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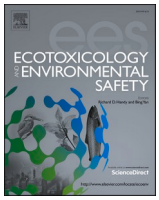

Correlation analysis of single- and multigenerational endpoints in *Daphnia magna* toxicity tests: A case-study using TiO_2 nanoparticles

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ABSTRACT

Multigenerational toxicity tests provide more sensitive measures of population-level effects than conventional single-generation tests. Particularly for stressors which exhibit slow uptake rates (e.g. nanomaterials), multigenerational tests may also provide a more realistic representation of natural exposure scenarios. To date, the inherently high costs and labor intensity have however limited the use of multigenerational toxicity tests and thereby their incorporation in environmental risk assessment. The aim of the present study was therefore to determine to what extent short(er) term endpoints which are conventionally measured in *Daphnia magna* toxicity tests hold predictive capacity towards reproduction measured over longer timescales, including multiple generations. To assess this, a case-study was performed in which effects of TiO_2 nanoparticles (0, 0.02, 0.2, 2 and 5 mg L^{-1}) on *D. magna* life-history traits were assessed over five generations. Additionally, it was determined whether offspring derived from exposed parents exhibited sustained adverse effects when rearing them in clean (non-exposed) media after each generation of exposure. The present study showed that although various life-history traits correlate with the total reproductive output in the same- and subsequent generation under non-exposed conditions, these correlations were decoupled in presence of exposure to $nTiO_2$. In addition, it was found that $nTiO_2$ can induce adverse effects on population relevant endpoints at concentrations 1–2 orders of magnitude lower than previously found (i.e. 0.02 mg L^{-1}), and close to the range of concentrations occurring in natural freshwater ecosystems.

1. Introduction

With an estimated global annual production of 60,000–150,000 tons, titanium dioxide nanoparticles ($nTiO_2$) rank amongst the most highly produced and applied nanomaterials (NMs) worldwide (Zheng and Nowack, 2021; Keller and Lazareva, 2014). Recent studies have shown that $nTiO_2$ is currently released to the environment at various stages throughout its lifecycle (e.g. Slomberg et al., 2021; Wigger et al., 2020), prompting the need for an adequate characterization of its potential environmental risks (Nielsen et al., 2021).

Performing a risk characterization requires predicted or measured concentrations for the environmental compartment of interest, which can then be compared with a relevant and representative measure of sensitivity (ECHA, 2016). Recent predicted environmental concentrations (PECs) of $nTiO_2$ in European surface waters are in the microgram per liter range (i.e. 0.70 μg L^{-1}; Hong et al., 2021) and measured concentrations in Dutch rivers have been reported to range between of 0.2–8.1 μg L^{-1} (Peters et al., 2018). Hong et al. (2021) demonstrate that these concentrations are 1–2 orders of magnitude lower than predicted no-effect concentrations (PNECs) derived from currently available toxicity data (i.e. 41.8 μg L^{-1}). In concordance with previous studies (Wigger and Nowack, 2019; Coll et al., 2016), this would suggests that environmental risks resulting from the release of $nTiO_2$ to freshwater ecosystems are currently minimal.

The outcome of risk characterizations such as performed by Hong et al. (2021) is crucially dependent on the available toxicity data used for deriving PNECs. For NMs in general and $nTiO_2$ specifically, this data is now predominantly derived from short-term tests conducted using (unrealistically) high exposure concentrations (Schwirn et al., 2020). To illustrate, 70% of the (no-)effect concentrations used by Hong et al. (2021) to derive PNECs for $nTiO_2$ were obtained from short-term toxicity tests, whilst it has been previously concluded that environmentally realistic concentrations of NMs rarely induce effects during such test durations (Novak et al., 2018; Valsami-Jones and Lynch,

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2015).

In addition to providing a less sensitive measure of toxicity, short-term tests of $nTiO_2$ do not realistically represent exposure durations as these are likely to occur in natural environments. This is due to the fact that NMs such as $nTiO_2$ exhibit the same inherent persistence as other (i.e. bulk- or dissolved) metallic pollutants, causing organisms in environments to which $nTiO_2$ is released to experience exposure over prolonged time periods, including multiple- (and potentially more sensitive) life stages (Rasmussen et al., 2019; Valsami-Jones and Lynch, 2015; Savolainen et al., 2013). In addition to resembling environmentally realistic exposure conditions more accurately, long-term ecotoxicological tests allow for more extensive assessments of effects on ecologically relevant life-history traits of test organisms. Amongst these traits, continuation of reproductive success (i.e. the perpetuation of production of offspring over multiple generations) may be considered as a key apical endpoint in ecotoxicological assessments, as this is the predominant factor that drives the longevity of natural populations (Straub et al., 2020). Within conventional long-term ecotoxicological tests (e.g. the *Daphnia magna* reproduction test, OECD test guideline 211, 2012), continuation of reproductive success over multiple generations is however rarely considered (ECHA, 2017). The relevance of assessing such multigenerational effects induced by NMs has been demonstrated in previous studies, in which it has been found that sensitivity towards NMs only manifested significantly after multiple generations of exposure (Ellis et al., 2021, 2020; Liu et al., 2017; Jacobasch et al., 2014; Arndt et al., 2014; Völker et al., 2013; Bundschuh et al., 2012). Importantly however, exposure concentrations applied in mutigenerational effect assessments of $nTiO_2$ in the aforementioned studies have been limited to concentrations which are over 2 orders of magnitude higher than those found in natural environments.

Evidently, multigenerational toxicity tests are more demanding in time- and sampling efforts, use of test materials, and require larger numbers of test organisms compared to conventional long-term ecotoxicological tests. Both from a practical perspective and in light of the principles of the 3Rs of animal testing (i.e. Replacement, Reduction & Refinement), each of these variables would preferably be minimized. Tackling this trade-off could be achieved by determining endpoints which can be measured within shorter timeframes and which hold the capacity to predict effects occurring over longer timeframes, including multiple generations. To date, studies which have assessed such correlations are rare and focused on correlations within a single generation only (Liu et al., 2019; Kim et al., 2014).

