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Exposure medium and particle ageing moderate the toxicological effects of nanomaterials to *Daphnia magna* over multiple generations: a case for standard test review?[†]

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Pristine engineered nanomaterials (NMs) entering the aquatic environment become ‘aged’ during their lifetime via chemical, physical and/or biological process. Therefore, traditional ecotoxicology tests which were designed for soluble chemicals prior to the emergence of NMs, use pristine NMs and salt-only media which are not representative of realistic NM exposure scenarios. Exposure medium and NM ageing moderation of NM toxicity were explored using *Daphnia magna* multigenerational studies aiming to determine whether the daphnids adapted to continuous exposure and/or if parent-only exposure resulted in epigenetic effects in subsequent generations. Daphnids were continuously, or parent-only, exposed to pristine and aged titanium dioxide (TiO₂) and silver (Ag) NMs, in a standard high hardness culture media and synthetic European Class V lowland water. Pristine NMs in the standard culture medium had the most severe toxic consequences, and displayed negative effects in two generations post exposure. NMs aged in the class V water had fewer overall toxic consequences on growth and longevity across all generations in both continuous and parent-only exposure scenarios. Overall, in the absence of environmentally relevant media and aged NMs, standardised *Daphnia* tests currently overestimate NM toxicity, and fail to consider potential impacts in subsequent generations. The results demonstrate the importance of updating standard testing to reflect scientific advances and increase stakeholder trust in regulation.

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Environmental significance

Environmental nanomaterial (NM) transformations can be diverse and the role of environmental conditions will strongly influence these processes. The majority of NMs ecotoxicology tests don't use environmentally transformed NMs in environmentally representative waters. Natural water standards containing natural organic matter (NOM) are far more representative for realistic exposure conditions than traditional toxicology test and culture media outlined in such protocols. To identify the effect of exposure media and NM ageing, *Daphnia magna* were exposed to a variety of pristine and 6 month aged titanium dioxide (TiO₂) and silver (Ag) NMs in a standard *Daphnia* culture medium and a European class V lowland natural water standard. Pristine NMs had less toxic consequences in environmentally realistic medium compared to the standard culture medium, suggesting that the presence of NOM alters both the NM physico-chemistry and the interactions with *Daphnia* that are persistent across multiple generations. Furthermore, the aged NMs exposed to the *Daphnia* in the class V water had fewer overall toxic consequences on growth, mortality and reproduction including of generations F1–F3, compared to both pristine and aged NMs in the standard *Daphnia* culture medium. Thus, standardised *Daphnia* tests following the OECD protocols overestimate NMs toxicity, which can be resolved through ageing of the NMs in the medium and/or use of representative natural water compositions. Assessment of reproductive success of offspring of exposed daphnids is recommended, as surprising effects of pristine NMs were determined that could not be predicted from the F0 data.

Introduction

The Organisation for Economic Co-operation and Development (OECD) recommends a defined synthetic media

for the harmonised test guidelines (TGs) of chemicals in acute and chronic ecotoxicity of *Daphnia magna* immobilization and reproduction tests. The TGs recommended not to include any additives (such as soil extracts) to avoid inconsistencies in the characterisation of complex media between laboratories. Therefore, the recommended media for standardized toxicity testing of *Daphnia* are the Elendt M7/M4 and the high hardness combo media (HH combo).^{1,2} Other commonly used synthetic waters include those advised by the Environmental

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Protection Agency (EPA).³ Natural water usage is not discouraged for regulatory tests as long as they are well characterized and meet the validity criteria for testing and culturing *Daphnia* (pH, hardness, heavy metal analysis and the total organic carbon (TOC) levels do not exceed 2 mg L⁻¹).² Thus, the goal of standard testing is to facilitate comparison of chemicals under identical conditions for ranking of toxicity, rather than to provide environmental realism. While this works well for soluble chemicals, the highly reactive surfaces of NMs mean that the current tests are not optimal for NMs whose properties (and toxicity) are typically determined by their surroundings, a feature referred to as having extrinsic properties as well as intrinsic (unchanging) ones.^{4,5} Indeed it is widely acknowledged that “the influence of the dispersion medium on facilitating the existence of single particles or agglomerates is important” and should be considered in revision of TGs for ecotoxicity evaluation of NMs, as noted by.⁶ In their review of the OECD's ongoing work on revising TGs for NMs to facilitate mutual acceptability of data. Thus, the enormous surface reactivity of NMs and their tendency to interact with themselves or surrounding biomolecules to form a corona, suggests that lack of dispersing agent in the OECD *Daphnia* tests compromises the ability of the tests to rank NMs toxicity.

Significant work is underway to assess the suitability of regulatory tests designed for (soluble) chemicals for use with nanomaterials (NMs) whose enormous reactive surface area makes them more challenging to assess. It is well known that media composition affects NM physicochemical properties^{7,8} and their toxicity,⁹ thus it is essential to consider more environmentally relevant waters for NMs ecotoxicology studies^{10,11} as well as assessment of appropriately transformed NMs under relevant conditions. Hammes, Gallego-Urrea¹² classified European water types (class I–VI) based on their chemical properties (pH, ionic strength, composition and natural organic matter (NOM) content) for prediction of the stability of NMs. Application of these water classes for ecotoxicology assessments would provide more representative conditions and allow comparison of differently stabilized and transformed NM variants based on core speciation, acquisition of a NOM or other biomolecule corona and other environmental transformations. Environmental exposure assessment should consider older and/or transformed NMs in addition to the pristine (often highly reactive) forms. In the absence of realistic media and environmental ageing, the data is neither predictive or reflective of real NM exposure scenarios nor appropriate for risk assessments.¹³

We demonstrate here that the current use of pristine NMs in simple synthetic media significantly overestimates the NM hazards and risks, and that these impacts persist over multiple generations, whether the offspring are themselves also exposed or are removed from the exposure conditions and allowed to recover. While multigenerational impacts to daphnids are not currently considered in REACH and other regulatory regimes, understanding of the mechanisms of action of NMs including longer term and multigenerational

effects are essential for future grouping, read-across and *in silico* predictions, as well as for population and ecosystems level understanding of the longer-term implications of exposure to NMs. Revisions to the harmonised TGs for NMs are underway currently (*via* the OECD) and data such as that reported here may drive further changes and adaptations in the longer term, including consideration of multi-generational effects especially in light of the drive to reduce animal testing and the need for alternative test systems. Indeed, the need to consider environmental coronas and feeding of the organisms during the acute *Daphnia* immobilisation TGs (OECD 202) have recently been suggested as adaptations needed for NMs testing.¹⁴

Few studies to date have investigated the long term effects of ‘aged/transformed’ NMs and those that have, concentrate efforts on NMs exposed to simple media between 24–48 hours and up to 21 days.^{15,16} Although both are necessary, they do not capture the long-term ageing of NMs, nor consider the effects of parental exposure to subsequent generations, whether in sustained multigeneration exposure or parental only exposure effects. The present study compares the toxicity of a panel of pristine and 6 month aged titanium dioxide (TiO₂) and silver (Ag) NMs, where the NM ageing and exposure took place in both a standard *Daphnia* culture media (HH combo) and a synthetic class V European lowland water containing NOM.¹² Effect concentrations, determined from acute 48 hour immobilization tests, were exposed to daphnids over 28 days (F0) with the F1–F3 generations either continuously exposed (F_{exp}), or removed for recovery in NM-free media (F_{rec}).

Experimental

Materials

Commercially available chemicals, solvents and humic acids (HA) were purchased from Sigma-Aldrich (Dorset, UK) and were of analytical reagent grade. Ultrapure water (UPW) with a maximum resistivity of 18.2 MΩ cm⁻¹ was used throughout the experiments.

