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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

Dr. L.-J. A. Ellis, Dr. E. Valsami-Jones, Dr. I. Lynch
School of Geography, Earth and Environmental Sciences
University of Birmingham
Birmingham B15 2TT, UK
E-mail: I.lynch@bham.ac.uk

Dr. S. Kissane, Dr. J. B. Brown, Dr. J. K. Colbourne
Environmental Transcriptomics Facility
School of Biosciences
University of Birmingham
Birmingham B15 2TT, UK

E. Hoffman, Dr. J. B. Brown
Genome Dynamics Department
Life Sciences Division
Lawrence Berkeley National Laboratory
1 Cyclotron Road, Berkeley, CA 94720, USA

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/anbr.202000083.

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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.[20,21] *Daphnia* also present an optimal genomic model, and are advantageous for monitoring stress/adaptive changes to their environments,[22] as disturbing the homeostasis of *Daphnia* leads to energy demanding responsive, compensatory, and adaptive processes.[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.[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.[26] Interactions among elements of human response pathways are strongly conserved among animal species. Consequently, a chemical hazard assessment framework built upon reactions (i.e., molecular key events) is likely to be informative for a greater diversity of species. Transgenerational inheritance can influence disease risk[27–29] and ageing.[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.[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.[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_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[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_{exp}) and recovery (F_{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_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_2 NMs

Multigenerational health effects and life history parameters assessed included survival, growth, reproduction, morphological traits, TiO_2 biodistribution, and expression of key stress response genes involved in compensational pathways that are highly conserved between species.[26] We hypothesized that the toxicity of pristine TiO_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_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_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 codependant effects.

2.2. Toxicity of the Pristine TiO_2 NMs (Single Generational Effects)

The most sensitive populations were those exposed to the pristine uncoated TiO_2 NMs in the simple salt-only culturing medium, with only 10% survival by day 25 (Figure 1a and SI.1 A, Supporting Information), significantly ($p < 0.05$, Table SI.1, Supporting Information) inhibited growth (Figure 1b and SI.2, Supporting Information) and altered structural morphology (**Figure 3**b, d, f and h). For all conditions, Log_{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_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.[35] In this study, control daphnids cultured in the simple culturing medium and the artificial river