The aim of the present study was therefore to determine whether endpoints measured in earlier stages of conventional *Daphnia magna* toxicity tests correlate with endpoints at later stages, including those occurring over multiple generations. We specifically decided to assess correlations of earlier measured endpoints with cumulative reproductive output as we consider reproductive success to be the most relevant apical endpoint with regard to population longevity, as also indicated by its central importance in the standardized OECD *D. magna* reproduction test (OECD TG 211, 2012). To address this, we applied a modified version of the *D. magna* reproduction test (OECD Test Guideline 211, OECD, 2012), in which we assessed effects of $nTiO_2$ on *D. magna* life-history traits over five generations at test concentrations of 0, 0.02, 0.2, 2 and 5 mg L^{-1}. At the low end, these concentrations more closely resemble those measured in European surface waters (i.e. up 8.1 μg L^{-1}) than applied in previous multigenerational toxicity assessments of $nTiO_2$. In addition to assessing effects of consecutive multigenerational exposure, we determined whether offspring derived from exposed parents exhibited sustained adverse effects when rearing them in clean (non-exposed) media after each generation of exposure.

2. Materials and methods

2.1. Test materials and exposure conditions

The $nTiO_2$ (JRCNM01005a, European Commission – DG JRC, also provided by Degussa/Evonik as AEROXIDE P25®) used in the experiments was obtained from the repository of representative industrial nanomaterials of the Joint Research Centre of the European Commission (JRC, Ispra, Italy). This repository consists of industrially relevant NMs that are derived from large single batch productions and which are distributed to the scientific community worldwide to promote reproducibility and reliability of nanomaterial safety testing. The $nTiO_2$ used in this experiment has a reported average primary particle size of 15–24 nm and consists of a mixture of ~85% anatase: 15% rutile crystalline forms (JRC, 2014). Preparation and characterization of $nTiO_2$ used was performed according to the OECD Guidance Document on Aquatic and Sediment Toxicological Testing of Nanomaterials (OECD GD 317, 2020) as much as possible, and any exceptions to this are delineated in respective method sections.

Stock- and exposure suspensions were prepared according to Nederstigt et al. (2022). In short, 100 mg L^{-1} stock suspensions were prepared in Mili-Q water (Milipore Milli-Q reference A+ system, Waters-Millipore Corporation, Milford, MA, USA) followed by 10 min of ultra-sonication using a bath sonicator with a calculated energy output of 27 ± 0.2 W s^{-1} (Sonicor SC-50-22, Sonicor INC. NY, USA). Exposure suspensions were subsequently obtained by dilution of stocks in Elendt M7 medium (Elendt, 1990).

2.2. Characterization of stock- and exposure suspensions

Size and shape of the $nTiO_2$ used in the experiment was confirmed through Transmission Electron Microscopy (TEM, JEOL 1010, JEOL Ltd., Tokyo, Japan) and hydrodynamic diameters (z-average), polydispersity indexes (PDIs), and zeta-potential were determined in stock suspensions (0, 0.5, & 1 h after preparation) and in Elendt M7 medium (0, 24 and 48 h after preparation) using a Malvern Zetasizer Ultra (Malvern, Malvern, UK) according to test conditions. Mass-based exposure concentrations over time between medium renewal (0, 1, 24 and 48 h) were measured by inductively coupled plasma atomic emission spectrometry (ICP-OES) (Agilent Technologies, Santa Clara, CA, USA). Additional extensive characterization data has been made publicly available by the JRC (2014).

2.3. Test organisms

Daphnia magna used in the experiment were collected from an in-house culture at Leiden University. This culture is maintained according to the conditions prescribed in OECD Test Guideline 211 (OECD, 2012) (climate room temperature 22 ± 1 °C, photoperiod 16:8 h light: dark) for over 10 years. Neonate sensitivity to toxicant stress is assessed each time after initiation of a fresh culture using $K_2Cr_2O_7$ according to a *Daphnia magna* Immobilization Test (OECD Test Guideline 202,OECD, 2004) and was found to be within the recommended range prior to the start of the experiment (24 h EC50: 1.6 ± 0.1 mg L^{-1}).

2.4. Experimental design

We previously applied a modified version of the *Daphnia magna* reproduction test (OECD Test Guideline 211, OECD, 2012) and demonstrated that environmentally realistic exposure concentrations of $nTiO_2$ induced a delayed time until maturity in the second generation of continuously exposed individuals (Nederstigt et al., 2022). In the current study, we extended this approach and assessed effects of $nTiO_2$ on *D. magna* life-history traits over five generations using exposure concentrations of 0, 0.02, 0.2, 2 and 5 mg L^{-1}.

The experiment was initiated by introducing 15 neonates per

concentration in individual glass beakers (i.e. one individual per beaker) containing 50 mL of Elendt M7 medium spiked with $nTiO_2$ at concentrations of 0, 0.02, 0.2, 2 and 5 mg L^{-1}. The exposure medium was refreshed every 48 h and aerated prior to addition of $nTiO_2$. Before and after every medium renewal dissolved oxygen (DO) concentrations and pH were measured in three replicates per test concentration (Hach hq40d, Hach Ltd., Colorado, USA). DO concentrations remained $\geq$ 3 mg L^{-1} (mean DO: 9.0 mg L^{-1}, minimum DO: 7.0 mg L^{-1}) and pH remained between 7.3 and 7.9 (mean pH: 7.7) in all measured test vessels throughout the experiment. Feeding took place directly after every medium renewal at a rate of 0.1–0.2 mg C/Daphnid/day by application of *Raphidocelis subcapitata*.

In total, 5 generations (F0-F4) of *D. magna* were tested for each treatment concentration in successive 21 day reproduction tests according to OECD Test Guideline 211 (OECD, 2012). To initiate subsequent generations, the neonates in the third brood of every treatment concentration were collected, pooled and randomly selected to be subjected to the following 21 day reproduction test. Each generation tested for each treatment concentration from the F1 onwards was divided into a treatment- and recovery population. Individuals in the treatment population received the same exposure as the parent generation, whilst individuals in the recovery population were reared in a clean (exposure-free) environment to assess recovery potential (or conversely, whether offspring derived from exposed parents exhibited sustained adverse effects) in absence of $nTiO_2$. As such, effect assessment for each generation from the F1 onwards was performed under both continuous multigenerational exposure and exposure for 1–4 generations followed by a recovery period of 21 days.

