Multigenerational Exposure to Nano-TiO$_2$ Induces Ageing as a Stress Response Mitigated by Environmental Interactions

Laura-Jayne A. Ellis, Stephen Kissane, Elijah Hoffman, Eugenia Valsami-Jones, James B. Brown, John K. Colbourne, and Iseult Lynch*

Despite their ubiquity in personal care products, the health implications of titanium dioxide (TiO$_2$) nanomaterials (NMs) are under strenuous investigation for their potential as a carcinogen, whereas other evidence has shown links with premature ageing. Both potential hazards are manifested after chronic exposure. To explore the chronic effects of TiO$_2$ NMs in the environment, a multigenerational study using the model test species Daphnia magna is conducted. Phenotypical characteristics associated with ageing are observed (loss or shortening of tails and lipid accumulation) with increased expression of highly conserved key stress response genes involved in inflammatory responses and oxidative stress. These responses are visible in continuously exposed daphnids over four generations and in daphnids removed from maternal exposure even three generations later. However, exposure to the “aged” variants of these NMs at the same concentrations significantly reduced these effects, and exposure in medium containing natural organic matter is less severe than in salt-only medium.

1. Introduction

Nanomaterials (NMs) are important to the healthcare and cosmetics industries, prompting concerns about their potential health effects as chemicals directly applied on skin and as pollutants in the wider environment. Titanium dioxide (TiO$_2$) as an example, is a photocatalyst producing hydroxyl radicals, superoxide anion radicals, and other reactive oxygen species (ROS) leading to DNA damage, lipid peroxidation,$^{[1]}$ and genotoxicity.$^{[2]}$ TiO$_2$ is classified as a group 2B possible carcinogen to humans for inhalation exposure$^{[3]}$ and is currently under review. Despite this potential hazard, European and international (FDA, USEPA) legislation$^{[4]}$ permits TiO$_2$ NMs in cosmetic products (max. 25% weight and 5.5% weight in aerosol products).$^{[5]}$ TiO$_2$ NMs are inevitably dispersed into the environment driving the need to study their whole life cycle from their “pristine” engineered form to environmentally “aged” transformed NMs. The environmental hazard potential of TiO$_2$ NMs has been previously investigated in different aquatic organisms, including algae$^{[6,7]}$, Daphnia$^{[8,9]}$, and zebrafish.$^{[10-12]}$ The common responses to TiO$_2$ NM exposure included inhibited growth,$^{[13]}$ oxidative stress,$^{[6,10,12]}$ and bioaccumulation.$^{[8,14]}$ However, these investigations only used pristine TiO$_2$ NMs and did not make comparisons with their aged NM counterparts, nor did they explore multigenerational effects.

Research into the modes of action suggest that TiO$_2$ NMs may trigger premature ageing,$^{[15]}$ although this hazard is expected to differ between different exposure scenarios as the transformations of NMs in the environment are found to generally reduce NM reactivity and thus ecotoxicity.$^{[16,17]}$ It has been proposed that environmental transformations homogenize the chemical and material structures of a diverse range of NMs by coating them with natural organic matter (NOM) for example.$^{[18]}$ We therefore studied the relative health hazards of these NMs, as a pristine-engineered product and as an environmental pollutant, using a model test species and observed the effects of continuous versus parent only exposure over multiple generations. The microcrustacean Daphnia magna (D. magna) are a foundational ecological species for toxicological studies that typically reproduce parthenogenetically (genetically identical clones).$^{[19]}$ The effect of NM
pollutants on life history traits, such as the timing of juvenile development, age of reproductive maturity, first reproductive cycle, number of offspring, senescence, and death, provides sensitive information regarding ecological stress and chemical toxicity.\textsuperscript{[20,21]} Daphnia also present an optimal genomic model, and are advantageous for monitoring stress/adaptive changes to their environments,\textsuperscript{[22]} as disturbing the homeostasis of Daphnia leads to energy demanding responsive, compensatory, and adaptive processes.\textsuperscript{[23]} Consequently, the genetic processes alter under chronic stress, which can be monitored by identifying epigenetic changes. Epigenetic changes can be transferred to subsequent generations resulting in altered phenotypes in later nonexposed (recovery) generations.\textsuperscript{[21,24,25]}

In addition to its role as a sentinel model species for ecotoxicity, Daphnia are increasingly being recognized as a surrogate model for human health as part of an alternative (animal-free) approach, with continued investigation into the human genome and the sequencing of whole animal genomes identifying highly conserved regions in the genes associated with homeostasis, growth, maintenance, and reproduction between animal genomes. Therefore, assessing the response outcome from ecotoxicology models such as Daphnia is significant in regards to model environmental and human health hazards, as similarities are shared within their genomes.\textsuperscript{[26]} Interactions among elements of human response pathways are strongly conserved among animal species. Consequently, a chemical hazard assessment framework built up upon reactions (i.e., molecular key events) is likely to be informative for a greater diversity of species, Transgenerational inheritance can influence disease risk\textsuperscript{[27–29]} and ageing.\textsuperscript{[15,30,31]} Senescent decline and ageing are driven from both internal (genetic) and external (environmental) factors, and the process of ageing is closely related to how effectively an organism can cope with induced stress.\textsuperscript{[32]} Long-term environmental stress disturbs the physiological functions, leading to disruption of cellular functions, and resulting in age-related stress responses as an adaptive response to accumulated damage.\textsuperscript{[31]}

Thus, understanding multigenerational effects arising from NMs released into the environment is important for environmental risk assessments of NMs, as parental exposure may compromise the sensitivity and tolerance of future generations. The results are also as a means to explore the process of chemical-induced ageing, providing insights relevant for other species including humans, as a result of the highly conserved genes and response pathways across species.

This study investigates the effects of exposure to uncoated and polyvinylpyrrolidone (PVP)-coated TiO\textsubscript{2} NMs as a “pristine”-engineered product and as an “aged” environmental pollutant, in two different media on four generations of Daphnia magna. HH combo represents a standardized salt-only culturing medium, whereas Class V water represents an artificial river water classification with the addition of NOM. Investigating the presence of NOM in the culturing media will demonstrate how the environment transforms the NMs\textsuperscript{[33,34]} through corona formation, and the resulting effect on ecotoxicity. For the multigenerational investigations, the offspring (F1) from the exposed parental generations (F0) were split into continuously exposed (F\textsubscript{exp}) and recovery (F\textsubscript{rec}) paired studies after direct F0 maternal exposure. Important insights regarding NM-induced accelerated ageing of the daphnids in response to the pristine uncoated and PVP-coated TiO\textsubscript{2} NMs are presented, which were ameliorated by environmental ageing of the NMs. Correlations with conserved genes and pathways in humans are identified suggesting that ageing in daphnia may be indicative of similar effects in humans.

2. Results

2.1. Adverse Health Outcomes from Exposure to TiO\textsubscript{2} NMs

Multigenerational health effects and life history parameters assessed included survival, growth, reproduction, morphological traits, TiO\textsubscript{2} biodistribution, and expression of key stress response genes involved in compensational pathways that are highly conserved between species.\textsuperscript{[26]} We hypothesized that the toxicity of pristine TiO\textsubscript{2} NMs would vary with surface chemistry (uncoated vs coated), with the medium type (salt-only Daphnia culturing medium vs organic matter containing artificial river water), and with environmental ageing of the NMs (for 6 months in the respective media) which would reduce their surface reactivity.