Media and representative waters

Experiments were conducted in *Daphnia* high hardness combo media (HH combo)¹⁷ and in a synthetic lowland class V artificial water;¹² compositions of both are given in the ESI† Tables SI.1, SI.1A and SI.1B. The HH combo media represents an average hard water standard found in the environment without any natural organic matter (NOM)¹⁷ and is commonly used for the culturing of *Daphnia*. The class V lowland water has high alkalinity, high ionic strength and 4.6 mg L⁻¹ NOM, and is typical of waters found in the southern UK, Poland, Greece, France, the Balearic countries and the Iberian Peninsula.¹²

Nanomaterials and characterization

The NMs used in this study include PVP-coated and uncoated TiO₂ NMs (both supplied by Promethean Particles Ltd.), Ag₂S



PVP coated Ag (AppNano Ltd, Spain), uncoated (bare) Ag (Promethean Particles Ltd.) and PVP coated Ag (Amepox Ltd, Poland) (see section 1.2 ESI†). Dynamic light scattering (DLS) was used to measure the hydrodynamic size of the pristine and aged NMs, using a Malvern Nanosizer 5000 instrument. Transmission electron microscopy (TEM) analysis (Table 1, Fig. 1 and 5) were performed using a JEOL 1200EX 80 kV and a JEOL 1400EX 80 kV Max system. Samples were prepared for TEM by depositing a 10 μL drop of the NM suspension onto a 300 mesh carbon-coated copper TEM grid (Agar Scientific, UK). TEM primary particle sizes were determined by counting at least 100 NMs.

Nanomaterial ageing

NMs were chemically aged by exposing stock solutions of 1000 mg L^{-1} to the HH combo and class V water for at least 6 months prior to *Daphnia* exposure (stored at 4 °C in darkness during the ageing). Aged samples were re-characterised by DLS and TEM (Table 1 and Fig. 1). Ageing and transformations of NMs is considered to reduce their reactivity and toxicity, and often results in sulfidation, phosphidation, chloride binding or other changes to the surface chemistry depending on the salts present in the media, and/or adsorption of biomolecules from their surroundings.^{18–20}

Daphnia maintenance, culturing and exposure conditions

The *Daphnia magna* Bham2 strain originated from the University of Reading.²¹ *Daphnia magna* Straus (Clone Type 5 – IRCHA) were originally obtained from the Water Research Centre (WRC), Medmenham, UK. Initial stocks of daphnids were maintained using pools of third brood Bham2 strain (genetically identical) and were kept in a 20 °C temperature-controlled environment with 12 hour light and dark cycles. Daphnids were cultured in HH combo media and synthetic class V lowland water using only third broods to create successive generations. Media was refreshed weekly to ensure healthy maintenance and cultures were fed *Chlorella vulgaris* algae daily (0.5 mg carbon for days 0–5 and 0.75 mg carbon from day 5).

Range-finding study (*Daphnia* acute immobilization test, OECD 202) and pre-testing conditions. Acute immobilization tests were conducted on the daphnids using only the pristine

NMs in the HH combo media to determine the initial effect concentrations (EC values) and establish the baseline toxicity of the NMs in the simplest medium (HH combo). This baseline NM toxicity was then used to highlight the differences between:¹ the toxicological effects of the pristine NMs in comparison to the aged NMs exposure in both the HH combo media and class V water (at the same effect NM concentration), and² to demonstrate that the acute exposures are not predictive of the NM impacts over longer term multi-generational exposures, where greatly enhanced sensitivities are observed even at the acute EC₆ (TiO₂) and EC₃₀ (Ag NMs). To determine the initial ECs for the multigenerational studies, a total of 60 neonates (<24 hours old) were exposed to a range of concentrations of NMs (uncoated Ag, PVP Ag Ag₂S, uncoated TiO₂, and TiO₂-PVP) over 24 and 48 hours to assess their immobilization and survival (Fig. SI.1A and SI.1B†). Note that the daphnids were not fed during these exposures in accordance with the standard OECD 202 test for acute immobilisation.¹

We understand there is a difference between using environmental concentrations and ECs. Our justification for using effect concentrations in this study is due to regulation and environmental risks being assessed by characterizing the overall effects in biological receptors. For this reason, pilot studies (using 10 daphnids/250 mL medium in three replicates) were conducted to identify any issues with study design and/or the NM concentrations selected, as described in section 1.2 of the ESI†. The final selected concentrations for the multigenerational reproductive studies were EC₃₀ values for the Ag NPs (20 $\mu\text{g L}^{-1}$ of PVP Ag, 20 $\mu\text{g L}^{-1}$ of uncoated Ag and 100 $\mu\text{g L}^{-1}$ of Ag₂S, respectively (Fig. SI.1B†)) and EC₅ values for the TiO₂ NMs (5 mg L^{-1} for each of the TiO₂ NMs).

Daphnia survival and growth

Daphnids were checked daily for survival, egg production and neonate release. Neonates of the third broods were used to set up the following generations (unless otherwise stated). Measurements of body length were taken every 3 days from release in accordance with the cycles of moulting of the carapace (between days 3–24).²² Body lengths were measured from the apex of the helmet to the base of the apical spine

Table 1 Characterization of TiO₂ and Ag NMs

Identifier	Pristine TEM individual particle size (nm)	Aged in HH combo TEM individual size (nm)	Aged in class V TEM individual size ^a (nm)	Pristine DLS particle size (nm)	Pristine PDI	Aged in HH combo DLS size ^a (nm)	PDI of NMs aged in HH combo	Aged in class V river water DLS size ^a (nm)	PDI of NMs aged in class V water	Surface coating ^b
TiO ₂ -Uncoated	9 ± 2	11 ± 3	11 ± 3	207 ± 11	0.5	305 ± 139	0.8	291 ± 149	1	Bare
TiO ₂ -PVP coated	9 ± 2	10 ± 3	10 ± 2	311 ± 43	0.4	305 ± 176	0.8	409 ± 289	0.9	PVP ₁₀
Ag ₂ S	44 ± 14	39 ± 15	45 ± 15	299 ± 6	0.4	145 ± 2	0.3	171 ± 22	0.3	PVP ₁₀
Ag PVP	18 ± 11	38 ± 19	105 ± 102	260 ± 180	0.3	129 ± 22	0.4	129 ± 141	0.7	PVP ₁₀
Ag uncoated	61 ± 36	36 ± 16	95 ± 111	120 ± 30.5	0.5	7363 ± 1054	0.9	1423 ± 545	0.8	Bare

Note: The TEM sizes are reported as the individual NM sizes. PDI = polydispersity index. ^a Aged in the respective media for 6 months.

^b According to manufacturer.