2.5. Endpoints

For each generation, mortality, time until sexual maturity (defined as days elapsed before release of 1st brood), number of neonates in 1st brood, number of broods, number of neonates per brood and the total number of neonates produced were recorded over 21 days. In addition, body size (measured from the top of the head, through the eye, to the base of the apical spine) of adults (n = 5) after 21 days exposure and of neonates (n = 5) in the 3rd brood (i.e. those used for initiating subsequent generations) was measured from pictures taken with a Leica MZ16FA stereomicroscope equipped with a Leica DFC420C digital color camera (Leica, Wetzlar, Germany) using ImageJ image analyzer software (ImageJ, 2018).

2.6. Data analysis

Data analysis was performed using R version 1.1.419 (R Core Team, 2017). To determine whether individuals exposed to $nTiO_2$ or derived from exposed parents differed with regard to the measured endpoints, data from each endpoint, generation and exposure/recovery population was analyzed separately. Mortality was analyzed using binomially distributed generalized linear models including treatment, generation and their interaction as explanatory variables (GLMs, package: *stats*, function: *glm*). Total number of neonates produced, time until maturity, number of neonates in first brood, number of broods, number of neonates per brood and body size were analyzed using anovas (package: *stats*, function: *aov*) followed by Tukey pairwise comparisons. Prior to analysis all data and models were checked for heteroskedasticity (Levenes test) and normal distribution (Shapiro Wilk test and visual inspection of histograms and QQ-plots) of the residuals. If assumptions for parametric tests were not met, the data was log10(x + 1) or square-root transformed or Kruskal-Wallis tests were performed followed by Bonferonni corrected Dunn's post-hoc tests.

Correlations between endpoints measured in earlier stages of the 21 days test period and the total number of neonates produced in a single generation were assessed using the pooled data of each tested generation. As body size measurements of adults and neonates were conducted on fewer (n = 5) individuals than measurements of reproduction, these data points were averaged for each treatment (including exposure/recovery) and generation separately and assigned to the corresponding reproduction data. Correlations between each measured endpoint and the total number of neonates produced over 21 days were assessed through linear models (package: *stats*, function: *lm*), which were fitted using the pooled and separate data of controls and treatments (including exposure/recovery). All models and data were checked for heteroskedasticity (Breusch Pagan test) and normal distribution (Shapiro Wilk test and visual inspection of histograms and QQ-plots) of the residuals. In case of heteroskedasticity, weighted linear models were fitted. In case of deviations from a normal distribution of model residuals, robust linear models were fitted (package: *MASS*, function: *rlm*).

To determine whether endpoints measured in parental generations correlated with the total number of neonates produced in the subsequent generation, data of consecutive generations was again merged into a single dataset. In this dataset, mean values for each measured endpoint in the parental generations were assigned to the corresponding reproduction data of the subsequent generations. Analysis of correlations was performed as described for correlations between endpoints measured within a single generation. Outcomes of statistical tests were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Characterization of $nTiO_2$ and fate in the experimental setup

TEM micrographs (SI Fig. 1) showed that $nTiO_2$ underwent rapid aggregation after dispersion into MilliQ water. The measured average particle diameter was 24 ± 5 nm (mean ± standard deviation measured over 15 particles/aggregates) and particles exhibited a predominantly angular shape. Hydrodynamic diameters, PDIs and zeta-potential measurements (−5.09 ± 0.58 mV) indicated that $nTiO_2$ underwent rapid aggregation in the test medium within the timeframe between medium replacements, in concurrence with TEM analyses (Table 1). The hydrodynamic diameter of the particles and PDIs generally showed an increase over time at each treatment concentration. Although hydrodynamic diameters were found to be higher at lower treatment concentrations, this may have partly been an artifact emerging from increased measurement uncertainty at lower concentrations, as also indicated by the high PDIs. ICP-MS measurements indicated that $nTiO_2$ concentrations in the water column decreased over time, and settling rates in the 5 mg L^{-1} treatments concentration were considerably higher than those in lower treatment concentrations (Table 1). After 24 h, this even resulted in lower measured water column concentrations in the 5 mg L^{-1} treatment concentration than in the 2 mg L^{-1} treatment concentration. This possibly resulted from increased aggregation rates at higher treatment concentrations, as is more often observed in exposure suspensions of NMs (Cuppen et al., 2016).

Instability of NM suspensions is a common issue in ecotoxicological experiments and may ultimately affect bioavailability and toxicity (Cuppen et al., 2016). Various strategies have been proposed to mitigate this phenomenon and these can be broadly categorized as either involving modification of exposure conditions or ensuring adequate characterization of NM fate over the course of the experiment. Both strategies and their implications have been extensively discussed in relation to the test setup of the current experiment in Nederstigt et al. (2022). To assure comparability with other studies, it was decided to avoid modification of the test medium (e.g. through introduction of stabilizing agents) within the current experiment and to instead provide extensive characterization data on exposure conditions over time.

3.2. Validity criteria of the experiment

In line with the criteria described in OECD TG 211 (OECD, 2012), control populations of all generations exhibited < 20% mortality. With

Table 1
Time-dependent concentrations, hydrodynamic size and polydispersity index (PDI) measured within the 48 h medium renewal intervals of $nTiO_2$ in samples collected from the center of the water column of the test vessels. (manuscript location = line 252).