The most severe effects were observed in Daphnia (F0) exposed to the pristine uncoated TiO\textsubscript{2} in the Daphnia culturing medium, with increased mortality, reduced fitness, and reproduction, and no surviving offspring for multigenerational monitoring (Figure 1). Both pristine PVP and uncoated TiO\textsubscript{2} NMs, in both media, were toxic, showing adverse effects to the maternal (F0) daphnids and to both the continuously exposed and removed (recovery) subsequent generations (F1–3) (Figure 2). Moreover, Daphnia exposed to the aged NMs in the environmentally realistic water had significantly reduced toxicological effects in both the continuously exposed and recovery generations, showing that NM physicochemistry and medium composition are codependent effects.

2.2. Toxicity of the Pristine TiO\textsubscript{2} NMs (Single Generational Effects)

The most sensitive populations were those exposed to the pristine uncoated TiO\textsubscript{2} NMs in the simple salt-only culturing medium, with only 10% survival by day 25 (Figure 1a and Table SI.1, Supporting Information), significantly (p < 0.05, Table SI.1, Supporting Information) inhibited growth (Table SI.2, Supporting Information) and altered structural morphology (Figure 3). For all conditions, log\textsubscript{10} transformations were used to assess the rate of change in the average daphnid growth over time to give a rate of change coefficient (Table SI.2, Supporting Information). Toxicity was considered if there was a reduction/increase relative to the control groups, which had coefficient values between 0.008 and 0.009. The F0 daphnids exposed to the pristine uncoated TiO\textsubscript{2} NMs in the salt-only culturing medium grew more slowly (0.007, Table SI.1, Supporting Information) and were on average 48% (p = 0.005, Table SI.1, Supporting Information) smaller on day 6 (Figure 1b) than the control populations.

Healthy juvenile daphnids are usually between 10 and 12 days old once the first neonates are released. Thereafter, daphnids produce a clutch of parthenogenetic eggs after every adult molt (every 3/4 days) until death.\textsuperscript{[35]} In this study, control daphnids cultured in the simple culturing medium and the artificial river
Figure 1. Aged TiO$_2$ NMs are less toxic than the pristine ones in the F0 generation exposure: a) survival (%) versus age, b) the size versus age, and c) the cumulative total of the average neonates produced per daphnia for each brood. Each of the graphs (a–c) show the results for each medium condition and NM type (pristine vs aged) for each of the two coated TiO$_2$ NMs. The data are expressed as the mean ± the standard deviation (represented by confidence bands).
Figure 2. Multigenerational effects of toxicity: F1–3 generations showing a) the survival, b) the size versus age, and c) the cumulative average total number of neonates per daphnid in each medium condition for each of the two TiO$_2$ NMs (uncoated and PVP coated). The data are expressed as the mean ± the standard deviation (represented by the confidence bands).
water representative (Figure SI.3, Supporting Information) released their first broods between days 11 and 12, and fifth broods between 24 and 26 days old.

The average number of offspring per daphnid was around six neonates per brood (Table SI.3 and SI.4, Supporting Information). The reproductive success of the Daphnia exposed to the pristine uncoated TiO$_2$ NMs in the salt-only culturing medium were severely affected. The first broods were not released until day 17 (Table SI.3 and Figure SI.3A, Supporting Information), whereas the fifth broods were delayed until day 36 (12 days later than the controls) with only an average of two neonates per daphnid.

Similarly, the pristine PVP TiO$_2$ NMs in the salt-only culturing medium had compromised survival, with only 27% of the daphnids alive by day 25 (Figure 1a; Figure SI.3A, Supporting Information). The reproductive success of the Daphnia exposed to the pristine uncoated TiO$_2$ NMs in the salt-only culturing medium were severely affected. The first broods were not released until day 17 (Table SI.3 and Figure SI.3A, Supporting Information), whereas the fifth broods were delayed until day 36 (12 days later than the controls) with only an average of two neonates per daphnid.

Similarly, the pristine PVP TiO$_2$ NMs in the salt-only culturing medium had compromised survival, with only 27% of the daphnids alive by day 25 (Figure 1a; Figure SI.3A, Supporting Information). The reproductive success of the Daphnia exposed to the pristine uncoated TiO$_2$ NMs in the salt-only culturing medium were severely affected. The first broods were not released until day 17 (Table SI.3 and Figure SI.3A, Supporting Information), whereas the fifth broods were delayed until day 36 (12 days later than the controls) with only an average of two neonates per daphnid.

The average number of offspring per daphnid was around six neonates per brood (Table SI.3 and SI.4, Supporting Information). The reproductive success of the Daphnia exposed to the pristine uncoated TiO$_2$ NMs in the salt-only culturing medium were severely affected. The first broods were not released until day 17 (Table SI.3 and Figure SI.3A, Supporting Information), whereas the fifth broods were delayed until day 36 (12 days later than the controls) with only an average of two neonates per daphnid.

Similarly, the pristine PVP TiO$_2$ NMs in the salt-only culturing medium had compromised survival, with only 27% of the daphnids alive by day 25 (Figure 1a; Figure SI.3A, Supporting Information). The reproductive success of the Daphnia exposed to the pristine uncoated TiO$_2$ NMs in the salt-only culturing medium were severely affected. The first broods were not released until day 17 (Table SI.3 and Figure SI.3A, Supporting Information), whereas the fifth broods were delayed until day 36 (12 days later than the controls) with only an average of two neonates per daphnid.

The results show inconsistences in the daphnid growth and development between the two differently coated TiO$_2$ NM exposures in the salt-only Daphnia culturing medium.

The same exposures with pristine NMs were conducted in an artificial river water containing NOM (Class V water), showing major differences in the fitness parameters of the Daphnia when compared with the exposures in the Daphnia culturing media. Daphnids exposed to pristine uncoated TiO$_2$ NMs in artificial water with NOM had 86% survival for 25 days (Figure 1c), which correlated with reduced body burden of Ti (Tables SI.5 and SI.6, Supporting Information), and brood timings that were comparable with the controls (Table SI.1, Supporting Information). The pristine PVP TiO$_2$ NMs had increased survival at day 25 (50%), and the brood timings that were also comparable with the controls. However, for both exposures, the growth was significantly affected, whereby the daphnids were between 10% and 31% (uncoated TiO$_2$) and 10% and 46% (PVP TiO$_2$) smaller than the controls. Here, the PVP TiO$_2$ NMs were the most toxic, further highlighting the codependant effects of medium composition and NM surface reactivity.