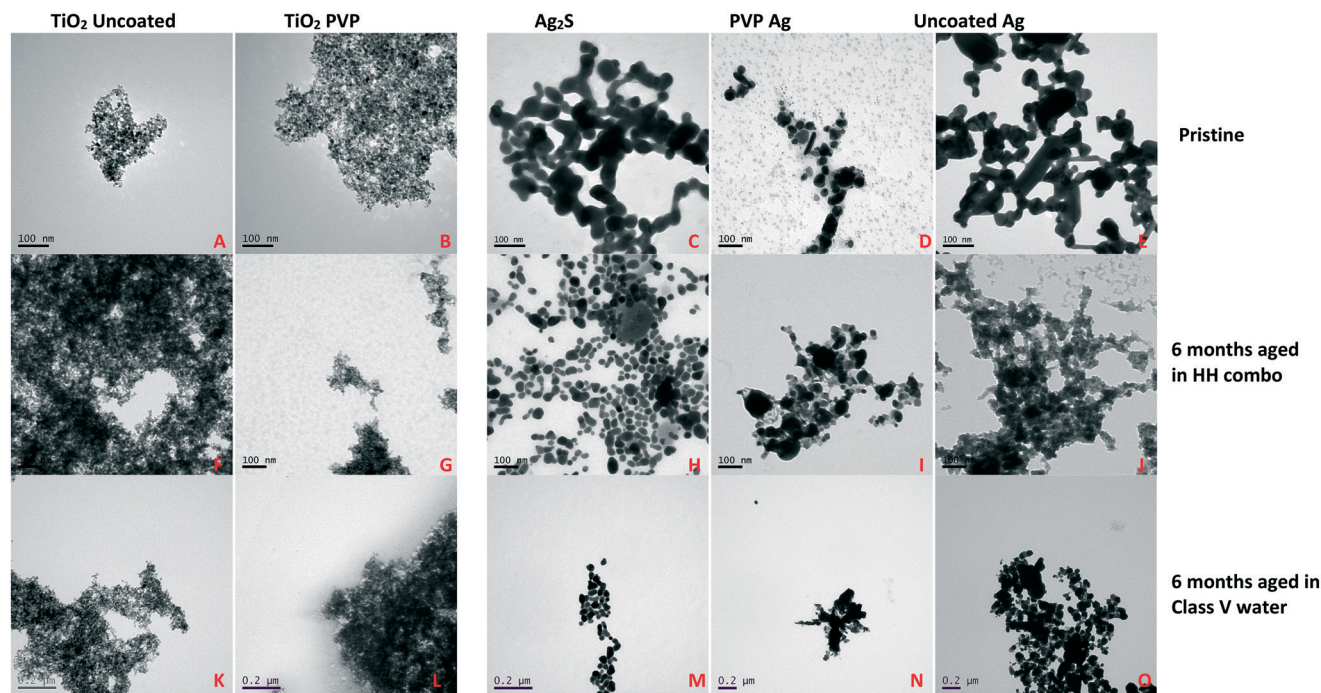


Fig. 1 Characterization of the NMs in the two media before and after 6 months ageing. A: Pristine uncoated TiO_2 in ultrapure water (UPW), B: pristine PVP TiO_2 in UPW, C: pristine Ag_2S in UPW, D: pristine PVP Ag in UPW, E: pristine uncoated Ag in UPW, F: aged uncoated TiO_2 in HH combo media, G: aged PVP TiO_2 in HH combo media, H: aged Ag_2S in HH combo media, I: aged PVP Ag in HH combo media, J: aged uncoated Ag in HH combo media, K: aged uncoated TiO_2 in class V water, L: aged PVP TiO_2 in class V water. M: Aged Ag_2S in class V water, N: aged PVP Ag in class V water and O: aged uncoated Ag in class V water. The pristine and aged TiO_2 dispersions in each of the media were visibly aggregated when imaged with TEM, although little difference was observed between the pristine and aged TiO_2 NM primary NM sizes. Ageing the Ag NMs in the two media had least effect on the Ag_2S NMs, whereas the uncoated Ag was the most unstable leading to hetero-agglomeration (Table 1).

using a Nikon (Japan) stereomicroscope, model SMZ800 Digital Sight fitted with a D5-Fi2 camera, using NIS Elements software.

Multigenerational study design

The goal of the multigenerational study was to assess in parallel the impacts of continuous exposure to the NMs in order to determine if there was any adaption to the exposure or a progressively severe response, *versus* the impacts of parent only exposure to determine if there were any epigenetic effects passed from exposed mothers to offspring grown under NM-free conditions, *i.e.* the recovery generations. Thus, the third brood neonates from the NM exposed F0 parent generations were split with half continuously exposed to the same NM and the other half grown in medium only, as shown schematically in Fig. SI.2.†

The F0 generation consisted of a total of 10 daphnids/250 mL in three replicates exposed to the EC_{30} (Ag NMs) and EC_5 (TiO_2) concentrations for 1 month. The third broods (F1) from the F0 generation were removed within 24 hours of birth and then split into two groups: one group were kept exposed to the same EC concentration of NMs to produce a continuously exposed group (F_{exp}) over four successive generations (F0, $F_{1\text{exp}}$, $F_{2\text{exp}}$ and $F_{3\text{exp}}$) (see section 1.4 ESI†). The second group were removed from the parental F0 exposure and maintained in the relevant media only (NM

free) for the three subsequent generations to produce the recovery sets ($F_{1\text{rec}}$, $F_{2\text{rec}}$ and $F_{3\text{rec}}$) (Fig. SI.2.†). The medium (with or without NMs for the exposed and recovery experiments) was refreshed once per week. The F1–3 generations were always made from the third brood of the previous generation (unless otherwise stated, where 3rd brood numbers were very low), and neonates were removed from the exposures within 24 hours of birth for set up of the next generation.

Sample preparation for determination of NM (bio) accumulation (TEM)

The TEM cross sections of the F0 generations after 7 days exposure were prepared by the Centre for Electron Microscopy at the University of Birmingham (UK). Briefly whole *Daphnia* (neonates <24 h) were euthanised and fixed immediately in a 2.5% glutaraldehyde in a 0.1 M phosphate buffer suspension. Daphnids were dehydrated in ethanol and embedded in epoxy resin before sectioning using a ultramicrotome with a diamond knife. Images were visualized using JEOL 1200EX 80 kV and JEOL 1400EX 80 kV microscopes.

Accumulation in daphnids and metal concentrations

At day 7, pools of 10 F0 daphnids were euthanized (using liquid nitrogen) and mechanically homogenised in 2% nitric



acid (HNO₃) using a Precellys 24 instrument (Bertin Technologies) with 2 cycles of 30 s at a 6000 pulse speed. Samples (in triplicate) were analysed for their (bio) accumulated concentration (accumulation in the gut and potential bioaccumulation inside the tissue) using inductively coupled plasma mass spectrometry (ICP-MS) (Nexion 300X instrument, Perkin Elmer). Samples were dissolved in 2% nitric acid and ran alongside an online germanium internal standard.

After the first 7 days of exposure only, when the media was refreshed, samples of the old media containing NMs were taken for single particle-ICP-MS (NexION 300D, Perkin Elmer) in order to quantify the dissolved and NM concentrations. Operating conditions were optimized to produce maximum Ag/Ti intensity using Ag/Ti NM and ionic standards. Instrument calibration was achieved by analysis of a blank and 3 dissolved Ag/Ti solutions ranging from 0 to 1 µg L⁻¹. Sample flow rates were measured prior to analysis. Transport efficiencies were determined using three standards of citrate stabilized gold NMs with diameters of 20, 40 and 80 nm. Time resolved signals were acquired using the Syngistix nano application module in the NexION software. All concentrations and particle diameters were determined from triplicate samples. To ensure no deviations of instrumental drift over time, Ag/Ti dissolved calibration check standard was also ran in between sampling. Experimental procedures were followed according to the Perkin Elmer application notes.²³

Statistical analysis

All experiments were repeated in triplicate, and the data was recorded as the mean with standard deviation. For the growth studies, a student's unpaired *t*-test for unequal variance was used to observe the significant difference between the control and all exposed/recovery generations. In all analyses, a *p*-value < 0.05 was considered to be statistically significant (ESI:† Appendix 1 Tables AP.1–5).

The linear model rate (using the slope coefficient to denote the rate of change) of daphnid growth between each population for their age *versus* time was analysed in RStudio using log₁₀ transformations. A positive correlation number shows an incline in the rate of growth and the further from 0 the slope is, the steeper the fits though the data. Therefore, all exposed and recovery generations were compared to the control slope coefficients to denote the rate of growth. ESI:† (Appendix 1 Tables AP.6–7).

Results and discussion

Characterization of NMs pre- and post-ageing

Differently surface coated Ag and TiO₂ NMs were specifically selected, since both materials are routinely used in health and fitness products, and have the most potential for widespread environmental release.^{24,25} The NMs were pre-

characterised using TEM and DLS for both the pristine and post 6 months aged NMs in the standard *Daphnia* culture media (HH combo) and synthetic class V water. The results are presented in Table 1 and Fig. 1. The individual reported TEM sizes for the aged TiO₂ were no different to the pristine dispersions. However, increased sizes and agglomeration were observed for all aged Ag NMs.

It is important to note that TEM only measures a very small fraction of the sample, and in order to be able to size the individual particles it is necessary to focus on areas that are not so highly aggregated. While the TEM images (Fig. 1) clearly show aggregation between the different sets of particles, in many cases the aggregates were so large it was impossible to fit them into a single image to allow for sizing. For this reason, only the individual (primary) particle sizes are reported from the TEM data. The DLS reports the *z*-average size, which by definition, is an average over all sizes detected and as scattering scales 1/*D*⁶ (where *D* is the particle diameter) it skews the size towards larger particles. Thus, the absolute numbers are not the main message from Table 1, but rather changes in the numbers as a result of the ageing processes in the different media are the important feature.