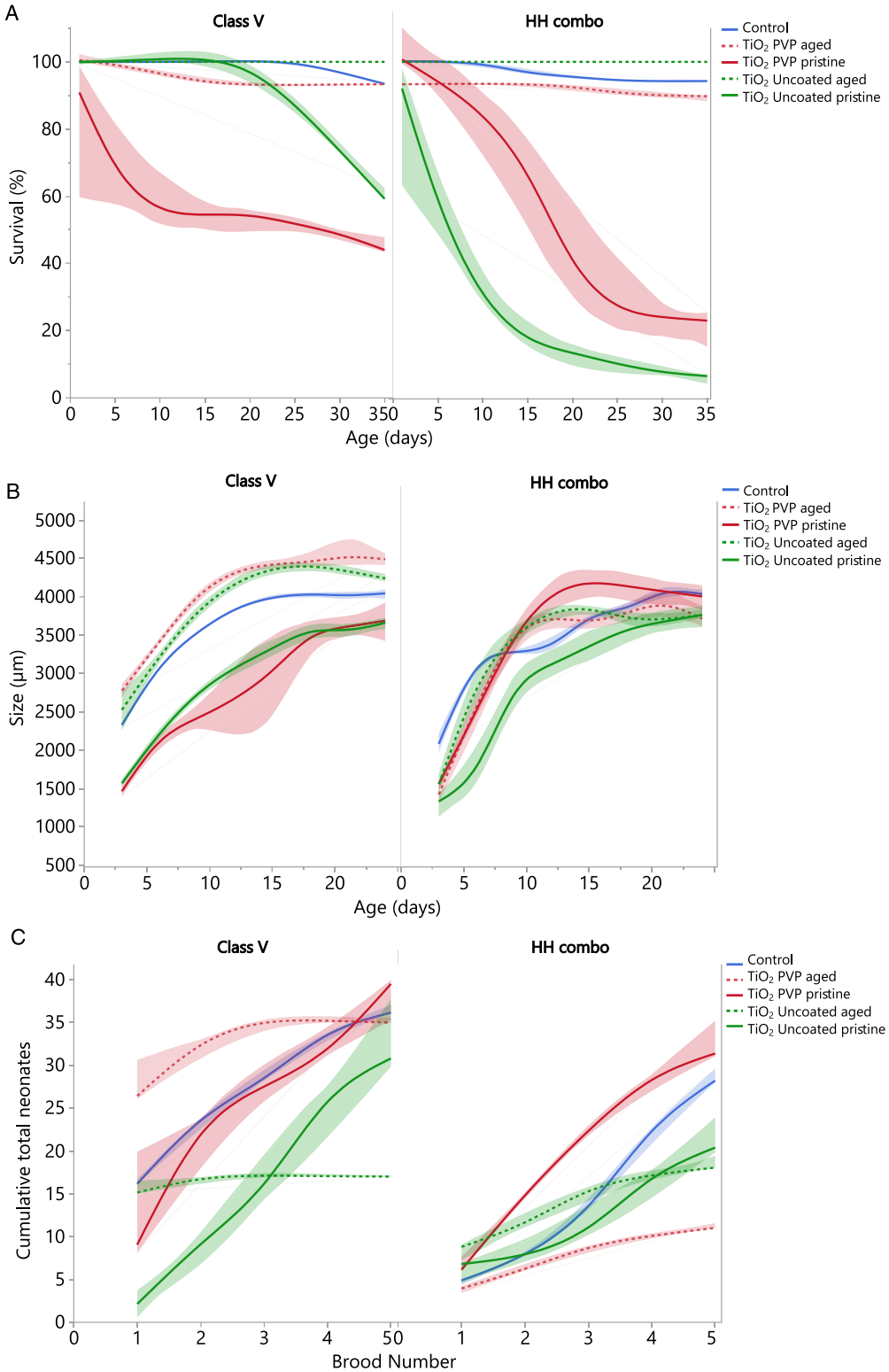

Figure 1. Aged TiO_2 NMs are less toxic than the pristine ones in the F0 generation exposure: a) survival (%) versus age, b) the size versus age, and c) the cumulative total of the average neonates produced per daphnia for each brood. Each of the graphs (a–c) show the results for each medium condition 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).