Figure 3. Morphological effects on daphnids from images of, a) day 3 F0 control, b) day 3 F0 daphnid exposed to pristine uncoated TiO$_2$ NMs in the salt-only culturing medium, c) day 3 F1 control to compare with d) day 3, two F$_{1_{rec}}$ individuals from the (pristine TiO$_2$, PVP NMs in salt-only culturing medium) showing morphological abnormalities and altered tail spines. e) Day 15 F0 control to compare with f) day 15 F0 daphnid exposed to pristine uncoated TiO$_2$ NMs in the artificial river water. g) F1 control to compare to h) Day 6 F$_{1_{rec}}$ (pristine TiO$_2$, PVP NMs in artificial river water) whose tail is absent and which has a larger abdomen. i) Day 15 F0 individual control compared with, j) day 15 F0 daphnid exposed to pristine uncoated TiO$_2$ NMs in the culturing medium with evidence of lipid accumulations around the abdominal claw and heart. k) Day 12 F1 control compared with, l) day 12 F$_{1_{exp}}$ aged uncoated TiO$_2$ NMs (culturing medium) showing evidence of lipid accumulation. m) A matched time point for day 12 F$_{1_{rec}}$ aged uncoated TiO$_2$ NMs (culturing medium) exposure, showing no lipid accumulations. Scale bars are 500 μm.
2.3. Aged TiO$_2$ NMs are Less Toxic than Pristine TiO$_2$ NMs: Single Generational Effects

Realistic exposure conditions combined with environmental transformation of the NMs reduces their toxicity. For both coated and uncoated aged TiO$_2$ NMs, increased survival showing 100% (uncoated TiO$_2$) and 93% (PVP TiO$_2$) were observed for the duration of the study in the F0 populations when exposed to the aged NMs in the artificial river water (Figure S1.1D, Supporting Information). The daphnids were on average larger than the controls, by between 2% and 7% (PVP TiO$_2$) and 1% and 10% (uncoated TiO$_2$) (Figure 1b). The reproductive success for the F0 Daphnia exposed to the aged uncoated TiO$_2$ NMs exposed in the artificial river water had comparable average number of neonates (Figure 1c) and brood timings to the unexposed control populations. Furthermore, daphnids exposed to the aged PVP TiO$_2$ NMs also had comparable brood timing for the first three broods, although some daphnids had failed to become gravid. Moreover, the fourth and fifth broods were delayed until day 31, highlighting effects of NM induced stress (Figure S1.3D and Table S1.4, Supporting Information).

2.4. Pristine Nano–Bio Interactions: Multigenerational Effects (F1–3 Generations)

The single-generational exposures provide strong evidence that the pristine NMs (before environmental transformation) are toxic to the Daphnia, showing reduced fitness, survival, and reproduction. Here, the chronic effects of both the pristine (as engineered) and aged (environmentally transformed) NMs to the transgenerational progeny (F1–3) from their maternal exposure (F0 as previously discussed) are investigated. The F1 progeny were split into a continuously exposed (F$_\text{exp}$) and recovery (F$_\text{rec}$) set of paired generations (Figure S1.9, Supporting Information). Monitoring the paired generations allows for the assessment of both the adaptability and recovery ability of the daphnids, to both pristine and aged NMs in each of the culturing media (salt only vs the artificial river water with NOM).

The sensitivity from the exposure to pristine uncoated TiO$_2$ NMs in the culturing media was inherited by all the F1 generations, irrespective of removal (F$_\text{1rec}$) or continued exposure (F$_\text{1exp}$), with 100% lethality observed by day 16 (Figure S1.1, Supporting Information; Figure 2a). Thereafter, there were no subsequent generations to monitor. Sensitivity was also inherent in the F1–3$_\text{exp}$ generations exposed to the pristine PVP TiO$_2$ NMs in salt-only culturing medium, with reduced survival (Figure 2a). The F1–3$_\text{exp}$ generations were also significantly ($p < 0.02$) smaller than the controls (Table S1.4, Supporting Information). The reduction in growth may be due to negative impacts on the feeding behavior, as food quality has an influence on life history traits (including growth and reproduction).38 The pristine uncoated TiO$_2$ NMs parent-exposed recovery generations (F1–3$_\text{rec}$) each had comparable sizes and growth relative to the unexposed controls (Table S1.1, Supporting Information).

Large green (algae) and white (TiO$_2$) agglomerates settled at the bottom of each of the exposure beakers, which was also observed in Daphnia TiO$_2$ NM exposure studies by Bundschuh et al.37 The sedimentation/agglomeration of the alage–TiO$_2$ suggests that the TiO$_2$ NMs reduced the maternal (F0) food intake leading to the observed toxicity in the lift history parameters. Zhu et al.39 reported that exposure to uncoated TiO$_2$ NMs also reduced the feeding and filtration of Daphnia, resulting in inhibited growth and reproduction. Our results agree with the previous findings, suggesting that TiO$_2$ NMs ingested complexed with food enhances the internal concentration of TiO$_2$ (Table S1.5, Supporting Information), leading to reduced longevity in the exposed Daphnia.

Further toxicological effects were apparent in F2$_\text{exp}$ generations exposed to the pristine PVP TiO$_2$ NMs in the salt-only culturing media, with reproductive delays (Figure S1.4, Supporting Information). The first broods were not released until day 16 (4 days later than the controls), producing on average (across the five measured broods) four neonates per adult. The F3$_\text{exp}$ generation brood timings were also significantly delayed, producing third broods on day 20 with an average offspring number per daphnid (across all five broods) of only 2. The average offspring numbers (per daphnid) for the fourth and fifth broods were 0.6 and 0.8, showing in some cases, daphnids failed to become gravid after 20 days of exposure to the pristine TiO$_2$ PVP NMs in the salt-only culturing media. Prolonged oxidative stress as a result of metal bioaccumulation is linked to reduced fitness, reduced survival, and the inability to produce offspring,38 all of which were observed in this study and the effects of which were inherited by the subsequent generations.

Major positive differences to the longevity of the daphnids exposed to pristine NMs were observed in the artificial river water (Figure 1c) compared with exposures in culturing medium (Figure 1a). Daphnids were more tolerant to the pristine uncoated TiO$_2$ NMs with successive F1–3 generations. However, the effects of the pristine NM exposure were evident in the F1–3 generations. First, the F1$_\text{exp}$ and F1$_\text{rec}$ generations were always smaller than the controls, and F2/3$_\text{rec}$ generations were mostly comparable with the control sizes at the same measured time points. In addition, irrespective of exposure or removal, in the F1–F3 generations body shape defects were observed along their posterior and anterior sides (Figure 3f and h), with tail losses and/or reduced tail size (Figure 3d,h, Figure S1.7, and Table S1.7, Supporting Information). Finally, the toxicological effects of the pristine NMs in the artificial river water were further evidenced by reproductive issues, as the F1$_\text{exp}$ and F3$_\text{exp}$ generations had large variances in brood numbers and timings (mainly delays), whereas F2$_\text{exp}$ were early in comparison with the control populations. For the F2–3$_\text{rec}$ generations, some recovery was observed with comparable number of days between the first and second brood releases, although this also declined after the third broods in the F2$_\text{rec}$ and F3$_\text{rec}$ generations.

In contrast, NM surface coating-specific behaviors were observed in daphnids exposed to the pristine PVP TiO$_2$ NMs in the artificial river water. The F1$_\text{exp}$ and F1$_\text{rec}$ generations had 97% and 93% survival, whereas none of the F2$_\text{exp}$ generations survived past day 19. Furthermore, the F2$_\text{rec}$ generation did not produce sufficient neonate numbers in their third broods, for successive F3$_\text{rec}$ generations. The results neither correlated with increased TiO$_2$ uptake when compared with the salt-only culturing medium exposures (Table S1.5-6, Supporting Information), nor could we explain it by displacement or loss of the PVP coating, as the PVP control exposures had
98–100% survival in both water conditions (Figure S1.1E, Supporting Information). The only noticeable difference was that the NMs were larger in size when compared with those exposed in the salt-only culturing medium, suggesting agglomeration of the PVP-coated TiO₂ NMs in the realistic water may have reduced the available food source due to TiO₂-algae sedimentation, resulting in a lack of nutrients and overall death of the organisms due to induced over-compensatory stress mechanisms.