Nanomaterial environmental transformations and their possible effects

It is important for risk assessment and regulation to consider the types of transformations that NMs can undergo in the environment, and the impacts of these on NMs reactivity and toxicity. Fig. 2(A and B) represents schematically the possible environmental transformations of pristine NMs, such as TiO₂ and Ag NMs, including altered surface speciation, acquisition of an environmental corona, or agglomeration, all of which reduce the surface reactivity and potential for oxidative dissolution (in the case of Ag NMs). Similarly, long-term ageing of the NMs in simple salt media lacking NOM, has also been found to passivate NMs surface chemistry (Fig. 2C) as demonstrated in this work, leading to dramatically reduced toxicity and impacts on *Daphnia* compared to the pristine NMs (Fig. 2D). Such transformations have previously been grouped into chemical, physical, biological (*i.e.* in the presence of microbes) and macromolecule binding, by Lowry *et al.*¹⁸ The fact that purely salt-based medium can result in reduced toxicity of the NMs is an interesting finding, and correlates with the observed changes in agglomeration and polydispersity (TiO₂ and uncoated Ag NMs, as per Table 1).

Moreover, initial pristine NM that are released into the environment is also an important aspect to consider, for this reason the pristine NM comparisons were conducted in both waters, to represent such interactions. The likelihood of contact between the pristine NMs with the *Daphnia*, or any other aquatic species would be negotiable due to rapid transformation, which would occur almost instantaneously due to the high surface reactivity of the NMs with the surrounding environment. Rapid transformation has also been heavily demonstrated in the literature in a variety of different media



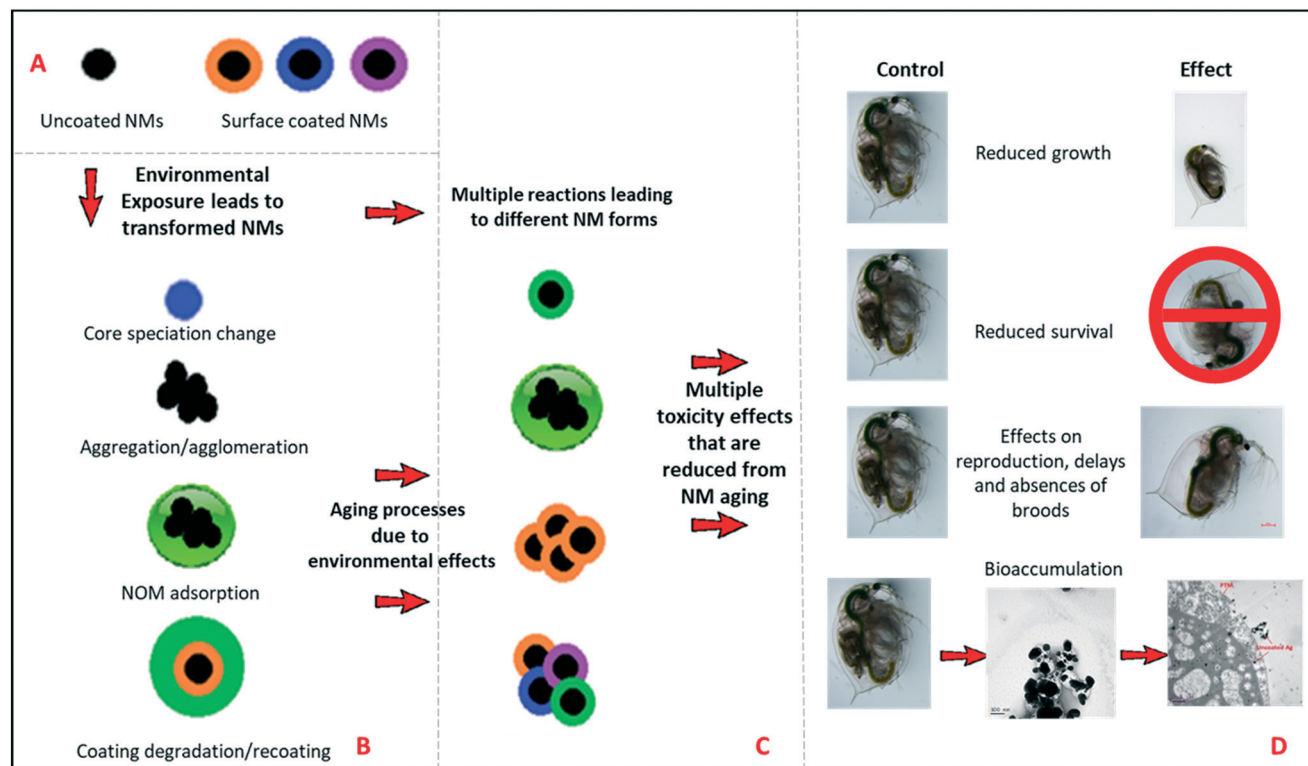


Fig. 2 Schematic diagram of the environmental transformations that NMs can undergo, leading to changes in surface chemistry and toxicological effects. A) Examples of coated and uncoated pristine NMs entering the environment. B) The multiple transformations to the pristine NMs once entering the environment, i.e., core speciation changes (oxidation and sulfidation), agglomeration, degradation of surface coating, photolysis, bacterial degradation and substitution with NOM. C) Ageing in the environment results in multiple NM forms with lower surface reactivity than their pristine counterparts. D) The multiple toxicological outcomes from exposure (D), which are more pronounced when the organisms are exposed to the pristine *versus* aged/NOM-coated NMs.

using a range of different surface coated NMs. For example multiple studies have focused on determining the physicochemical changes undergone by Ag NPs during and after their use,²⁶ assessments of their stability in various test media,^{7,27} fate/transformations²⁸ and bioavailability.²⁹

Longevity

The effect of pollutants on life history traits such as survival (Fig. 3 and SI.3†), growth (Fig. 4 and SI.4†) and reproduction (Fig. SI.3†), provides sensitive information for ecological stress and chemical toxicity.³⁰ The results from the multigenerational study show that the NM composition (TiO₂ *versus* Ag), age (pristine/aged), surface coating and water condition (without/with NOM) significantly affected survival in each of the continuously exposed generations (Fig. 3 and SI.3†). By day 25 the F0 parent generation cultured in HH combo medium exposed to pristine Ag NMs had overall survivals of 73% (uncoated Ag), 53% (PVP Ag) and 83% (Ag₂S) (Fig. 3A). The PVP Ag NMs were more tolerated in the subsequent generations with survivals of 97% (F1_{exp}) and 63% (F2_{exp}) by day 25, suggesting some adaption to the continuous exposure, although there were no surviving populations in the F3_{exp} generations, whereas the F1–3_{exp} generations continuously exposed to the uncoated Ag NMs

had much lower total survivals of 25% (F1_{exp}), 3% (F2_{exp}) and 27% (F3_{exp}) by day 25 (Fig. 3A).

