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Results obtained from parametric analyses (two-way anovas) and non-parametric analyses (Kruskal-Wallis) comparing total number of neonates produced, time until maturity, number of neonates in first brood, number of broods and body size between nanomaterial and in presence and absence of UV radiation in the F0 and F1.

Explanatory variable(s)		Df	Sum Sq / Chisq	Mean Sq	F value	Pr(>F) / Pr(>Chisq)
Total number of neonates produced						
F0	$nTiO_2$	3	297	98.87	1.95	0.13
	UV * $nTiO_2$	3	340	113.28	0.61	0.09
	$nCeO_2$	3	267	89.15	1.57	0.20
	UV * $nCeO_2$	3	246	81.89	1.44	0.24
F1	$nTiO_2$	3	0.22	0.07	2.85	**0.04**
	UV * $nTiO_2$	3	0.18	0.01	0.11	0.74
	$nCeO_2$	3	0.10	0.03	1.05	0.37
	UV * $nCeO_2$	3	0.17	0.06	1.84	0.15
Time until maturity						
F0	$nTiO_2$	3	1.09	–	–	0.78
	UV * $nTiO_2$	3	75	–	–	0.48
	$nCeO_2$	3	0.39	–	–	0.94
	UV * $nCeO_2$	3	78	–	–	0.48
F1	$nTiO_2$	3	42.57	–	–	**$3.0e^{-9}$**
	UV * $nTiO_2$	3	91	–	–	0.48
	$nCeO_2$	3	1.28	0.43	0.37	0.77
	UV * $nCeO_2$	3	9.66	3.22	2.81	**0.04**
Number of neonates in first brood						
F0	$nTiO_2$	3	3.7	–	–	0.29
	UV * $nTiO_2$	3	76	–	–	0.48
	$nCeO_2$	3	1.06	0.35	0.13	0.94
	UV * $nCeO_2$	3	14.57	0.03	1.78	0.16
F1	$nTiO_2$	3	0.22	0.07	2.85	**0.04**
	UV * $nTiO_2$	3	0.18	0.01	0.11	0.74
	$nCeO_2$	3	0.10	0.03	1.05	0.37
	UV * $nCeO_2$	3	0.17	0.06	1.84	0.15
Total number of broods						
F0	$nTiO_2$	3	1.13	0.37	0.55	0.65
	UV * $nTiO_2$	3	3.07	1.02	1.49	0.23
	$nCeO_2$	3	4.02	–	–	0.26
	UV * $nCeO_2$	3	81	–	–	0.48
Bodysize						
F0 (Adults)	$nTiO_2$	3	19.70	–	–	**$1.9e^{-4}$**
	UV * $nTiO_2$	3	21.87	–	–	**$2.6e^{-3}$**
	$nCeO_2$	3	3.05	1.02	52.42	**$1.8e^{-12}$**
	UV * $nCeO_2$	3	1.92	0.64	32.97	**$6.5e^{-10}$**
F1 (neonates)	nTiO2	3	0.02	0.01	3.39	0.03
	UV * $nTiO_2$	3	0.01	0.01	0.68	0.57
	$nCeO_2$	3	0.01	0.01	0.97	0.42
	UV * $nCeO_2$	3	0.01	0.01	0.73	0.54

Df = Degrees of freedom, Sum Sq = Sum of Squares, Mean Sq = Mean Squared error, Chisq = Chi-squared; P-values ≤ 0.05 are indicated in bold.

suspensions may be amended with natural organic matter (NOM). In the current study however, following this protocol was considered unfeasible as this would have resulted in either drastically reducing the exposure concentrations, or performing the exposure using a medium composition which would be diluted with ultra-pure water to such an extent that it is likely to result in sub-optimal performance of the test organisms (i.e. containing fewer trace elements and micro-nutrients than advised in OECD TG 211, OECD 2012). Furthermore, amendment of stock suspensions with NOM as suggested in the NanoReg dispersion protocol (2015) was avoided, as this could reduce comparability between tests (OECD GD 317, OECD 2021). To this end however, the extent to which amendment of test medium with algae suspensions, as is required in long-term *D. magna* toxicity tests, acts as a similar stabilizing agent for NMs as NOM, remains a topic for further consideration.

According to the NanoReg dispersion protocol (2015), and as also prescribed in OECD TG 318, OECD 2017, it was decided to adopt the recommendation to calibrate the used sonication device in order to determine the acoustic energy delivered during the sonication process (Kaur et al., 2017). Additionally, exposure dynamics (i.e. aggregation and settling rates, water column concentrations and zeta potential) were monitored by performing measurements at multiple time points within the 48h medium renewal intervals, and TWA exposure concentrations were reported as suggested in OECD GD 317 (OECD 2021). Ultimately, it must be noted that the NanoReg dispersion protocol (2015) provides a highly valuable option for preparing stable stock- and exposure suspensions of NMs, and enhances reproducibility and translatability of (standardized) ecotoxicological tests.