Measured concentration mean ± SD (mg L^{-1}) n = 3					
Nominal conc. (mg L^{-1})	T = 0 h	T = 1 h	T = 24 h	T = 48 h	TWA
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
5	5.00 ± 0.00	4.68 ± 0.05	0.97 ± 0.28	0.41 ± 0.06	0.78 ± 0.17
	PDI mean ± SD n = 2				
	T = 0 h		T = 24 h	T = 48 h	
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	
5	0.40 ± 0.05		1.60 ± 0.11	1.97 ± 0.08	
	Hydrodynamic size mean ± SD (nm) n = 2				
	T = 0 h		T = 24 h	T = 48 h	
0.02	4304 ± 116		7298 ± 78	5393 ± 206	
0.2	1353 ± 334		4459 ± 101	4206 ± 1220	
2	521 ± 57		6608 ± 853	6224 ± 739	
5	348 ± 31		3734 ± 606	6738 ± 501	

SD = standard deviation, n = replicates, TWA = time weighted average, nominal conc. = nominal treatment concentration. Data of 0.02, 0,2 and 2 mg L-1 treatment concentrations has been reported in Nederstigt et al. (2022)

exception of the F3 (mean number of living offspring per parent 58), individuals in all generations produced an average number of living offspring > 60 over 21 days.

3.3. Multigenerational effects of $nTiO_2$ exposure and recovery

Apart from mortality rates, *D. magna* in exposure and recovery populations exhibited differences from the controls for all measured life-history traits in at least one generation, including in the lowest (0.02 mg L^{-1}) treatment concentration. Notably however, no dose-related responses were observed, and differences between treatments and controls showed no consistent (i.e. increasing or decreasing) trend over generations.

3.3.1. Mortality and body size

Daphnia magna exhibited no statistically significant differences in mortality rates in any of the treatments or generations relative to controls ($p = 0.92$ $\chi^2_{(9)} = 3.16$). Body size of both adults collected after 21 days and neonates in the 3rd brood showed slight differences between treatments and controls (Table 2), but this was limited to generations from the F2 onwards and did not show a dose- or generation dependent trend.

3.3.2. Reproduction related endpoints

When measured as the total number of neonates produced over 21 days, reproduction from the F1 onwards was lower in exposure than in control populations, although the exposure concentrations at which effects were observed differed depending on the generation considered (Fig. 1, Table 2). Interestingly, recovery populations appeared to follow roughly similar trends as exposure populations derived from the same parental generations, with the exception of the F4 (Fig. 1E). Analysis of cumulative reproduction rates over time show comparable but more pronounced trends (SI Fig. 2, SI Table 1).

Whether expressed as total reproduction or cumulative reproduction rates, reproduction of *D. magna* is governed indirectly through (1) age-dependent mortality rates, and directly through (2) the required time to reach sexual maturity, (3) the timing of reproductive events (i.e. number of released broods over 21 days) and (4) the number of offspring produced per brood. The absence of differences in mortality rates between treatments and controls suggests that in the current experiment, mortality can be excluded as a contributing factor to observed differences in reproduction rates. In contrast, all three aforementioned parameters directly related to reproduction were found to be adversely affected in both consecutively exposed and recovery populations (Table 2). The largest consistency in effects of $nTiO_2$ between these parameters and total reproduction was found for the number of neonates produced per brood (Table 2). Overall however, as reductions in total reproduction were strongest when measured as the cumulative sum of neonates produced over time, it can be concluded that $nTiO_2$ affected reproduction of *D. magna* through a combined effect on all reproduction related parameters. Interestingly, the number of neonates in the first brood showed the fewest differences between treatments and controls of all reproduction related endpoints (Table 2).

3.3.3. Variation in magnitude of effects and effect concentrations

The results of the current experiment suggest that $nTiO_2$ can induce adverse effects on reproduction related parameters of *D. magna* at 10–100 times lower concentrations than previously found, and at concentrations close to those measured in the natural environment (Peters et al., 2018; Jacobasch et al., 2014). In addition, *D. magna* derived from exposed parents were found to exhibit adverse alterations in life-history parameters even when reared in $nTiO_2$ free medium. This adds to previous findings of Ellis et al. (2021), who reported reduced longevity and reproduction in *D. magna* derived from parents exposed to (higher) concentrations of $nTiO_2$ (5 mg L^{-1}). In principle, such multigenerational effects may either be the result of biological alterations in individuals derived from exposed parents or from maternal transfer of the NM (i.e. the transfer of the NM from mother to offspring). Ellis et al. (2021) demonstrated that biological alterations (i.e. accelerated aging) in offspring of *D. magna* exposed to $nTiO_2$ may indeed occur. To our knowledge however, there is no experimental evidence which demonstrates that maternal transfer of NMs takes place in *D. magna*.

In consecutively exposed generations, *D. magna* exposed to the higher treatment concentrations (i.e. 2 and 5 mg L^{-1}) in the current experiment showed less pronounced effects on mortality and reproduction than reported in other studies (Jacobasch et al., 2014). Large variations in magnitude of effects and effect concentrations in ecotoxicological studies of NMs, such as observed both between generations tested in our study as well as between our findings and those of Jacobasch et al. (2014), have been reported previously (Book and Backhaus, 2022). It is possible that such discrepancies arise from differences in stability of exposure suspensions, which can result in altered bioavailability and thereby toxicity (i.e. through differences in particle sizes and/or sedimentation rates) (Cuppen et al., 2016). Although Jacobasch et al. (2014) used the same NMs as used in the current study, both experiments were conducted in different test media (i.e. Elendt M4 and Elendt M7 respectively, Elendt, 1990). In the current study, hydrodynamic sizes of particles in suspension immediately after preparation of the test medium were within the range of those reported by Jacobasch et al. (2014). However, as Jacobasch et al. (2014) provide no further data on particle sizes throughout the exposure period (i.e. covering the time-frame between medium renewals), a full comparison to this end is not possible. As such, the contribution of differences in aggregation rates to discrepancies in observed magnitudes of effect at higher (i.e. 2 & 5 mg L^{-1}) test concentrations in the current study and those found by Jacobasch et al. (2014) remains inconclusive.