Growth and morphological observations were also affected with the PVP-coated TiO₂ NMs, as the F₁ exp generation were overall 11% larger (day 24) than the controls, despite being smaller for the first 9 days. Irregularities in body shape and tail lengths were observed in the F₁ rec generations, despite removal from maternal exposure at birth (± 8 h). The subsequent generations (F₂) for the removed and continued exposure had comparable growth trends to the pristine uncoated TiO₂ NMs despite no reproductive success in the third broods. Losses of tails and body abnormalities were observed in all the F₀ and F₁ populations (Figure 3 and 4).

2.5. Aged Nano–Bio Interactions: Does the Environment Protect Against Multigenerational Effects? (F₁–3 Generations)

Exposure to the aged uncoated TiO₂ NMs in salt-only culturing media resulted in lower mortality and the environmental ageing also had considerable positive effects on the reproductive ability of the successive generations (F₁–F₃) compared with the pristine NM exposures (Figure 2b). Although the exposed generations (F₁–F₃) only produced an overall average of three neonates per daphnid, those in the recovery generations had an average of six neonates per brood. However, maternal effects of NM exposure were still prominent with initial delays between each of the broods (compared with the control) irrespective of continuous exposure or removal (Table S1.4, Supporting Information). Negative effects on the growth of the continuously exposed F₂ exp and F₃ exp generations were observed (up to 33% smaller in the F₃ exp population). In all recovery populations, all daphnids sizes were comparable with the controls, further showing some restoration after the initial F₀ maternal exposure (Figure 2b).

Daphnids exposed (F₁–3 exp) to the aged uncoated TiO₂ NMs in the artificial river water were all significantly larger (Table S1.1, Supporting Information) than the controls, and had deviations in tail lengths (Figure 4) and morphology, with negative effects on reproductive succession (Figure S1.3D and Table S1.4, Supporting Information) also observed. The recovery generations (F₁–3 rec) were comparable with the control populations with no morphological differences in the tails observed or effects on the reproductive success. However, there were phenotypic characteristics of lipid deposits visible around the heart and abdominal claw of all generations (exposed and recovery). The F₁–3 exp generations exposed to aged PVP TiO₂ NMs in the artificial river water were, on average, between 2% and 7% larger than the controls but smaller than the former pristine PVP TiO₂ exposures under the same conditions (Figure 2d). There were also morphological effects to the aged TiO₂ PVP transgenerational exposure in the artificial river water showing NM surface coating-specific behaviors.

In contrast, the recovery generations (F₁–F₃ rec) were significantly larger (up to 29%) than the controls in all generations. The reproductive success was also poor in all the following F₁–F₃ rec generations, however showing some recovery, with shorter times between broods and increasing average neonate numbers per Daphnia. The decline in offspring and reduced reproductive success in the F₁–3 generations may be linked with reduced maternal feeding rates, due to the internalization of TiO₂ NMs in the gut (Figure 5b). Maternal feeding rates have also been documented to affect offspring growth and reproduction. A decline in fecundity is also linked with a deterioration in fitness and is linked with ageing. Although there were transgenerational sensitivities to the NM exposure, realistic exposure conditions combined with aged NMs reduces the toxicity that creates prolonged stress and reduced survival, when compared the pristine NM exposure and the aged NMs in the standard salt-only culturing medium.

2.6. Ageing Phenotypes as a Response to Nano–Bio Interactions

We hypothesized that the bioaccumulation of TiO₂ NMs (Tables S1.5-6, Supporting Information) leads to excessive oxidative stress in the Daphnia. Prolonged oxidative stress is further linked to ageing, reduced fitness, reduced survival, and the inability to produce offspring, all end points which were observed in this study from the pristine NM exposures and the aged NMs in the culturing medium. The phenotypical characteristics associated with ageing are typically reduced growth with tail losses which steadily decline with age and lipid deposition.

A dramatic and unexpected finding was the evidence of tail loss or tail length reductions in both the pristine (in culturing medium and artificial river water) and aged NM exposures (in culturing medium only) from very early in the exposure duration (Figure 5d,h). Loss of tail is a type of phenotypic plasticity with indicates the disruption of the embryonic development from midembryonic maturation onward, as the tail spines are developed during these stages (between stages 4 and 5). The first instance of the tail abnormalities was seen in the F₁ rec and F₂ rec generations of those daphnids exposed to pristine PVP TiO₂ NMs in the salt-only culturing medium. Complete loss of tails was also observed in both the pristine PVP and uncoated TiO₂ NMs in the exposed and recovery generations in culturing medium. Notably, these abnormalities were only found in the pristine TiO₂ PVP recovery exposures in the salt-only medium. No abnormalities were seen in the aged NM exposures under the same conditions.

Previous work investigating daphnia longevity and ageing found a correlation between tail length and Daphnia age as one of the morphological indicators of the rate of ageing. Therefore, as the daphnids age, growth, and tail loss theoretically should reduce at a steady rate and be comparable with the control populations. Using this theory, NM-induced changes in tail length should lead to predictions of daphnid ages higher than their actual ages, indicative of an accelerated ageing phenotype. Using the equation of the line of best fit from the plot of tail length versus age measurements of healthy daphnids (i.e., the
Figure 4. Box plots of the tail lengths compared with age over time for daphnids exposed to a) pristine TiO$_2$ NMs in the salt-only culturing media, b) aged TiO$_2$ NMs in the salt-only culturing media, c) pristine TiO$_2$ NMs in the artificial river water containing NOM, d) aged TiO$_2$ NMs in the artificial river water containing NOM and e) survival for one generation of daphnids only exposed to the PVP surface coating in left: culturing media and right: the artificial river water.
control populations in this study, (Figure 4), the apparent ages of the TiO$_2$ NM-exposed daphnids were determined relative to their actual ages to produce the predicted age values (Tables SI.8 and SI.9, Supporting Information). Using this assessment, the predicted ages of the exposed daphnids were significantly ($p \leq 0.05$) (Table SI.7, Supporting Information) higher than their actual ages based on the measured tail lengths when compared with the control populations. These phenotypic effects were also present in the F2 and F3 recovery generations, indicating epigenetic traits.

Observations of tail abnormalities were also reported by Djekoun et al.\cite{42} who exposed $D. magna$ to different concentrations of cadmium over time and assessed the developmental stages of the eggs and neonates for morphological and toxicological effects. They identified inhibition of various developmental stages, releases of broods, and abnormalities in the carapase, eyes, and caudal spine. Notably, these morphological malfunctions were observed in both the continuously exposed and recovery generations in this study suggesting epigenetic changes following maternal exposure. Literature shows that heavy metal exposure effects the carapase shedding which can affect growth\cite{20,41,42}. However, F2–3$_{rec}$ generations were never directly exposed, only via the maternal line, therefore the tail losses need to be explained by other mechanisms.