The F0 generation exposed to the pristine uncoated TiO₂ NMs in the HH combo media were also extremely sensitive. After 5 days, only 50% survival was observed, which decreased to 17% by day 11 (Fig. 3A). Due to the delay between egg clutch production and the time taken to release the fifth brood, the F0 generational study continued for a total of 36 days (compared to between 25–28 days for controls). Due to poor neonate numbers produced by F0 daphnids exposed to uncoated pristine TiO₂ NMs, the F1 generations were taken from the fourth broods (rather than the third broods), and within 24 hours post birth the F1_{exp} generation had 100% mortality. Neonates removed from exposure to uncoated pristine TiO₂ NMs in the F1_{rec} generation survived for only 16 days before 100% mortality was observed. Due to reduced longevity, there were not enough individuals (for statistical significance) in the subsequent broods to produce the F2–3 generations thereafter. Reduced longevity and delays between broods were also observed for daphnids exposed to the pristine PVP TiO₂ in the HH combo medium, with a total population survival 27% at day 25 and 24% by day 34. The F1 generations were also taken from the fourth broods due to poor neonate numbers in the third. The F1_{exp} generation continued for 31 days and had survival of 70% (by day 31), showing some



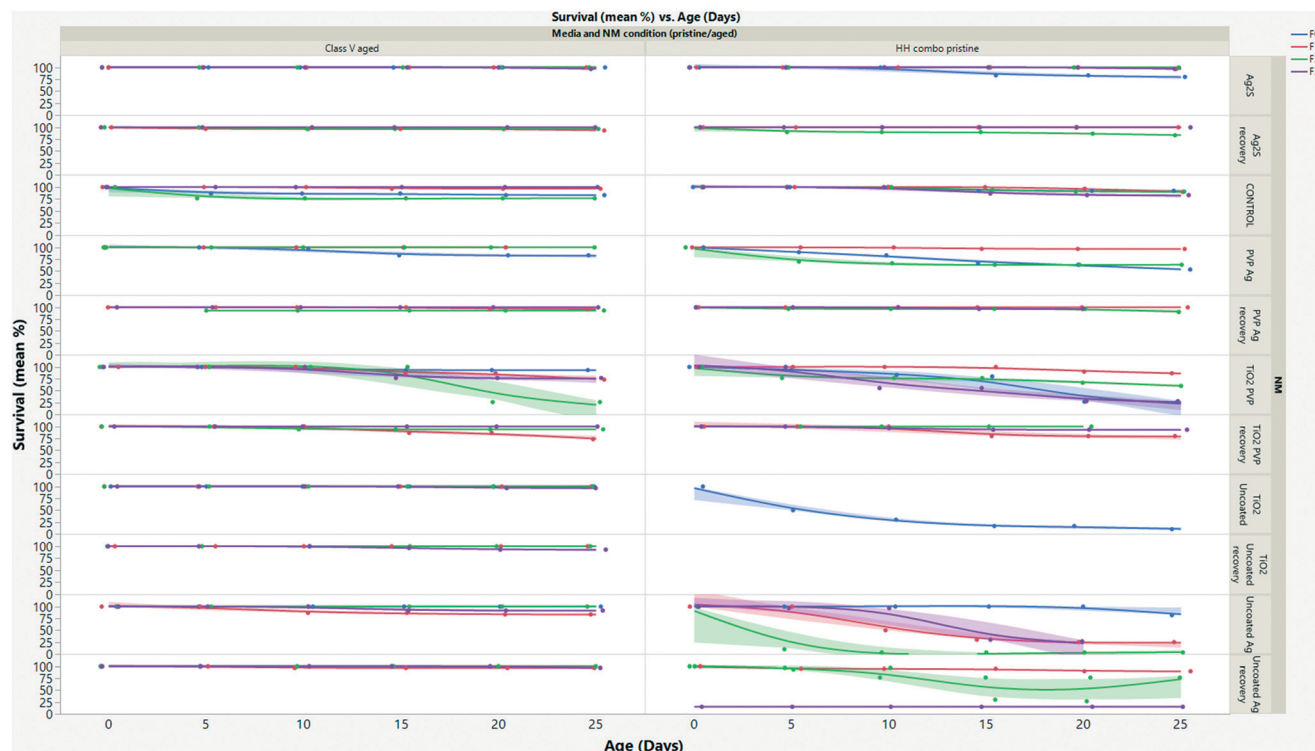


Fig. 3 Multigenerational survival (%) for daphnids exposed to left) aged NMs in class V water and right) pristine NMs in HH combo media. The results show that the multigenerational survival of the daphnids increased in the presence of aged NMs in the class V water.

adaptation to the exposure conditions (Fig. 3A). Those in the $F2_{exp}$ and $F3_{exp}$ populations had 60% (day 29) and 50% (day 28)

survival evidencing increased sensitivity to the exposures as the subsequent generations continue.

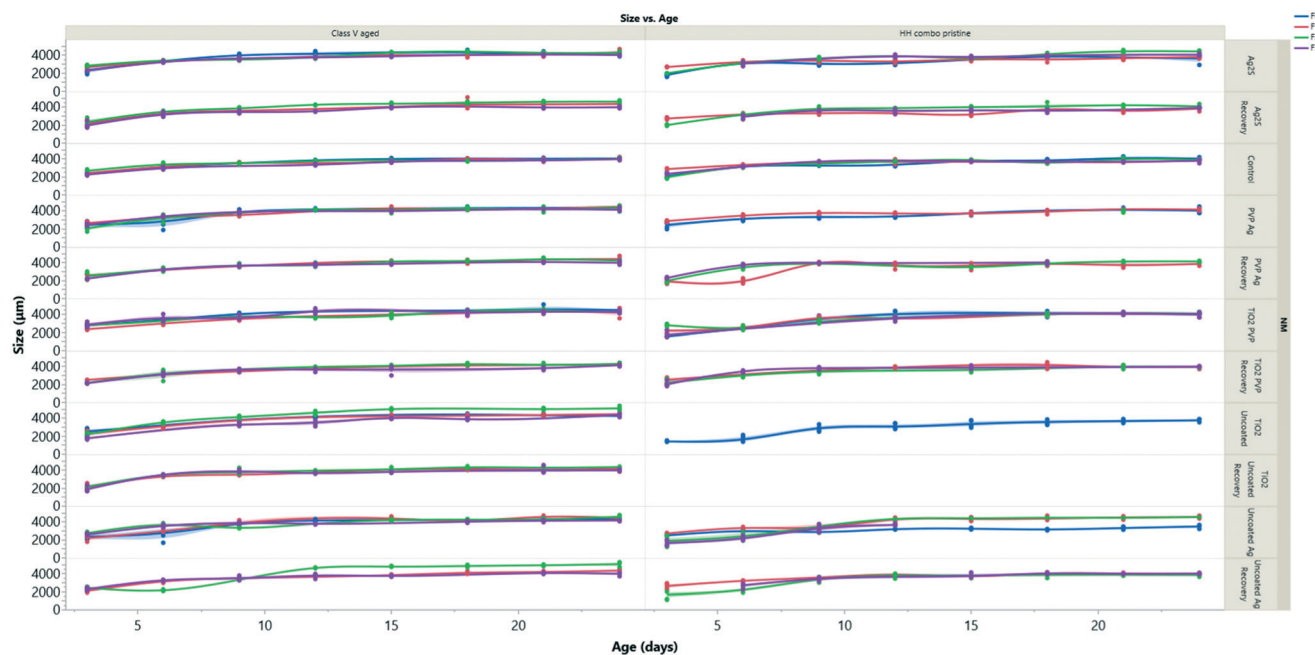


Fig. 4 Size of daphnids over time in all generations following exposure to left) aged NMs in class V river water, right) pristine NMs in HH combo media. Exposure to the pristine NMs had the most negative effects on the daphnids body length in the HH combo. Interestingly these effects were also trans-generational and reductions in sizes were still observed after 2 generations removed from exposure, suggesting that the NMs have an epigenetic effect on the development of the daphnids.



Explanations for the high mortality observed in the pristine NM exposure studies must be related to differences in exposure conditions between acute and chronic exposure studies. Food is added during the chronic multigenerational studies which may enhance the uptake of the NMs by ensuring a constant passage of material through the organism's guts^{31,32}. Feeding is not recommended in the OECD acute immobilization TG,¹ although it has been suggested that this is not ideal for NM exposures to filter feeding organisms since the EC can significantly change in chronic studies in the presence of food and NOM.^{31,32} Zhu³³ and Das³⁴ suggested that the presence of food during chronic exposure tests reduces the retention of TiO₂ by accelerating excretion, thus lowering toxicity. This appears not to be true with the present study since there were higher mortalities in the presence of food (chronic exposures) than in its absence (acute exposures). It is possible that the TiO₂ NMs can adsorb to the food sources accelerating their ingestion/retention and become trapped in the gut (Fig. 5B), leading to increased uptake and localized toxicity as observed by Nasser and Lynch.^{14,35}

The initial uptake *via* food sources may explain the high population mortality in the F0 generations, leading to interrupted metabolic pathways, cellular signalling, and enzyme function.³⁶ Zhu³³ also reported from acute studies, that daphnids exposed to 50 and 100 mg L⁻¹ uncoated TiO₂ NMs (21 nm) had between 10 and 20% immobilization with no significant mortality. However, in chronic exposures (3–21 days) to 0.1 mg L⁻¹ uncoated TiO₂, the mortality increased significantly ranging from 13–100%, in strong agreement with our study. Chronic 21 day exposures by Kim *et al.*³⁶ further observed 90% mortality at concentrations of 2.5 mg L⁻¹ uncoated TiO₂. Those experiments were conducted in moderately hard water (pH 7.6) using uncoated TiO₂ (40 nm). The original 48 hour EC₅₀ was 4.5 mg L⁻¹ whereas the EC₅₀ values for 7 and 14 day exposures were 2.7 and 1.9 mg L⁻¹, respectively, thus, as with our pilot acute studies (ESI,† section 1.3), the EC₃₀ values significantly underestimated the toxicity in the chronic exposures.