Table 2
Output (p-values) of Bonferroni corrected Dunn's multiple comparisons and Tukey's Honest Significant Difference tests comparing body size of adults (21 days old) and neonates (3rd brood), and reproduction related endpoints of *Daphnia magna* undergoing continuous multigenerational exposure to $nTiO_2$ (exp) and *D. magna* undergoing exposure for 1–4 generations followed by a recovery period of 21 days (rec). p-values displayed are derived from comparisons between controls and respective treatments. (manuscript location = line 399).

Generation	Nominal treatment concentration $nTiO_2$ (mg L^{-1})							
	0.02		0.2		2		5	
	exp	rec	exp	rec	exp	rec	exp	rec
Adult body size								
F0	0.99	na	0.46	na	0.08	na	0.22	na
F1	0.96	0.07	0.15	1.0	0.12	1.0	1.0	0.99
F2	0.68	0.07	0.49	0.98	0.83	0.94	0.98	0.23
F3	0.87	**0.02***	**$1e^{-4}$*****	**$9e^{-4}$*****	**$5e^{-3}$*****	0.43	0.13	0.56
F4	0.23	0.10	1.0	0.99	0.26	0.93	1.0	0.80
Neonate body size								
F0	0.05	ns	0.27	ns	0.39	ns	0.93	ns
F1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.85
F2	0.38	0.79	0.48	**$2e^{-3}$****	1.0	0.79	0.99	0.56
F3	0.49	1.0	1.0	1.0	0.51	0.43	1.0	0.21
F4	1.0	1.0	1.0	1.0	**0.03***	0.56	0.61	**$1e^{-3}$****
Total number of neonates produced (21d)								
F0	0.98	na	0.99	na	0.15	na	0.12	na
F1	**0.03***	0.05	1.0	1.0	0.22	1.0	**$9e^{-4}$*****	0.07
F2	0.23	**0.04***	0.07	0.45	**$1e^{-4}$*****	**$1e^{-4}$*****	**$1e^{-4}$*****	0.36
F3	0.98	0.96	0.94	0.96	1.0	0.83	**$1e^{-4}$*****	**$1.0e^{-4}$*****
F4	**$1e^{-4}$*****	1.0	**$5e^{-4}$*****	**$4e^{-3}$*****	**< 0.001*****	1.0	1.0	1.0
Time until maturity								
F0	1.0	na	1.0	na	1.0	na	0.77	na
F1	0.06	**$1e^{-3}$****	**$3e^{-4}$*****	**$2e^{-4}$*****	**$2e^{-3}$****	0.27	**< $1e^{-4}$*****	**< $1e^{-4}$*****
F2	1.0	1.0	1.0	0.08	1.0	1.0	1.0	1.0
F3	1.0	1.0	1.0	1.0	1.0	0.31	**$9e^{-3}$****	0.09
F4	1.0	1.0	1.0	1.0	0.26	**0.04***	0.96	**$2e^{-3}$****
Total number of broods								
F0	ns	ns	ns	ns	ns	ns	ns	ns
F1	1.0	0.18	1.0	1.0	1.0	**$7e^{-3}$****	0.10	**$9e^{-3}$****
F2	1.0	1.0	1.0	0.30	1.0	**$9e^{-3}$****	**$3e^{-3}$****	1.0
F3	1.0	1.0	0.32	1.0	1.0	1.0	**$9e^{-3}$****	0.09
F4	0.06	1.0	1.0	**< $1e^{-4}$*****	1.0	0.78	1.0	0.13
Neonates in 1st brood								
F0	1.0	na	1.0	na	1.0	na	0.77	na
F1	1.0	1.0	1.0	1.0	1.0	1.0	0.73	1.0
F2	0.41	**0.02***	1.0	1.0	1.0	1.0	**0.01***	0.11
F3	1.0	0.68	1.0	0.99	0.23	0.99	1.00	0.99
F4	0.31	0.72	0.99	0.99	0.74	0.14	0.12	**0.01***
Neonates per brood								
F0	0.99	na	0.65	na	0.51	na	**0.01***	na
F1	1.0	1.0	1.0	1.0	0.38	0.32	1.0	1.0
F2	1.0	0.05	1.0	1.0	0.06	0.22	1.0	1.0
F3	0.94	0.63	**$1e^{-4}$*****	**$7e^{-3}$****	0.99	0.06	**$1e^{-4}$*****	**< $1e^{-4}$*****
F4	**$5e^{-3}$****	1.0	**< $1e^{-4}$*****	1.0	0.5	1.0	**$5e^{-3}$****	1.0

Legend: exp = consecutive exposure, rec = recovery. Asterisks indicate statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001). p < 0.05 are indicated in bold. na = not available (no recovery populations were tested in the F0)

Although $nTiO_2$ is amongst the few anthropogenic NMs which has been detected at measurable concentrations in natural freshwater ecosystems, little is known about its long(er) term fate under natural conditions and to what extent this resembles its fate in ecotoxicological test setups (Peters et al., 2018). Similarly, although continuation of reproductive success across multiple generations in laboratory studies is a relevant endpoint with regard to population dynamics of test organisms, extrapolation of deviations from control groups to this end to predict equivalent alterations in populations living under natural conditions will come with uncertainty (e.g. due to the absence of natural processes such as resource limitation or predation in laboratory settings). This is inherent to any extrapolation of responses measured under controlled laboratory conditions to those in natural settings. In addition to these caveats, the variability present both within the results of the current experiment and between ecotoxicological studies of NMs in general urges for caution when extrapolating results to natural conditions.

3.4. Correlations between life-history traits and reproduction within a single generation

To asses to what extent reproduction over 21 days correlated with other life-history traits measured in conventional *D. magna* toxicity tests, regression models were fitted using each measured trait and the total number of neonates produced over 21 days (i.e. the total reproductive output).

In controls, all traits except for time until sexual maturity correlated positively with the total reproductive output (Fig. 2, SI Table 2). The strongest correlation ($p = 0.001$, $R^2 = 0.11$) was observed for the number of neonates in the first brood (Fig. 2J). Interestingly, this correlation was absent in exposure (Fig. 2K) and recovery populations (Fig. 2L). Similarly, whereas adult body size correlated positively with the total reproductive output in controls, this correlation was not observed in exposure populations (Fig. 2E). For neonate body size, this difference was even more pronounced, showing a positive correlation for controls (Fig. 2A) and negative correlation for exposure (Fig. 2B) and recovery populations (Fig. 2C).