4.2. (Multigenerational) effects of $nTiO_2$ & $nCeO_2$ in Daphnia magna

In the present study, *D. magna* exposed to $nTiO_2$ and $nCeO_2$ showed impairment of reproduction related endpoints both in presence and absence of UV radiation. In the F0, cumulative reproduction over time decreased in *D. magna* exposed to $nTiO_2$ and $nCeO_2$ at treatment concentrations of 0.2 mg L^{-1} (in presence of UV radiation) and 0.02 mg L^{-1} (in absence of UV radiation) respectively. Interestingly, UV radiation showed opposite results with regard to this endpoint between NMs, where $nTiO_2$ treatments responded more strongly in presence of UV, and $nCeO_2$ responded more strongly in absence of UV. A more distinct effect of co-exposure to UV radiation and NMs was observed in the body size measurements of adults at the end of the exposure period of the F0, where both NM treatments induced reductions in presence, but not in absence of UV radiation. With regard to multigenerational effects, *D. magna* exposed to $nTiO_2$ showed a concentration-dependent increase in time until maturity in the F1, whilst this effect was not observed in the F0. For $nCeO_2$ and other endpoints measured in the F1 exposed to $nTiO_2$, no multigenerational effects were observed. Overall, observed effects differed between $nTiO_2$ and $nCeO_2$, despite application of equal nominal

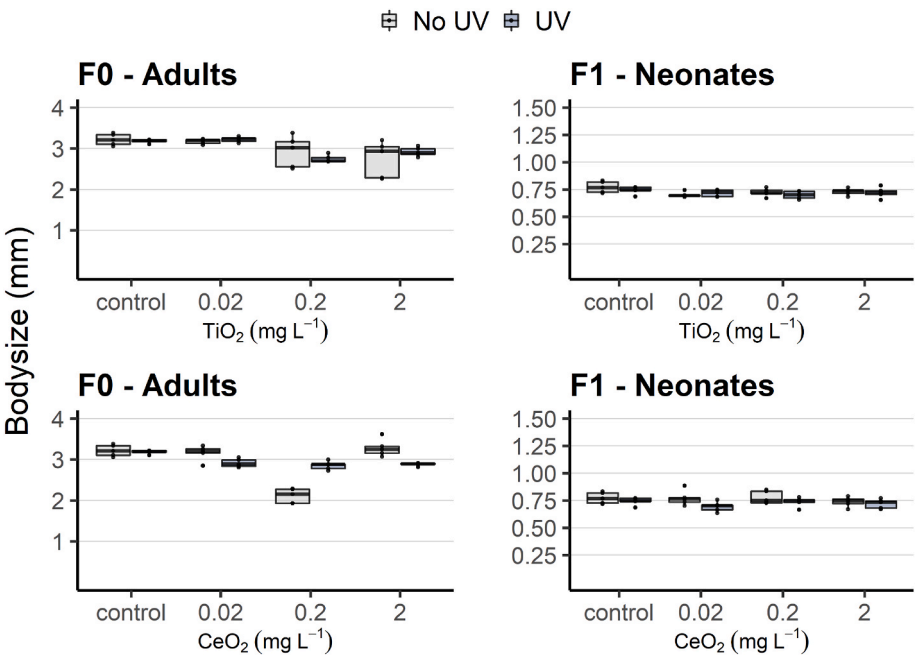

Fig. 4. Body size of adult *Daphnia magna* (F0 after 21 days exposure) and neonates (F1). (Use of color requested for printing of figure). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

test concentrations. In addition, lower nominal test concentrations of both NMs were found induce more pronounced effects than higher concentrations on several endpoints, including the number of neonates produced over 21 days. Regarding these findings, future studies on mechanistic understanding of modes-of-action of each NM and their relation to differences in exposure dynamics (e.g. aggregation and sedimentation rates) may provide valuable insights into translatability of test results of different NMs.

Due to absence of standardization of multigenerational effect assessments of NMs in *D. magna*, comparisons between studies should be made with caution. Our findings with regard to reproductive delays in the F1 exposed to TiO_2 for example are in line with those of Ellis et al. (2021), who found that reproductive delays in *D. magna* exposed to $nTiO_2$ are exacerbated by exposure over multiple generations of exposure. In their study however, Ellis et al. (2021) assessed effects at a single treatment concentration of 5 mg L^{-1}, and exposed individuals in groups of 10 in a single container, rather than in separate containers. Reproduction rates of *D. magna* are known to be affected by crowding, and as such, the choice of experimental setup may induce cofounding effects on reproduction related endpoints (Lowes et al., 2021). To this end, the experimental setup applied by Jacobasch et al. (2014) more closely resembles the setup used in the present study, where *D. magna* were exposed separately. The $nTiO_2$ used by Jacobasch et al. (2014) was the same material as used in the present study, and multigenerational effects on reproduction related endpoints were found at similar concentrations as well. An important distinction however is that in the present study, effects were observed at treatment concentrations which were approximately 10–100 times lower (i.e. 0.02 & 0.2 mg L^{-1}) than those applied in the study by Jacobasch et al. (2014). Anthropogenic $nTiO_2$ has been detected in natural surface waters at concentrations of 0.2–8.1 μg L^{-1} (average particle sizes of 300 nm), and $nCeO_2$ has been found in concentrations ranging from 0.4 to 5.2 ng L^{-1} (average particle size of 19 nm) (Peters et al., 2018). In the present study, treatment concentrations of $nTiO_2$ which induced adverse effects were thus fairly within the range of concentrations present in the natural environment, suggesting that similar effects as found in these studies could occur in the natural environment as well.