We hypothesize that the toxic effects were mediated from the direct exposure of accumulated uncoated TiO₂ as shown in Fig. 5B, leading to ROS production *in vivo*. We further consider that the uptake of TiO₂ with food leads to accumulation in the gut. Heavily agglomerated TiO₂ has been shown to disturb chemical transformations in the digestive tract, and the accumulation can interfere with food intake.³⁷ Therefore, as a result the accumulation causes localized toxic effects when the daphnids fail to excrete the TiO₂, which would not have been observed in the initial OECD acute range finding tests.

When the NMs were chemically aged in the HH combo media, the survival across the exposed generations was significantly increased when compared to the pristine NM exposures in HH combo media (Fig. SI.3B†), showing that NM aging even in simple salt medium reduces their toxicity. The F0 generations exposed to the aged Ag NMs, had overall increased survival at day 25 at 97% (uncoated Ag), 97% (PVP Ag) and 90%

(Ag₂S). Survival of daphnids continuously exposed to the aged uncoated Ag was 86% (F1_{exp}), 97% (F2_{exp}) and 77% (F3_{exp}) between days 25–30. For the aged uncoated TiO₂ exposures (Fig. SI.3B†), the F0 generation had 97% survival, and successive F1–3 generations (continuously exposed) had total survivorships of 100% (F1_{exp}), 93% (F2_{exp}) and 70% (F3_{exp}) at day 25. Survival was also increased for populations exposed to aged PVP TiO₂ NMs, (F0 had 90% survival at day 25) when compared to the pristine NM exposures in HH combo medium. The F1–3_{rec/exp} generations had ≥90% survival by day 25, showing that the parental exposure had very little effect on the subsequent non-exposed generations for the aged NMs. In all cases, the increased survival of exposure generations shows positive adaptations to continuous exposure and of recovery generations suggests that parental exposure to the aged NMs does not significantly affect fitness or survival of subsequent unexposed generations, thus further indicating that ageing the NMs greatly reduced the toxic effects compared to the pristine (highly reactive) NMs.

To mimic realistic environmental conditions, the pristine and aged NM exposures were repeated in synthetic class V European lowland water (Table SI.1†), in order to assess the media composition effects on NM toxicity. Significant differences were observed in the daphnid survival in the class V water compared to the HH combo medium studies (Fig. 3 and SI.3B†). The survival of the F0–F3 generations in the controls was ≥96% which was notably higher than those in the HH combo medium (although those were within the OECD guidelines for control survival), suggesting that more realistic medium supports healthier daphnids. Daphnids exposed to pristine uncoated Ag in the class V water were more sensitive to toxic effects (Fig. SI.3C†) compared to the pristine NM exposures in the HH combo medium. Reduced survival was observed for each of the exposed generations, with 10% (F0), 40% (F1_{exp}), 7% (F2_{exp}) and 13% (F3_{exp}) survival at day 25. These findings are consistent with previous research, which also observed increased toxicity in the presence of pristine NMs in the presence of NOM,³⁸ or a secreted biomolecule corona²⁵ which was hypothesised to be a result of NM agglomeration and the texture of the corona layer resulting in the particles appearing more like the daphnids typical food source of algae.

The recovery generations had survivals of 63% (F1_{rec}), 93% (F2_{rec}), and 13% (F3_{rec}) between days 25–28 showing that maternal exposure to pristine uncoated Ag in class V media had some epigenetic consequences with effects from maternal exposure continuing into the subsequent recovery generations. On the other hand, the pristine PVP Ag and the uncoated TiO₂ NMs exposures evidenced significantly increased survival in the class V exposures (compared to the HH combo), showing surface coating and media specific differences. Daphnids exposed to the pristine uncoated TiO₂ in the class V media were able to produce successive offspring showing positive outcomes using the same pristine NMs in realistic waters. The survivorship of the F0–3_{exp} generations was 86% (F0), 67 (F1_{exp}), 41% (F2_{exp}) and 83%