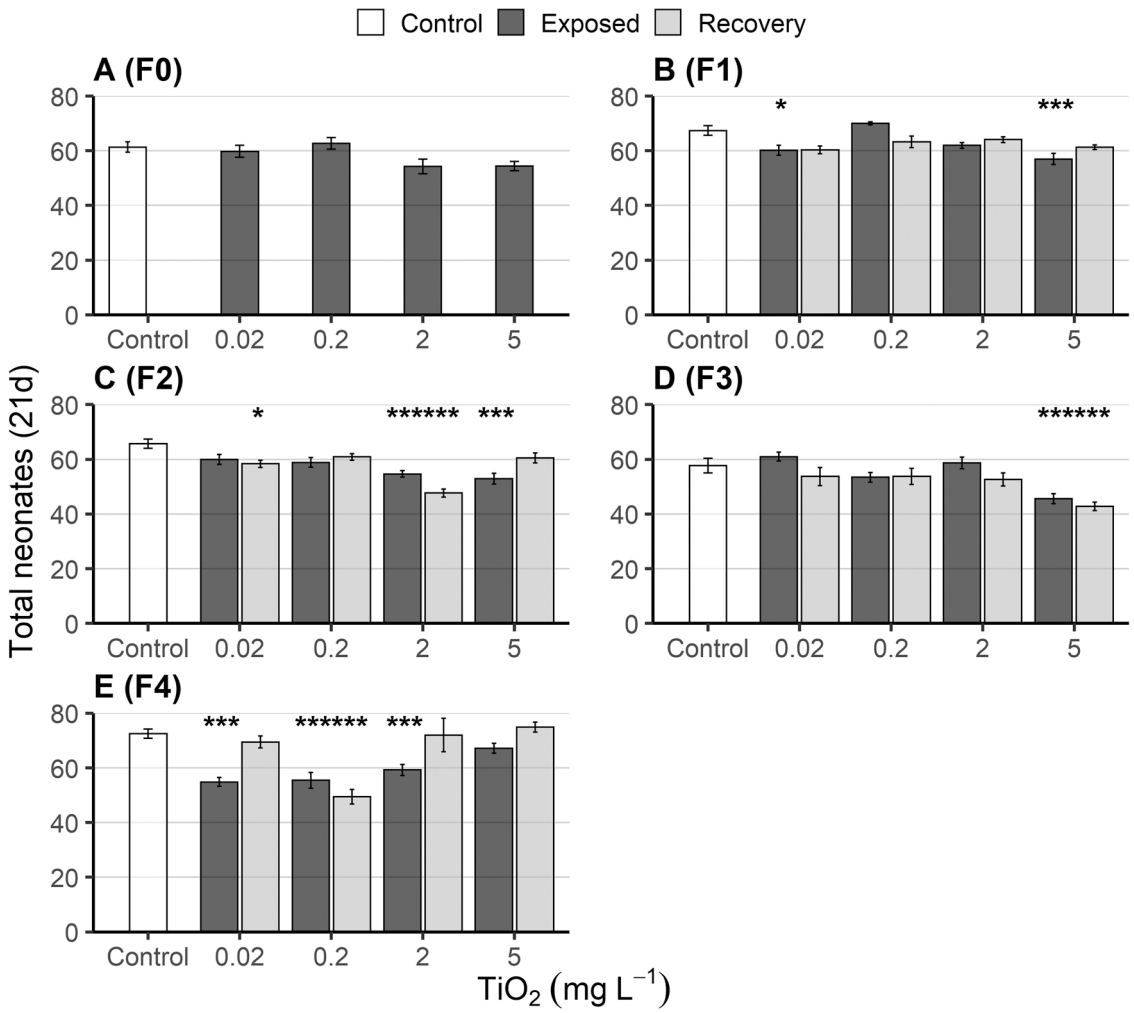

Fig. 1. Total number of neonates produced by *Daphnia magna* (mean ± standard error) undergoing continuous multigenerational exposure to $nTiO_2$ for 21 days (exposure) and *D. magna* undergoing exposure for 1–4 generations, followed by a recovery period of 21 days (recovery). Asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) of comparisons between controls and respective treatments derived from Bonferroni corrected Dunn's multiple comparisons and Tukey's Honest Significant Difference tests.

These findings indicate that under stress-free conditions (i.e. in control populations in the current experiment), the total reproductive output of *D. magna* over 21 days correlates well with endpoints which can be measured at shorter timescales. Under exposure to $nTiO_2$ and in recovery populations however, these correlations are significantly reduced, and in the case of neonate body size, even reverse in direction. As such, it appears that under stressed conditions, a negative trade-off is present between investments in offspring quantity (i.e. total reproductive output) and quality (i.e. neonate body size). Overall, this implies that effects of $nTiO_2$ on reproduction of *D. magna* manifest throughout the 21 day test period, rather than in the early stages of the exposure period. This may be partly explained by relatively slow uptake rates of $nTiO_2$, which are reported for poorly dissolving NMs in general (Scott-Fordsmand et al., 2017).

Liu et al. (2019) found that when combining the data of controls and exposure groups from a $nTiO_2$ experiment on *D. magna,* adult body size and neonates in the first brood correlated positively with the total number of neonates produced over 21 days. This is in agreement with the findings of the present study (SI Table 2 & SI Fig. 3). Crucially however, the results of the current study demonstrate that when considering control-, exposure- and recovery populations separately, correlations may differ in strength and direction. Based on these findings, it is likely that using short-term endpoints as predictive proxies in long-term toxicity tests of *D. magna* may lead to an underestimation of overall toxicity.

Within the current experiment, the only endpoint that showed a consistent correlation with the total reproductive output in control-, treatment- and recovery populations was the number of broods produced over 21 days (Fig. 2, SI Table 2, SI Fig. 3). From a practical perspective, using the number of broods produced over 21 days as a predictive proxy for overall reproductive performance would provide a means for reducing labor intensity of a *D. magna* reproduction test, but would not result in a reduction of time needed to conduct the experiment.