The daphnids exposed to the pristine uncoated TiO$_2$ NMs (in the salt-only $Daphnia$ culturing medium) had significantly shorter tails (comparable with the reduced body length) until day 18 where their predicated and actual ages were comparable by the average tail length measurements. The effects of NM exposure were also evident in the daphnids exposed to the aged uncoated TiO$_2$ NMs (in the salt-only culturing medium).

Maternal and direct exposure effects were seen throughout the F1–2 recovery and exposed generations, although after days 15–18 in both the F3 adaptation (F3$_{exp}$) and recovery (F3$_{rec}$) sets, there was evidence of the predicted tail ages matching their actual ages (Table SI.8-9, Supporting Information). Another phenotypical characteristic associated with ageing is lipid deposition.\cite{40} The accumulation of lipid deposits has been previously linked with ageing in multiple species,\cite{43-46} as a response to exposure from environmental toxicants.\cite{40} These findings may also explain the enlarged sizes of the daphnids at the earlier time points for those exposed to the aged NMs in the present study. Although we did not quantify lipid deposition, morphological analysis (Figures 3f,j and l) identified fatty deposits around the heart, brain, and abdominal claw, similar to those identified by Jordão et al.\cite{47} These lipid deposits were not present in the control populations at earlier developmental stages (Figure 3a,g) but were present in the pristine uncoated TiO$_2$ NMs exposures from day 15 (F0) and from day 9 (F0) in the aged exposures (in $Daphnia$ culturing media).

Using artificial intelligence and deep learning methodologies, the adverse effects of the TiO$_2$ NMs on daphnids were classified in terms of the several possible malformations (tail length, overall size, and uncommon lipid concentrations and lipid deposit shapes) by comparing the experimental images of the NM exposed daphnids to control daphnid images (which presented no damage). The developed nanoinformatics models were able to detect, isolate, and classify regions of interest on the $Daphnia$ images where specific malformations occurred, and assessments were made of the type and the severity of malformations compared with the control daphnids, including the increased lipid concentrations in exposed daphnids, enhancing...
our understanding of the possible age-related effect of NM exposure.\textsuperscript{48}

Antioxidant mechanisms are vital to maintain and protect from intracellular redox homeostasis, detoxify oxidants, and repair the damage caused by them. The eight genes used in this study were selected to provide mechanistic insights into NM–organism interactions and represent pathways encoding for cellular functions known to be induced by other environmental pollutants (metal detoxification, oxidative stress, energy production, DNA repair [Poynton et al., 2007] and general maintenance\textsuperscript{49,50}). The most important of these are GST and CAT, MET, and HO1 genes which code for proteins associated with removal of ROS and methionine sulfoxide species.\textsuperscript{51} These genes were significantly increased when exposed to the pristine uncoated and PVP TiO$_2$ NMs in both media conditions (Figures S1.4–6, Supporting Information).

Increased ROS is directly linked to reproductive inability due energy diversion to fight oxidative stress\textsuperscript{30} in response to the uptake and bioaccumulation of TiO$_2$ NMs (Table S1.5, Supporting Information). Oxidative stress and ROS production were also observed as a direct effect of TiO$_2$ exposure in studies by Li et al.\textsuperscript{6}

Prolonged oxidative stress and disruption of the melatonin may also be linked with ageing as a stress response\textsuperscript{15,30} As GSH is the most important and abundant antioxidant, ROS accumulation may be a contributor to the ageing process rather than a consequence of the apoptotic phenotype of cells that cease to divide.\textsuperscript{52} Barata et al.\textsuperscript{11} also identified that age-related decline was associated with increasing oxidative stress/damage. Age-related impairments failing to eradicate the induced ROS would cause saturation of transcription for these genes which would in turn affect homeostasis, increase ROS, increase lipid deposition, and eventually lead to fatality,\textsuperscript{53} all of which were endpoints observed in this study.

Exposure to xenobiotics/toxicants requires significant energy expenditure by the organism to deal with the biological processes induced, and as a result energy metabolism occurs following subsequent exposure.\textsuperscript{40} Studies by Bündschuh et al.\textsuperscript{37} support that energy is required in the early life stages to support growth, which supports reproductive success. Exposure to toxicants can then result in diversion of energy away from growth and reproduction, as proposed by the dynamic energy budget model.\textsuperscript{53,54} Both growth and reproduction were inhibited due to exposure to both pristine uncoated and PVP coated TiO$_2$ in the salt-only culturing medium. The mechanisms that maintain the normal redox homeostasis are important to protect against oxidative stress\textsuperscript{52} and include cellular maintenance and homeostasis, both of which were disrupted for all NM medium exposures, further evidenced with increased expression of β-actin. Furthermore, cellular energy demands caused by oxidative stress were highlighted in the increased expression of NADH for all exposures.

NM functionalization, ageing, and medium type were important in governing the chronic responses at the gene response level when exposed to both pristine and aged uncoated and PVP TiO$_2$ NMs in the different media conditions. The results show different gene expression patterns between the exposed and recovery populations. Analysis of the gene expression of key genes evidenced the phenotype and life history data (Figure 4 and 5 and Figure S1.3, Supporting Information). For each gene, the pristine uncoated TiO$_2$ NMs in the salt-only culturing medium was the most sensitive to increased expression (F0), which was reflected in the reproductive decline and zero survival of the following generations. As there were no successive offspring, it is difficult to observe the medium-specific changes to the generational gene responses for both the exposed and recovery generations. However, there is a trend, whereby exposure to the pristine uncoated TiO$_2$ NMs induced higher expression of all genes in all exposed generations when compared with the aged uncoated TiO$_2$ NMs in the artificial river water. The effects of the exposure to pristine uncoated TiO$_2$ NMs in the artificial river water was evident in all generations with increased gene expression in all three recovered generations, further highlighting maternal stress influence on the epigenetics in two generations removed from exposure. Exposure to the pristine PVP TiO$_2$ NMs also showed increased expression for all genes analyzed in both culture medium and artificial river water. The F1$_{rec}$ were also highly sensitive to the pristine PVP TiO$_2$ NMs in artificial river water exposures. Importantly, the elevation of DNA polymerase highlights the depletion of antioxidant repair mechanisms leading to the increase in DNA damage repair processes commonly reported with premature aging.\textsuperscript{53}

Environmentally aged uncoated and PVP TiO$_2$ NMs in the artificial river water had the least stressful effects on Daphnia generations compared to the pristine NMs under the same conditions. The recovery generations were also negligibly affected when compared with the control expression levels. NM surface-coating differences were also reflected in the life history data. Overall, reduced fitness, reduced survival, and the inability to produce offspring\textsuperscript{38} were all end points observed in this study. Therefore, the evidence presented suggests that pristine TiO$_2$ NM exposure, irrespective of medium conditions, induces ageing as a stress response in Daphnia. However, ageing of the NMs in environmentally relevant water significantly reduces the organism toxicological responses to the NM exposure.