4.3. Effect of co-exposure to UV radiation on $nTiO_2$ & $nCeO_2$ toxicity in Daphnia magna and mitigating effects of natural organic matter (NOM)

The present study provides no indication that co-exposure to UV radiation in the form of artificial daylight universally drives or enhances effects induced by photocatalytic NMs. Previous studies have shown that in acute toxicity tests, UV-induced generation of ROS can be an important part of the mode of action of photocatalytic NMs such as $nTiO_2$ and $nCeO_2$ in *D. magna* (Coral et al., 2021; Farner et al., 2019). To our knowledge however, the present study is the first to assess effects of co-exposure to photocatalytic NMs and UV radiation under chronic test conditions and on endpoints other than acute immobilization. It was found by Coral et al. (2021) that amendment of exposure medium with NOM significantly reduces the additive effect of UV radiation on $nTiO_2$ toxicity in *D. magna* (i.e. by reducing light transmission, altering particle surface charge and/or by acting as a quenching agent for ROS). Similarly, the absence of large differences in toxicity of $nTiO_2$ and $nCeO_2$ between UV and non-UV treatments in the present study may, in addition to differences in assessed endpoints, partly be explained by amendment of exposure medium with algae suspensions for feeding. Ultimately, this adds to the extensively discussed issue of whether toxicity tests should be performed under worst-case or under realistic exposure conditions, of which the decision may have significant implications on the findings of the study. Accordingly, mitigating effects of NOM on ROS induced toxicity may be considered as an additional mechanism of interest within the larger debate on effects of NOM on NM toxicity, as discussed in detail by Ellis et al. (2021 & 2020) and Ekvall et al. (2021).

5. Conclusion and outlook

By applying the extension to the *D. magna* reproduction test as suggested by Castro et al. (2018), the current study demonstrates multigenerational effects resulting from exposure to environmentally-realistic concentrations of $nTiO_2$ in the form of delayed time until maturity in the second generation of continuously exposed *D. magna*. ROS-induced toxicity through co-exposure to UV radiation showed varying effects on the assessed long-term endpoints, and was found to be significantly

less pronounced than observed in acute toxicity tests reported by Coral et al. (2021). To this end, the extent to which feeding conditions in long-term tests, and more generally the introduction of NOM to exposure media, affect (ROS induced) toxicity remains to be investigated in more detail prior to consideration as an element to be included in regulatory effect assessment of photocatalytic NMs.

At present, it cannot be stated with certainty that the effects observed in the present study translate to effects on population longevity beyond the time-scale that was tested, as studies which did conduct assessments on such time-scales have been limited to test concentrations exceeding those at which effects were observed in the current study by at least 50 times. When performing (multigenerational) toxicity tests with NMs, potential cofounding effects resulting from choices made with regard to test setups can be minimized by conforming to OECD TGs and accompanying guidance (such as OECD GD 317, OECD 2021) as much as possible, and by assuring adequate characterization of exposure conditions during the test. The current study demonstrates that when combined with the extended *D. magna* reproduction test as proposed by Castro et al. (2018), this allows for a standardized, low-demand and sensitive assessment of multigenerational effects of NMs which meets the criteria as specified for regulatory risk assessment.

In particular for NMs, short-term environmental effects are generally of limited relevance in the assessment of anthropogenic impacts, and long-term exposures to relatively low concentrations appears to hold more relevance for ERA. Nevertheless, data on such long-term exposures and related multigenerational effects of stressors is currently scarce and incidental. Without a clear incentive, e.g. a regulatory demand, it is likely that this scarcity remains, due to the resource-intensiveness of multigenerational testing. As such, it is unlikely that sufficient data will become available to allow multigenerational effects to become part of regulatory risk assessment in the near future. Further validation of the extended approach of OECD TG 211 applied in the current study (i.e. an additional assessment of the reproductive performance of the first generation of offspring) may provide a first step towards inclusion of multigenerational effects in regulatory risk assessment. In addition, further exploration into predictability of multigenerational effects from available data (e.g. derived from a conventional OECD TG 211 test) may contribute to a better inclusion of such long-term effects and the improvement of risk assessment in this regard, e.g. resulting in the adjustment or addition of (supplementary) assessment factors.

Author contributions

TN: Conceptualization; Investigation; Methodology; Conceptualization; Data curation; Formal analysis; Visualization; Writing - original draft; WP: Conceptualization; Supervision; Resources; Funding Acquisition; Project administration; Writing-review & editing; EB: Writing-review & editing; Regulatory aspects; MV: Conceptualization, Supervision, Resources, Funding acquisition, Project administration, Writing-review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2022.105156.

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