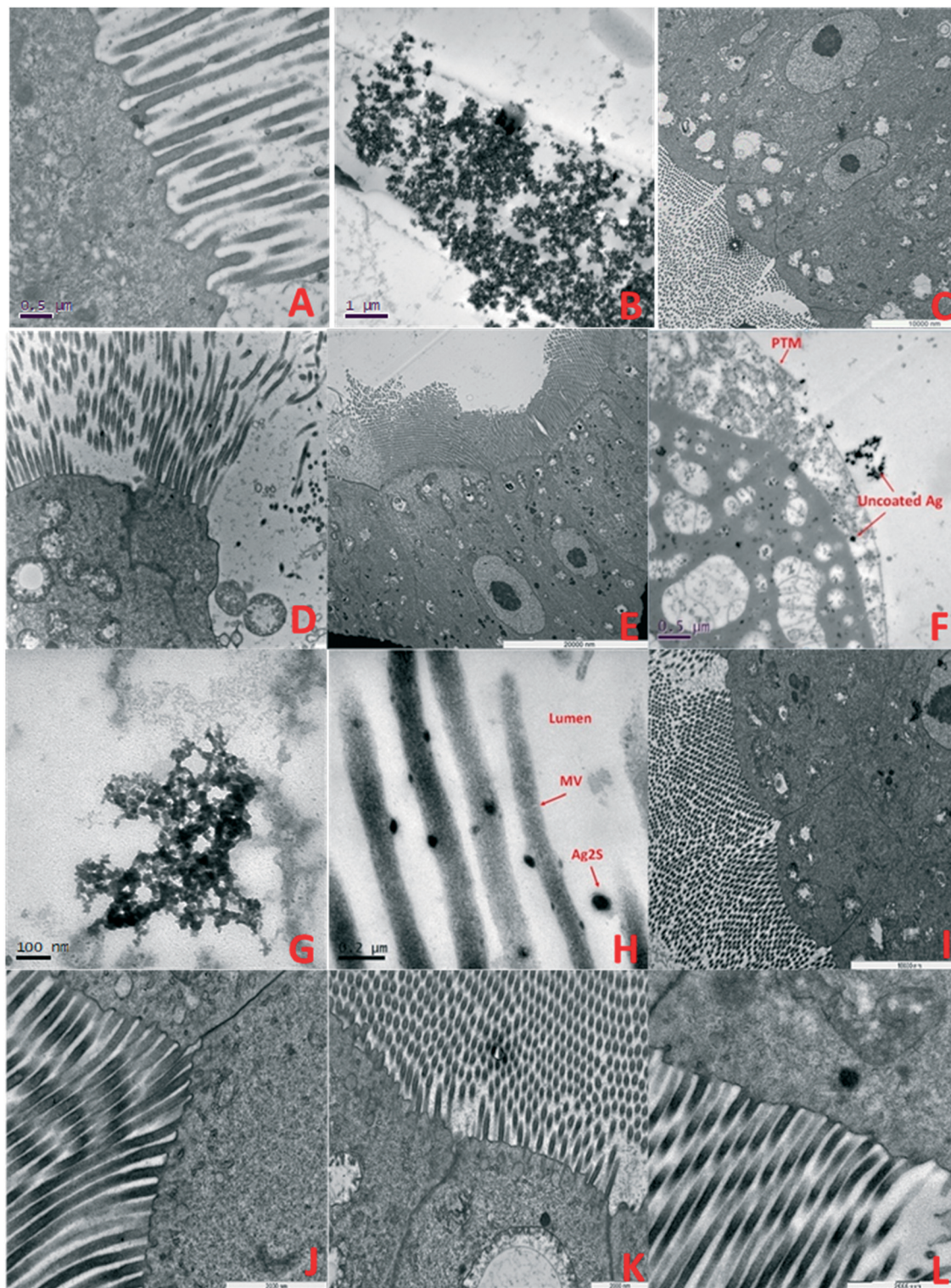


Fig. 5 TEM images of F0 generation daphnid guts showing accumulation and impacts of NMs after 7 days exposure: A) F0 control: B) pristine PVP TiO_2 in HH combo; showing accumulated TiO_2 NMs in the gut lumen contained by the PTM, C) aged PVP TiO_2 NMs in HH combo media, ordered cellular structures, evidence of lipofuscin and AV, D) pristine PVP TiO_2 NMs in class V water showing losses of structural membranes, enlarged mitochondria and disorganisation of the MV, E) aged PVP TiO_2 NMs in class V water showing a regular structured cell alignment, with evidence of enlarged mitochondria and AV, F) pristine uncoated Ag NMs in HH combo interacting with the cell, with lipid like cytoplasmic inclusions and large vacuole structures. Such interactions which may have disturbed metabolism and chemical transformations⁴⁸ which is consistent with reduced growth and delayed reproduction (Fig. S1.3†), G) pristine PVP Ag NMs in HH combo, H) pristine Ag_2S NMs in HH combo, I) aged PVP Ag NMs in HH combo, J) aged Ag_2S Ag NMs in HH combo, K) aged uncoated Ag NMs in class V, showing disorganization to the mitochondria, appearing lysed with empty internal spaces and disorganization of their cristae, and L) aged Ag_2S NMs in class V. Key: mitochondria (M), cell junctions (CJ), autophagy vacuoles (AV) nucleus (N), nucleolus (n), microvilli (MV), apical membrane (AM) and the peritrophic membrane (PTM).



(F3_{exp}) and for the recovery generations was between 90–100% between days 25–30.

Based on the HH combo results, high survival for the daphnid generations was expected for those exposed to pristine PVP TiO₂ NMs (Fig. SI.3C†). Intriguingly, only 50% survived (F0) the duration of the study (until day 28), and both the exposed and recovery F2 generations did not produce sufficient neonate numbers in their third broods to produce the F3 generations. Thus the PVP–NOM interactions and resulting corona may have led to increased uptake and retention, and thus toxicity. Interestingly, the uncoated Ag NMs under the same conditions also had increased toxicity which in this case may be linked to NOM-enhanced dissolution (Table SI.4†). The adsorption process of NOM to the NM surface is not fully clear (given the qualitative difference between the NOM and extracted humic substances used in many studies), and the mechanisms of toxicity between the uncoated Ag and TiO₂ NMs in both water types is observably different. The difference is perhaps linked to the surface chemistry and stability issues with Ag, which is known to produce ionic Ag from dissolution *in vivo*^{39,40} and in different media.⁷

The toxicity of the pristine PVP TiO₂ and Ag exposures was high in the class V water. To rule out the possibility of this being PVP surface coating toxicity resulting from the PVP being displaced by NOM, studies exposing only PVP (at concentrations equal to those coating the NMs) to the daphnids for the same length of time were conducted, resulting in no significant differences compared to control populations (Fig. SI.3B and D and SI.4B and SI.4B–D†). Apart from the aged PVP Ag which showed some toxicity, most of the aged NM exposures in the class V media had little to no mortality in the multigenerational exposures (Fig. 3). These results emphasize that NM ageing/transformation and environmentally suitable media are key for realistic exposure scenarios when addressing the potential hazards of NMs, and that more work is needed to really understand these time-resolved transformations and their consequences for toxicity. Previous studies have highlighted that NMs that were thought to be stable when coated in PVP⁷ are not when exposed in realistic waters, as evidenced also by the differences in survival for daphnids exposed to pristine TiO₂ PVP exposures in the two different media. Furthermore, feeding the animals in the acute immobilization tests may avoid underestimation of the acute EC₅₀ from the initial range finding studies.

Growth effects

The growth measurements over time for all generations are presented in Fig. 4 following exposure to pristine/aged Ag and TiO₂ NMs in both water conditions. log₁₀ transformations were used to create linear models to assess the rate of daphnid growth over time (using the equation of the line of best fit to determine the slope) determined as the coefficient of change (Appendix 1: Table AP.7†) as shown in Constantinou, Sullivan.⁴¹ NMs are considered to be toxic if

there is a reduction in the coefficient relative to the control groups, which had values between 0.008–0.009.

When compared to the control populations, the daphnids exposed to pristine uncoated Ag NMs (F0) in the HH combo medium were on average 5% larger than the controls after 24 hours but were significantly smaller ($p < 0.05$) from day 9 onwards (Fig. 4A). The rate of growth (log₁₀ transformation of the slope) was 0.011, evidencing the accelerated growth up to day 9. The F1–3_{exp} populations had values closer to 0 (0.003–0.006) evidencing the decrease in size and the fact that they grew slower than the controls. The F1–3_{rec} also had variances in their growth rates (0.003–0.0110) compared to the controls, despite being removed from the exposure scenario, showing epigenetic traits from maternal exposure stress. Similarly, exposures to pristine uncoated TiO₂ NMs in the HH combo medium under the same conditions also significantly inhibited the growth of the daphnids (Appendix 1: Table AP.6†) (Fig. 4). The F0 populations grew slowly (0.006 Appendix 1: Table AP.6†) relative to the control populations (0.008 Appendix 1: Table AP.6) and were on average 48% smaller by comparison on day 6 of the exposure.

The inhibition in growth over the exposure period correlates well with the survival data and the delays between broods observed (Fig. SI.5 ESI†) and suggest that the effects may be due to negative impacts on the feeding behaviour. As previously discussed, food quality has an influence on life history traits such as growth,⁴² and maternal feeding has been documented to affect offspring growth and reproduction.⁴³ It should be noted that there were large green (algae) and white (TiO₂) aggregates at the bottom of each of the beakers (observed for all TiO₂ exposure conditions), which was also observed in studies by Bundschuh, Vogt,⁴⁴ who also observed size reductions of juvenile daphnids exposed to TiO₂ NMs. The sedimentation/hetero-aggregation of the algae–TiO₂ complexes suggests that the TiO₂ NMs reduced the maternal (F0) food availability and likely also food intake. Zhu³³ reported exposures to uncoated TiO₂ NMs reduced the feeding and filtration behaviour of daphnids, resulting in inhibited growth and reproduction, as observed in the present study. Despite visual sedimentation of the NMs with algae, our results agree with the previous findings and suggest that TiO₂ NMs ingested with food enhances the internal concentration (ESI† Table SI.4) and associated toxicity compared to NM exposure in the absence of food.

Daphnids (F0) exposed to pristine PVP TiO₂ in HH combo medium (Fig. 4) between 24 hours and day 6, were significantly (23%, $p = 0.004$ and 0.0002) smaller than the control populations. After day 9, there was no significant difference between the growth of the control and daphnids exposed to the pristine PVP TiO₂ NMs, indicating that NM-exposed juveniles do reach full size, but more slowly, which is confirmed by the growth correlation coefficient value of 0.006 compared to the control of 0.008 (Appendix 1 Table AP.6†). The F1–3_{exp} generations were also, on average, significantly ($p < 0.02$) smaller and grew slowly compared to the controls, whereas the F1–2_{rec} generations were



comparable with the control populations indicating no impacts on growth or growth rate from material exposure to pristine PVP TiO₂ NMs in HH combo medium in subsequent generations.