2.7. Linking NM Exposure to Human Health

It is generally understood that phenotypes may vary substantially because of gene and environment interactions that were essentially shaped by evolution, or by environmental stress that predictably disrupts the normal functioning of genes.\textsuperscript{56–58} Over the past decade, there is growing knowledge that distantly related species share many ancestral genes by common descent that serve the same biochemical pathways.\textsuperscript{59} Comparative genomics studies have confirmed that crustaceans retain a greater number of ancestral gene families that are shared with humans than insects (such as Drosophila), including genes responsible for growth, reproduction, and maintenance.\textsuperscript{26,59,60} Research by Colbourne et al.\textsuperscript{26} and others\textsuperscript{61} confirms that invertebrates including Daphnia retain a disproportionately large number of ancestral gene families that are linked to human diseases, despite more than 780 million years since present day crustaceans and mammals last shared a common ancestor. With this knowledge in mind, the study presented here attempts to make a preliminary link between the ecological model test species Daphnia and human health by exploring the use of homology to define the understanding of the original molecular interactions as a result
of exposure-related harm. To this end, we demonstrate that it is possible to use gene expression to help bridge the gap between distantly related species by understanding how exposure to pollutants disrupts key conserved biological processes.\textsuperscript{[63]} By searching a gene orthologs database,\textsuperscript{[61]} a catalogue of genes shared among species by descent across the animal kingdom to infer functional conservation (https://www.orthodb.org/), it was possible to highlight some of the highly conserved key genes involved in stress response pathways, such as ageing, between our test model species (\textit{D. magna}) and humans (Table 1). Comparing common genes shared by common descent among species offers meaningful insights into the connections between model test species and human and environmental health exposure.\textsuperscript{[62,64]} Future work (in progress) is to make genome-wide assessments of the daphnids exposed to the variety of TiO$_2$ NMs (and others) to provide a greater knowledge transfer between ecological stress and potential ageing pathways.

3. Discussion and Conclusions

Using four conditions (pristine and transformed/aged NMs in salt-only medium vs environmentally relevant NOM-containing artificial water), we have demonstrated that even where toxicity is apparently reduced, some lasting effects persist in the subsequent generations affecting their apparent age and their reproductive success. Sublethal effects from exposure to “pristine” TiO$_2$ NMs in both the \textit{Daphnia} culturing medium and the artificial river water representative were observed. Identical exposures of the aged TiO$_2$ NMs in the artificial river water representative resulted in dramatically decreased effects on the \textit{D. magna}. However, although the aged NMs presented here appeared “safe” with fewer toxic consequences in the F0 generations, when the progeny were investigated (F1–3) as paired continuously exposed versus removed from the maternal exposure, there was still evidence of inherited dysfunction.

We have argued previously that the presence of biological macromolecules is essential for meaningful NM ecotoxicity assessment, and that the formation of the eco-corona in the test medium reduces the surface energy of the NMs as would occur instantaneously in the environment.\textsuperscript{[65,66]} Proteins present on the surface of the TiO$_2$ NMs used in this study were determined after 7 days incubation with the F0 \textit{Daphnia} as part of a larger published study\textsuperscript{[66]} to identify the biological interactions of the NMs, including uptake and toxicity. Ellis and Lynch\textsuperscript{[66]} demonstrated that the surface bound eco-corona compositions changed between the pristine and aged NMs, and between the environmental media in which the NMs were incubated, thus, changing the NMs biological identity and interactions. The proteins in the eco-corona on the pristine NM surfaces were largely associated with metabolic and cytotoxic damage and there were significantly less proteins were bound to the aged NMs in all medium conditions.\textsuperscript{[66]} Proteins bound only to the aged NMs were indicative of significantly lower toxic responses, thus showing that the NM eco-corona composition can facilitate detection of organism responses to NM exposure and potentially the identification of the molecular initiating events in adverse outcome pathways. Furthermore, the results demonstrated that the risk of over estimating the toxicity of pristine NMs if using salt only medium, as NOM and secreted biomolecule binding to the NMs reduces their surface reactivity and consequent toxicity to aquatic organisms.

This study confirms that exposure to pristine TiO$_2$ NMs in simple \textit{Daphnia} culturing media results in very dramatic multi-generational life history changes, with indications of accelerated ageing and epigenetic effects that propagate though the subsequent three generations. The morphological traits were consistent with ageing phenotypes, evidenced by shortened tail lengths and lipid deposits, which will be investigated further in our ongoing research analyzing the complete genome. These effects are substantially ameliorated through utilization of the artificial river water representative and utilization of environmentally transformed (aged) NM forms typical of environmental pollutants that have undergone other physical and/or chemical transformations that generally reduce, but do not completely alleviate their toxicity to \textit{Daphnia}.

Furthermore, this study attempts to transform the practice of safeguarding health, by ensuring exposure and risk assessments of NMs added to personal care products represent the entire life cycle of the materials from usage through disposal. Chemical hazard assessment is also starting to shift toxicological focus using model species and exploring their highly conserved biochemical pathways based on the use of homology, to understand if exposure induced harm may be representative of effects on human health also. As this study has shown phenotypical ageing stress responses following exposure to the NMs, complete genome sequencing is now underway for daphnids exposed under the same four conditions to shed additional light on the signaling pathways perturbed and to understand the biological pathways involved in the observed accelerated ageing and to tease out the Adverse Outcome Pathways involved in pollution-induced ageing. The conservation of genes, and likely their functions, between \textit{Daphnia} and humans suggests that \textit{Daphnia} could potentially be a model for exposure-related conditions of ageing and that NMs might provide an important tool for exploring ageing pathways in humans also, opening up avenues for intervention and reversal of ageing in the future.

4. Statistical Analysis

All experiments were repeated in triplicate, and the data were recorded as the mean with standard deviation. For the growth studies, a student’s t-test was used to detect any significant difference between the control, treated, and recovery groups. In all analyses, a p-value <0.005/0.05 was considered statistically significant (Appendix 1 Table AP.1, Supporting Information). The linear model rate (slope) of daphnid growth between each population for their age versus time was analyzed in RStudio using Log$_{10}$ transformations. A positive number shows an incline in the data and the further from 0 the slope is, the steeper the correlation fits though the data. (Appendix 1 Table AP.2, Supporting Information). Gene expression levels were normalized to 18S expression levels as previously described by Andersen, et al.\textsuperscript{[72]} Statistical significance of changes in gene expression were computed in RStudio, as follows: models were fit using lmit, eBays was used to compute the significance of parameters, resulting p-values that were corrected for multiple testing (false discovery rate) using the Benjamini–Hochberg (BH) method. Main effects
| Gene                              | Group at metazoan level | Homo sapiens | Daphnia magna | Phyletic Profile | Link                                                                 |
|----------------------------------|-------------------------|--------------|---------------|------------------|----------------------------------------------------------------------|
| Glutathione s-transferase (GST)  | 3208                    | GPA045       | CRUS          | 682 genes in 321 species (out of 450), single copy in 125 species, multi-copy in 196 species | https://www.orthodb.org/?level=33208&species=73337_0,29078_0,9793_0,9798_0,186990_0,9974_0,9402_0,112908_0,89399_0,9407_0,9606_0,35525_0&query=510902at33208 |
| Dehydrogenase (NADH)            | 3208                    | GPA045       | CRUS          | 483 genes in 435 species (out of 450), single copy in 392 species, multi-copy in 43 species | https://www.orthodb.org/?level=33208&species=73337_0,29078_0,9793_0,9796_0,9798_0,186990_0,9974_0,291302_0,109478_0,225400_0,59463_0,9402_0,112908_0,89399_0,9407_0,9606_0,35525_0&query=510902at33208 |
| β-Actin (act)                    | No matches              | No matches   | No matches    | No matches       | No matches                                                            |
| Catalase (cat)                   | 3208                    | CAT          | CRUS          | 739 genes in 440 species (out of 450), single copy in 313 species, multi-copy in 127 species | https://www.orthodb.org/?level=33208&species=73337_0,29078_0,9793_0,9796_0,9798_0,186990_0,9974_0,291302_0,109478_0,225400_0,59463_0,9402_0,112908_0,89399_0,9407_0,9606_0,35525_0&query=73337at33208 |
| DNA Polymerase                   | No matches              | No matches   | No matches    | No matches       | No matches                                                            |
| Hemoglobin RNA                   | No matches              | No matches   | No matches    | No matches       | No matches                                                            |
| Hemoglobin oxygenase-1           | 3208                    | GPA045       | CRUS          | 738 genes in 386 species (out of 450), single copy in 142 species, multi-copy in 244 species | https://www.orthodb.org/?query=H1&level=33208&species=73337_0,29078_0,9793_0,9796_0,9798_0,186990_0,9974_0,291302_0,109478_0,225400_0,59463_0,9402_0,112908_0,89399_0,9407_0,9606_0,35525_0&query=510902at33208 |