3.5. Correlations between life-history traits and reproduction of subsequent generations

As observed for within generation correlations between life-history traits and total reproductive output, between generation correlations were more pronounced in controls than in treatment- and recovery populations, suggesting a decoupling of life-history traits as a result of exposure to $nTiO_2$ (Fig. 3, SI Table 3). For controls specifically, an increase in quantity related reproductive parameters (i.e. total number of neonates produced, number of neonates in 1st brood, and number of broods) in previous generations correlated negatively with reproductive output in subsequent generations (Fig. 3A, D & J). This suggests that although neonate body size did not exhibit a trade-off with reproductive

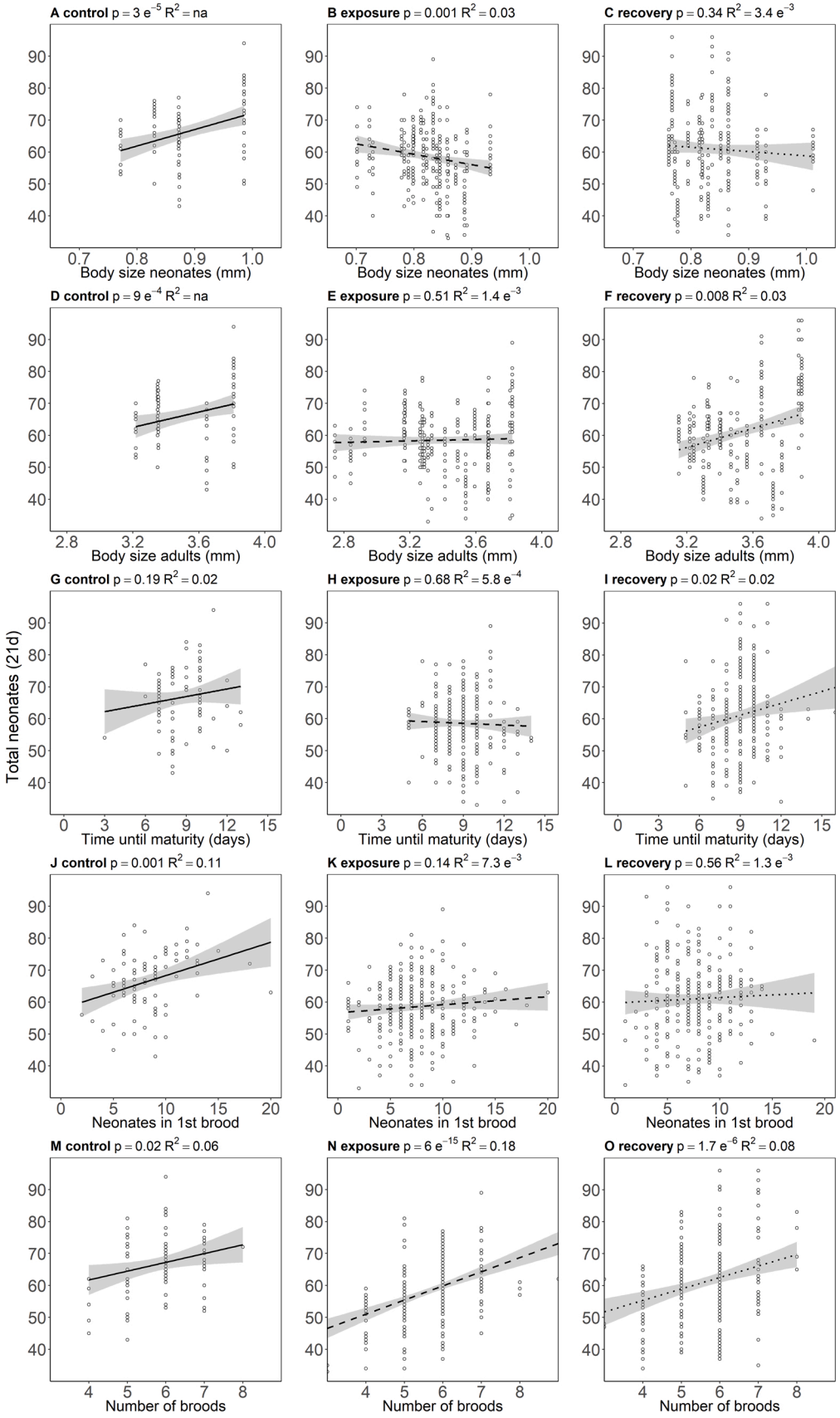

Fig. 2. (weighted) Linear regression models of life-history traits vs. total neonates produced by *Daphnia magna* over 21 days for control (solid lines), exposure (dashed lines) and recovery (dotted lines) data separately. Correlations are calculated using data derived from endpoints measured within a single-generation. Ribbons indicate 95% confidence intervals. When robust linear regression models were fitted R^2 values were not calculated (indicated with na).