Comparing the pristine and aged NM exposures under the same conditions, daphnids exposed to the aged Ag and TiO₂ NMs had less variance and less significant size differences between each of the different coated particles compared to the pristine NMs (Fig. SI.4B†). The most significant differences were observed at day 3 where the NM exposed daphnids (F0) were up to 11% (uncoated Ag), 18% (PVP Ag) and 4% (Ag₂S) smaller than the controls and grew more slowly (Appendix 1: Table AP.7†). Interestingly, daphnids (F0) exposed to the uncoated TiO₂ NMs aged in HH combo media (Fig. SI.4B†) exhibited accelerated growth (up to day 15), where they were on average 10–33% larger than the control populations which was evidenced by increased growth (Appendix 1 Table AP.6†). Accelerated growth has been documented to occur under increased stress situations.⁴⁵ In most cases the F1–3_{exp} generations were significantly smaller than the controls, with the most extreme being 35% smaller in the F3_{exp} population, for the aged uncoated TiO₂, which also grew more slowly in HH combo media (0.006: Appendix 1 Table AP.6†). The decline in growth in the multigenerational exposures may be due to an energy trade-off whereby growth is sacrificed to support basic survival under toxic conditions.⁴⁶ In all recovery populations (F1–F3_{rec}), the sizes and growth rates (ESI† Appendix 1 Table AP.6) were comparable with the controls, showing regeneration after the F0 maternal exposure.

The F0 generation exposed to the aged PVP TiO₂ NMs were also larger than the control populations in HH combo media. The F1–3_{exp} generations had comparable growth rates to the controls with corresponding log₁₀ transformation coefficients of 0.008 (ESI† Appendix 1 Table AP.6). To exclude the possibility of the PVP surface coating affecting growth, measurements were made to daphnids exposed only to the PVP coating in both the HH combo and class V media (Fig. SI.4B† and 4D), showing little to no differences from day 6.

The pristine NM exposure (TiO₂ and Ag NMs) in the class V water, had a similar reduced daphnid size effect which was also observed in the pristine NM exposures in the HH combo media. However, despite reduced size effects fewer toxic consequences were observed when exposed to the pristine uncoated TiO₂ NMs. Unlike the uncoated TiO₂ in the HH combo, the F0 generation in class V water were able to produce successive F1–3 generations.

For all conditions (TiO₂ and Ag exposures), the successive F1–3_{exp} generations were on average always smaller than the controls and grew slower (Appendix 1: Tables AP.6 and AP.7†). The daphnids exposed to the aged NMs in the class V water had even less variance between their body sizes compared to the controls. Therefore, the combination of both NM ageing and realistic exposure media show fewer toxic consequences to the daphnids when compared to the pristine exposures in the HH combo media.

(Bio)accumulation

Understanding the life cycle and interactions of NMs with biological matter has an important role in nanosafety and regulation. Although individual organ distributions of the TiO₂ and Ag NMs were not identified, the average (bio) accumulation concentration of Ag and Ti per daphnid in the F0 generation was determined (ESI† Tables SI.2–SI.5) 7 days after initial exposure. Measurements further include the average NM size, NM concentration (particles per mL) and dissolved metal concentrations (μg L⁻¹) in solution after the 7 days. In all cases, the behaviours of the NMs between the pristine/aged and different waters were surface coating and media specific. The internalized concentrations were always lower in the aged NMs than the pristine exposures. In the pristine HH combo medium, the highest retained concentration of total Ag was in the pristine Ag₂S exposures at 0.716 μg L⁻¹ (F0), compared to 0.174 μg L⁻¹ (F0) after exposure to aged Ag₂S NMs. Interestingly, the aged uncoated Ag NMs had higher concentrations of total Ag compared to the pristine exposures (Table SI.2†), although less toxic effects (growth/mortality) were observed, due to the reduction of ionic Ag arising from NOM stabilization of the NMs. The internalized concentration of pristine uncoated Ti was on average 4.279 μg L⁻¹ per exposed daphnid in the HH combo medium. This was particularly high when compared with the internalized concentrations identified in the daphnids exposed to aged uncoated TiO₂, which ranged between 1.078–1.754 μg L⁻¹ under the same conditions. The pristine uncoated and PVP TiO₂ NMs in class V river water also had high accumulation of Ti compared to the aged TiO₂ NMs (Table SI.5†).

TEM cross sections revealed localised accumulation in the daphnid guts of all NMs. Pristine PVP TiO₂ can be seen along the peritrophic membrane (PTM) Fig. 5B showing disorganisation of the gut microvilli (MV) and the presence of autophagic vacuoles (AV). Autophagic vacuoles contribute to homeostasis by degrading damaged organelles, proteins and lipid droplets, and are involved in development and growth regulation. In response to stress, AV have been linked to survival mechanisms during short terms starvation, by degrading nonessential components from which the cells can continue to sustain nutrients for biosynthetic processes.⁴⁷ This may be true for the conditions observed in our experiments, since the food source intake may have been disrupted by TiO₂ and Ag NM accumulation in the gut and/or the agglomeration and precipitation of NM-algal complexes noted previously.

Conclusion

Clear differences between the toxicological consequences of pristine and aged TiO₂ and Ag NMs were observed in the two different media conditions – salt only (HH combo) or NOM-containing (class V) water. Pristine NMs exposed in the standard culture media had the most toxic consequences to survivorship and growth and the highest (bio)accumulation,



and furthermore displayed negative effects in daphnids two recovery generations post the parental exposure. The F0 generations exposed to the pristine uncoated TiO₂ NMs in the HH combo medium were the most sensitive to chronic exposure, resulting in decreased longevity, reduced growth and dramatically inhibited reproductive success. For example, the survival of the F1 generations from parents exposed to pristine TiO₂ NMs in HH combo was less than 24 hours in the continuously exposed group, and just 16 days in the recovery generation. Since the reproductive TG as currently designed (OECD 211) does not look at the survival of the F1 generation, nor its ability to reproduce, these longer-term effects of chronic exposure to NMs are completely missed. The poor survival of the F1 recovery generation, where the daphnid neonates were removed from exposure within 24 hours of birth and subsequently cultured in medium free of NMs, suggests that the effects are sufficiently dramatic as to warrant further investigation. The results warrant serious consideration of whether multiple generations should be incorporated into the reproductive assay since collapse of F1 populations would have dramatic effects on food-chains and the ecosystems services provided by crustaceans. The evidence of epigenetic effects is also interesting in the context of developing alternatives to animal testing.

NM ageing in the salt-only HH combo medium reduced the toxic consequences for both the exposed and recovery generations, whereas the NMs aged in the NOM-containing class V water had fewest overall toxic consequences on growth, longevity and survivorship, reproduction, and (bio) accumulation across all exposed generations, with no observable differences between the recovery generations post parental exposure and the controls. The results suggest a mediating role of the ionic strength and NOM in the realistic exposure medium. In addition, particle ageing reduces the toxicity *via* transformations to less reactive surfaces, through interactions with salts or with NOM. Analysis of the toxicity data provides very strong evidence that the pristine NMs are more toxic compared to their aged counter parts, with the NMs being especially toxic in the HH combo medium.

Current harmonised TG for *Daphnia* acute and chronic toxicity tests have herein been demonstrated to overestimate NM toxicity to the F0 generation but also to completely ignore the potentially even stronger toxic effects on subsequent generations arising from parental and/or continuous exposure to NMs. The results show the importance of updating harmonised TG documents to reflect scientific advances and increase stakeholder trust in regulation. Based on the data presented here, standardised *Daphnia* tests following the OECD TGs greatly overestimate the toxicity of pristine NMs in the absence of an environmentally relevant media, and ignore potential epigenetic and/or cumulative effects in subsequent generations. When making a regulatory assessment it is necessary to obtain a complete understanding of NMs toxicity, which requires comparison of the differences between the pristine and transformed/aged NMs under a

range of media conditions that span the range of transformations/extrinsic properties of the NMs in order to facilitate toxicity ranking. The fact that the media conditions are closer to 'real' or environmentally relevant form of these materials that would actually be encountered by target organisms, is an additional benefit, but is not the central goal. Longer term considerations and test revisions would ideally also ensure that the offspring are themselves able to survive and reproduce in order not to cause catastrophic population collapse. Further revision of harmonised TGs are to consider multi-generational effects is likely to be required as further weight of evidence is accumulated.

Conflicts of interest

There are no conflicts to declare.

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