### Table 1. Orthologs: mapping genomics to functional data.

**Gene** | **Group at metazoan level** | **Homo sapiens** | **Daphnia magna** | **Phyletic Profile** | **Link**
--- | --- | --- | --- | --- | ---
Glutathione s-transferase (GST) | 3208 | GPA045 | CRUS | 682 genes in 321 species (out of 450), single copy in 125 species, multi-copy in 196 species | https://www.orthodb.org/?level=33208&species=73337_0,29078_0,9793_0,9798_0,186990_0,9974_0,9402_0,112908_0,89399_0,9407_0,9606_0,35525_0&query=510902at33208 |
Dehydrogenase (NADH) | 3208 | GPA045 | CRUS | 483 genes in 435 species (out of 450), single copy in 392 species, multi-copy in 43 species | https://www.orthodb.org/?level=33208&species=73337_0,29078_0,9793_0,9796_0,9798_0,186990_0,9974_0,291302_0,109478_0,225400_0,59463_0,9402_0,112908_0,89399_0,9407_0,9606_0,35525_0&query=510902at33208 |
β-Actin (act) | No matches | No matches | No matches | No matches | No matches |
Catalase (cat) | 3208 | CAT | CRUS | 739 genes in 440 species (out of 450), single copy in 313 species, multi-copy in 127 species | https://www.orthodb.org/?level=33208&species=73337_0,29078_0,9793_0,9796_0,9798_0,186990_0,9974_0,291302_0,109478_0,225400_0,59463_0,9402_0,112908_0,89399_0,9407_0,9606_0,35525_0&query=73337at33208 |
DNA Polymerase | No matches | No matches | No matches | No matches | No matches |
Hemoglobin RNA | No matches | No matches | No matches | No matches | No matches |
Hemoglobin oxygenase-1 | 3208 | GPA045 | CRUS | 738 genes in 386 species (out of 450), single copy in 142 species, multi-copy in 244 species | https://www.orthodb.org/?query=H1&level=33208&species=73337_0,29078_0,9793_0,9796_0,9798_0,186990_0,9974_0,291302_0,109478_0,225400_0,59463_0,9402_0,112908_0,89399_0,9407_0,9606_0,35525_0&query=510902at33208 |

### Key
- **Homo sapiens** and **Daphnia magna** are the species names.
- **Phyletic Profile** indicates the number of genes and copy numbers across species.
- **Link** provides a direct access to the ortholog information on OrthoDB.
were also evaluated using ANOVA in R (and corrected for multiple testing as above). Significance thresholds were applied to BH-adjusted p-values.

Further information about the reported methodologies are discussed in the Supporting Information.

5. Experimental Section

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

Media and Representative Waters: Experiments were conducted in Daphnia high hardness combo medium (HH combo) and in an artificial river water representative (Class V artificial water). The Daphnia culturing medium (HH combo) represented an average hard water salt-only standard without any NOM and was commonly used for the culturing of Daphnia. The artificial river water representative had high alkalinity and high ionic strength and 4.6 mg L⁻¹ NOM, typical of waters found in the southern UK, Poland, Greece, Frann, the Balkan countries, and the Iberian Peninsula. A description of the water combinations is shown in Table S1.11/1Table S1.11A–C, Supporting Information.

NM Characterization and Aging: The NMs used in this study include anatase uncoted TiO₂ (9 ± 2 nm) and PVP-coated TiO₂ (9 ± 2 nm) both obtained from the EU H2020 NanoFASE project partner Promethean Ltd. Dynamic light scattering (DLS) was used to measure the “pristine” and “aged” (for 6 months in the two media) TiO₂ NMs hydrodynamic diameters using a Malvern Nanosizer 5000 (Table S1.10, Supporting Information). Transmission electron microscopy (TEM) analysis of the TiO₂ NMs (Figure 5) was carried out using JEOL 1200EX 80 kV and JEOL 1400EX 80 kV microscopes. TiO₂ NMs were prepared by drop casting, by depositing a 20 μL drop of the NM suspension on a 300 mesh carbon-coated copper TEM grid (Agar Scientific, UK).

NMs were chemically aged by exposing stock solutions of 1000 mg L⁻¹ to the Daphnia culturing media and the artificial river water representative (Tables S1.11, Supporting Information) for at least 6 months before Daphnia exposure. Stock solutions were always stored at 4°C (refrigerated).

Daphnia Maintenance and Culturing: Initial stocks of D. magna were maintained using protocols 3rd brood Bham2 strain (genetically identical), which originated from the University of Reading (UK) and the Water Research Centre (WRc), Medmenham, UK. D. magna were kept in a 20°C temperature-controlled environment with 12-h light and dark cycles. D. magna were cultured in a standard high hardness Daphnia culturing media and the artificial river water representative which was refreshed weekly to ensure healthy culture maintenance. D. magna cultures were fed Chlorella vulgaris algae daily, to a total of 0.5 mg carbon between days 0–7 (750 μL) and 0.75 mg (1.5 mL) carbon from day 7.

Range-Finding Study (daphnia Acute Immobilization test, OECD 202): The acute immobilization tests were conducted on D. magna exposed to the pristine NMs in the salt-only Daphnia culturing medium only. This was to highlight the differences between: (1) feeding during long-term exposure versus lack of feeding in the acute study, (2) Daphnia culturing medium (salt-only) and artificial river water representative containing NOM and the resulting toxicity difference at the same NM concentration exposure, and (3) the pristine and aged NMs in terms of their toxicity to the exposed generation and their progeny. To determine the initial effect concentrations (ECs) for the multigenerational studies, a total of (20 x 3) 60 neonates (<24 h) were exposed to a range of concentrations of pristine uncoated TiO₂ and pristine TiO₂ PVP NMs over 24 and 48 h to assess their immobilization and survivability (Figure S1.8, Supporting Information). The Daphnia were not fed during these observations in accordance with the standard OECD test for acute immobilization 202. An EC₅₀ value was established for pilot studies at 45 mg L⁻¹ (TiO₂ PVP) and 30 mg L⁻¹ (TiO₂ uncoated). The pilot studies were conducted to identify any issues with study design and/or the NM concentrations used. The EC₅₀ also showed high mortality in pilot studies with almost 100% mortality after 5 days exposure. For this reason, the EC₅₀ was used throughout the experiments.
K with 400 μL lysis buffer (per sample), followed by mechanical homogenization using a Precellys 24 instrument (Bertin Technologies) using 1 cycle of 20 s at a 6400 pulse speed. The samples were then incubated at 37 °C for 25 min and transferred to a 96 well plate.