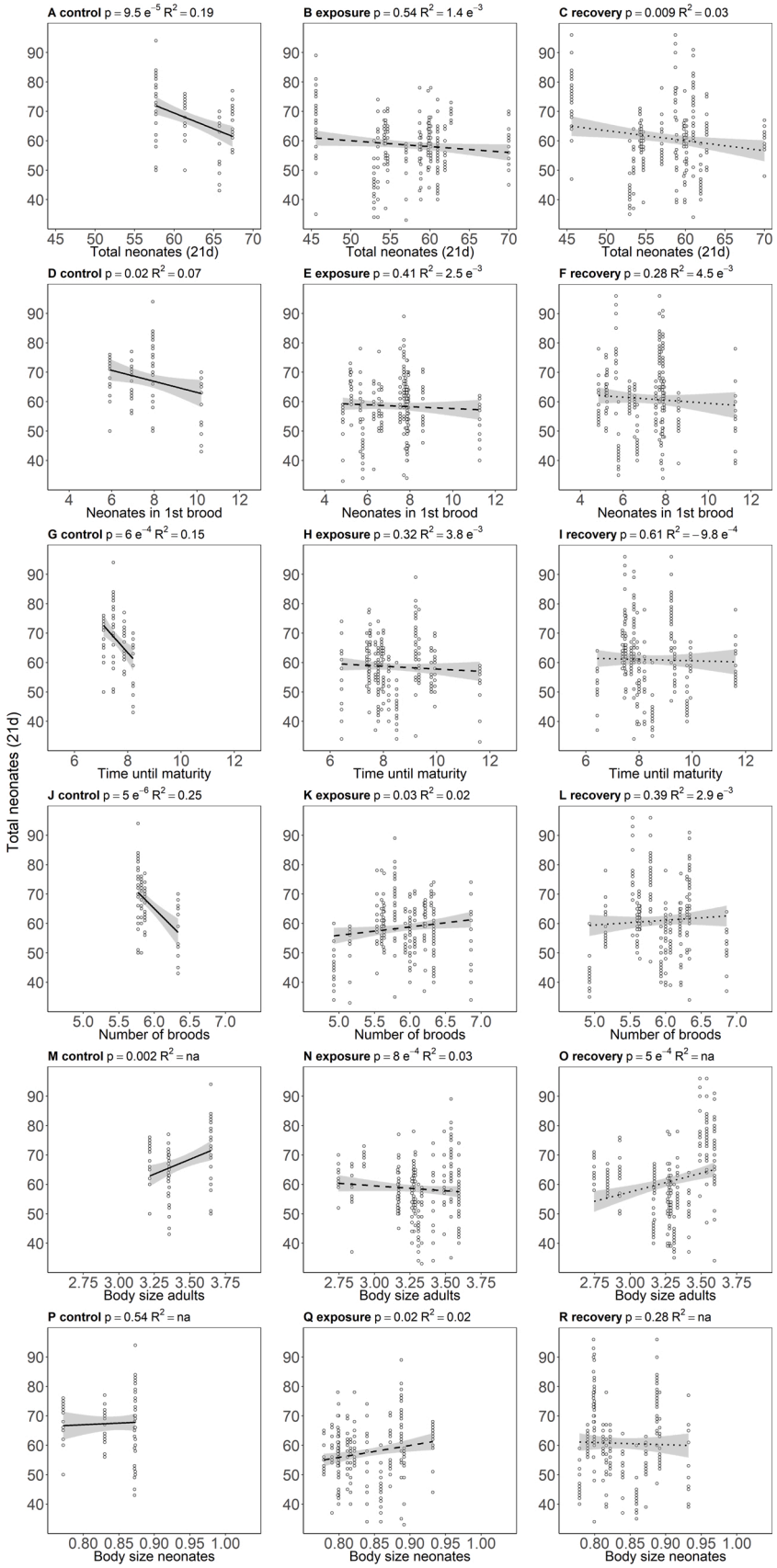

Fig. 3. (weighted) Linear regression models of life-history traits of 1st generation individuals vs. total neonates produced in the subsequent generation in *Daphnia magna* for control (solid lines), exposure (dashed lines) and recovery (dotted lines) data separately. Ribbons indicate 95% confidence intervals. When robust linear regression models were fitted R^2 values were not calculated (indicated with na).

output within generations of control populations (Fig. 2A), overall offspring quality (i.e. reproductive capacity) correlated negatively with offspring quantity in both the presence and absence of exposure to $nTiO_2$, which is in line with previous observations of trade-offs between offspring quantity and quality in *D. magna* (Cleuvers et al., 1997). As such, further investigation into offspring quality related parameters other than neonate body size could provide a valuable option for identifying predictive proxies for multi- and transgenerational effects in *D. magna*. To this end, Castro et al. (2018) suggest the use of time until (sexual) maturity and the number of neonates in the first brood. The results of the current experiment however suggest that correlations between these life-history traits and total reproductive output are decoupled in both treatment and recovery populations (Fig. 2H, I, K & L), and as such are likely to hold little to no predictive power under stressed conditions.

3.6. Conclusion and outlook

The results of the current experiment suggest that $nTiO_2$ can affect population-relevant endpoints of *D. magna* at exposure concentrations close to those present in natural environments and orders of magnitude lower than previously reported for multigenerational toxicity tests (Hong et al., 2021; Peters et al., 2018; Jacobasch et al., 2014). In addition, effects of exposure to $nTiO_2$ were found to remain present in subsequent generations, even when reared in exposure-free medium. Although we believe that extrapolations to natural conditions should be made with caution, the findings of the present study suggest that both under continuous and intermittent exposure (e.g. as a result of peak exposure), natural populations of *D. magna* exposed to concentrations as low as 0.02 mg L^{-1} could potentially be adversely affected by $nTiO_2$. These conclusions demonstrate the importance of considering multigenerational exposure- and effect studies in risk assessment of $nTiO_2$ and NMs in general.

Based on the decoupling of life-history traits and total reproductive output observed in treatment and recovery populations in the current experiment, assessing long-term and multigenerational effects of NMs on the basis of short(er) term endpoints should be performed with prudence. To this end, energy-related parameters (as proposed by e.g. Kim et al., 2014), molecular biomarkers (as proposed by e.g. Qi et al., 2021) as well as novel methods including automated image analysis (as proposed by Karatzas et al., 2020) may comprise valuable approaches for identifying predictive proxies which show higher consistency. In combination with extensive exposure characterization, this could improve both reliability and sensitivity of studies which support environmental risk assessment of NMs.

CRediT authorship contribution statement

TN: Conceptualization; Investigation; Methodology; Conceptualization; Data curation; Formal analysis; Visualization; Writing - original draft; **WP**: Conceptualization; Supervision; Resources; Funding Acquisition; Project administration; Writing-review & editing; **RB**: was during the execution trainee at ULEI; assisted in experiment performance; Methodology; Writing-review & editing; **MV**: Conceptualization, Supervision, Resources, Funding acquisition, Project administration, Writing-review & editing.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113792.

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