A Beckman Coulter Biomek Fxp was used to automate the immobilization and isolation of the RNA onto magnetic particles, separating it from any other contaminants in the samples. RNA isolation was completed by adding 400 μL bind buffer (containing 80 μL of bind buffer and magnetic beads with 320 μL of isopropanol) to each sample, while shaking to mix thoroughly for 5 min. The plate was then placed on the magnet for 6 min to separate the beads from the mixture, where the supernatant was removed from each sample. The plate was removed from the magnet and the samples were washed with ethanol (70%), covered with plate seal and stored at −80 °C until required. RNA yield was quantitated by a NanoDrop ND-8000 (ThermoFisher ND-8000-CL). Aliquots of each sample were diluted to ≈5 ng μL−1, and tested upon the Agilent Tape station 2200 (Agilent G2964AA) with High-Sensitivity RNA screentapes (Agilent 5067-5579) to ascertain the RNA Integrity Number.

**Preamplification/Reverse Transcription**: A OneStep qPCR kit (Qiagen) was used in accordance to the manufactures guidance. Briefly 800 ng from each sample was combined with primers for each gene (from a 100 μM stock) and added to 10 μL buffer, 2 μL dNTP mix, and 2 μL enzyme mix. Water was added to bring the final volume to 20 μL. Reverse transcription was facilitated by a 30 min incubation at 50 °C. Following reverse transcription, the samples were used to setup two separate preamplification plates (due to differences in primer annealing temperatures) (Table S1.13, Supporting Information).

Gene assay mixtures were created by mixing 1 μL of a 100 μM stock of each primer. Set A consisted of CST, NADH, and HO1 (note that 18S did not undergo preamplification due to its high level of expression), set B consisted of B-actin, DNA polymerase, catalase, and metallothioenen. Gene assay mixtures were then diluted adding sufficient DNA suspension buffer (TEKnova T0221) to bring the volume to 200 μL. For each sample, 1.25 μL cDNA was transferred to a clean plate. To this, 1 μL Pre-amp master mix (Fluidigm 100-5580), 0.5 μL Pooled Gene assay mix, and 2.25 μL DNase-free water was added. The plate was placed in an Eppendorf Mastercycler nexus gradient (Eppendorf 6331000017) on the protocol outlined in Table S1.13, Supporting Information. Following preamplification, the reaction mixtures (Table S1.14, Supporting Information) were cleaned up using Exonuclease I (Table S1.15, Supporting Information). To each sample, the following was added: 1.4 μL DNase-free water, 0.2 μL Exol reaction buffer, and 0.4 μL Exol (NEB M0293L). Samples were mixed, and then thermal cycled at the following conditions: (1) 37 °C for 30 min, (2) 80 °C for 15 mins. (3) Hold 4 °C (until sample removed from cycler).

After Exol treatment, the samples were diluted with 25 μL DNA suspension buffer (TEKnova T0221).

Diluted samples were stored at −200 °C until ready for Fluidigm Gene Expression. The Preampl gene assay Master Mix was combined to produce the preamplification premix (Table S1.13, Supporting Information), which was individually separated into inlets of a 96-well plate sample. Each individual sample (measured in triplicate) containing cDNA was transferred to a 96 well plate. The samples were added into one-well inlet (1 inlet per sample). The preamplification was conducted on an Eppendorf Mastercyler Nexus eco gradient model with a 2 min hold at 95 °C, 50 °C for 30 min, and held at 4 °C. RNA integrity was measured using an Agilent Technologies 2200 TapeStation. Specific target amplification products were then treated Exonuclease I (Exo I) (New England BioLabs) to degrade any unbound primers (Table S1.15, Supporting Information).

**qPCR**: Gene expression was conducted using Flex Six Integrated Fluidic Circuit (IFC) Delta Gene Assay (72 × 72) in combination with a DX Prime (153×) system and a Fluidigm BioMark (Standard) Real-time PCR instrument, as per the manufacturers recommended protocol. The purified Exo I-treated samples were mixed with the EvaGreen supermix and 2× loading assay (Table S1.16, Supporting Information) to produce the sample premix. The samples were then utilized for high-throughtput qPCR on 72 independent samples across 72 qPCR assay probes, equivalent to 5184 independent reactions. The IFC Delta Gene Assay partitioned the sample into 72 microfluidic chambers and performed qPCR detection and quantification for each specific gene in each chamber. For each Dynamic Array used, we enriched each sample and gene in triplicate using a 12 × 12 format to utilize the 72 assay chambers.

The Flex Six IFC was primed with 150 μL of the control line fluid for 15 min before loading the samples, using the prime script (153×) feature on the DX instrument (BioMark, Fluidigm). Samples and gene assays were loaded into the IFC and the “run script” on the DX instrument enabled the loading of the samples and assays into the chamber for 50 min before being run on the Biomark instrument according to conditions outlined in Table S1.17, Supporting Information.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

**Acknowledgements**

This work was funded via a Natural Environment Research Council Highlights grant (NE/N006569/1), with additional support from the H2020 project NanoSolveIT (Grant Agreement No. 814572). The authors acknowledge the use of the UoB Daphnia facility and the Environmental Transcriptomics facility, and the use of the School of Materials and Metallurgy TEM center. The authors further acknowledge Paul Stanley and Theresa Morris (School of Materials and Metallurgy) for assistance with the TEM Daphnia specimen preparations.

**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

Data openly available in a public repository that issues datasets with DOIs - currently generating the DOI.

**Keywords**

cotoxicology, epigenetic effects, nanoparticle transformations, reproductive effects

Received: January 11, 2021
Revised: February 13, 2021
Published online: April 8, 2021

[1] J. Virkutyte, S. R. Al-Abed, D. D. Dionysiou, Chem. Eng. Technol. 2012, 191, 95.
[2] L. Armand, A. Tarantini, D. Beal, M. Biola-Clier, L. Bobyk, S. Sorieul, K. Pernet-Gallay, C. Marie-Desvergne, I. Lynch, N. Herlin-Boime, M. Carriere, Nanotoxicology 2016, 10, 913.
[3] International Agency for Research on Cancer (IARC), Evaluation of Carcinogenic Risks to Humans, Vol. 1a-100, IARC, Lyon, France, 2011.
[4] T. G. Smijis, S. Pavel, Nanotechnol., Sci. Appl. 2011, 4, 95.
[5] Scientific Committee on Consumer Safety (SCCS), OPINION on Titanium Dioxide (TiO2) Used in Cosmetic Products that Lead to Exposure by Inhalation, European Commission, 2018.
[6] F. Li, Z. Liang, X. Zheng, W. Zhao, M. Wu, Z. Wang, Aquat. Toxicol. 2015, 158, 1.
[7] K. Hund-Rinke, M. Simon, Environ. Sci. Pollut. Res. 2006, 13, 225.