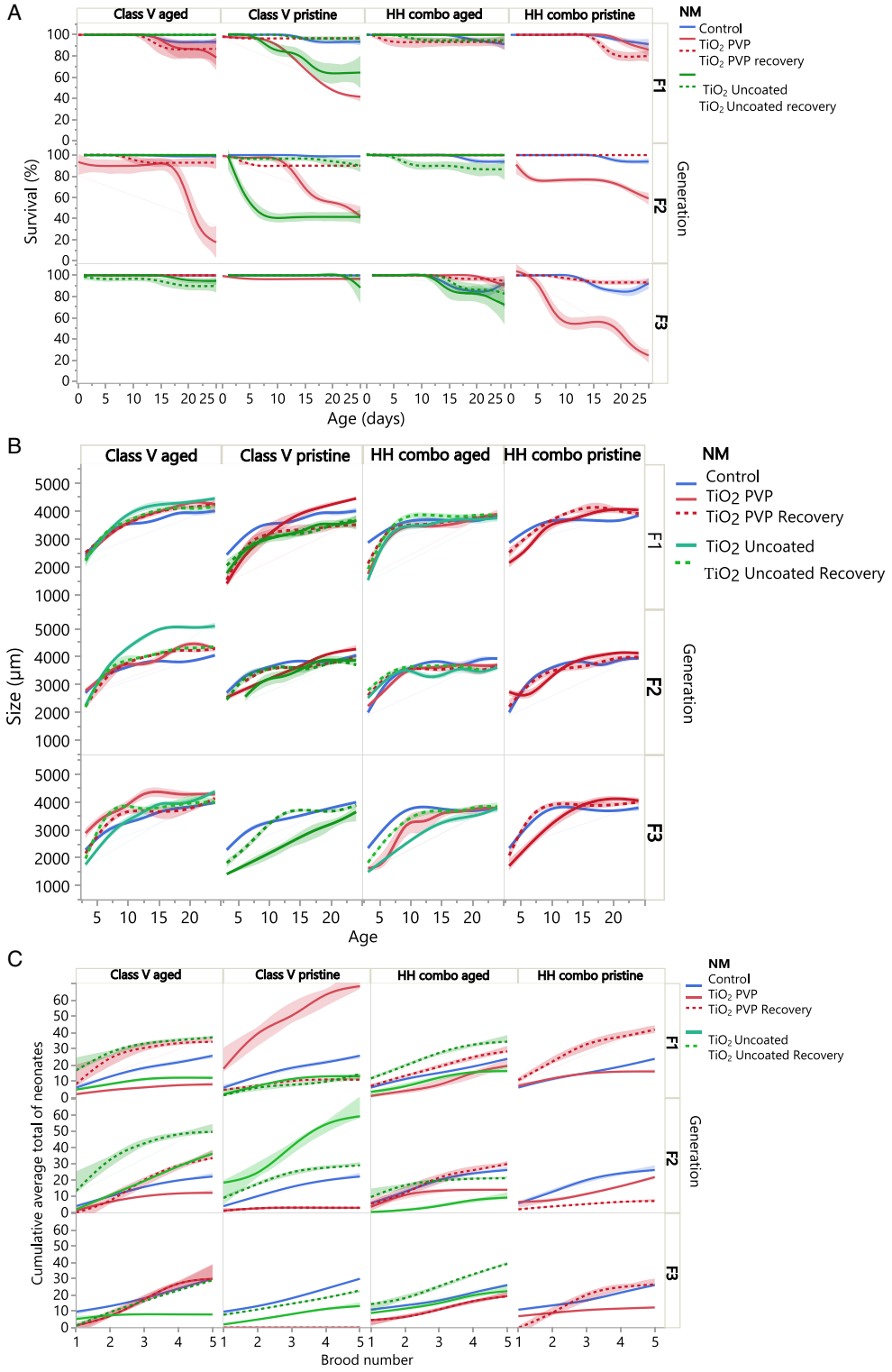

Figure 2. Multigenerational effects of toxicity: F1–3 generations showing a) the survival, b) the size versus age, and c) the cumulative average total number of neonates per daphnid in each medium condition for each of the two TiO_2 NMs (uncoated and PVP coated). The data are expressed as the mean ± the standard deviation (represented by the confidence bands).

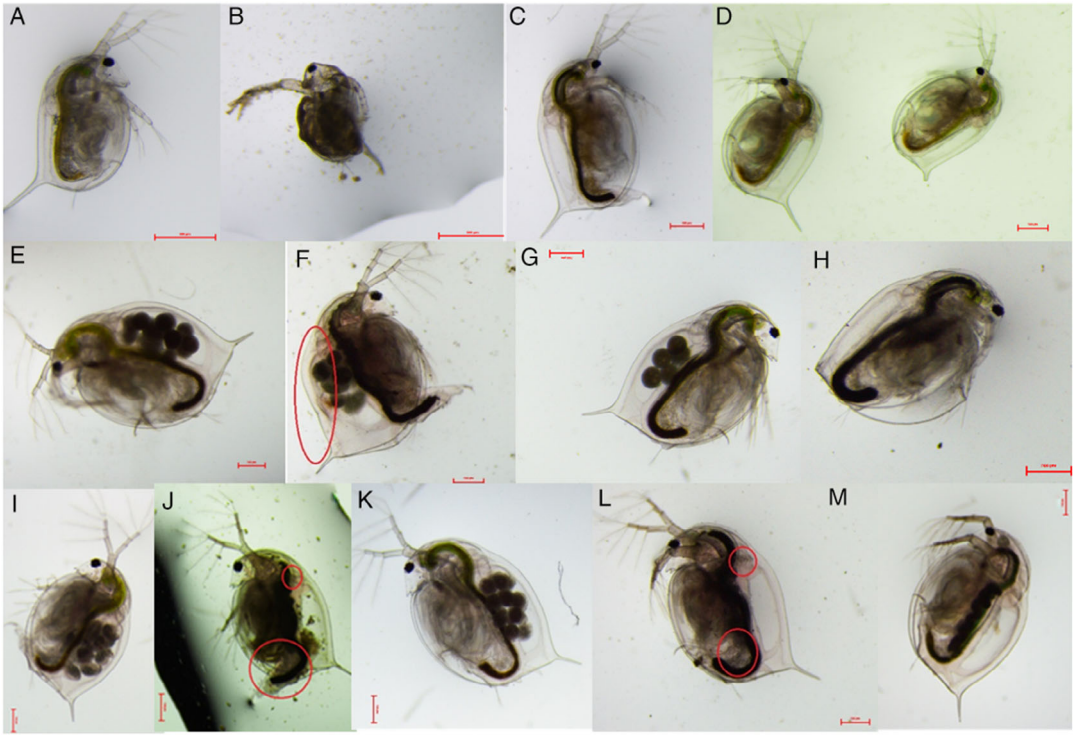

Figure 3. Morphological effects on daphnids from images of, a) day 3 F0 control, b) day 3 F0 daphnid exposed to pristine uncoated TiO_2 NMs in the salt-only culturing medium, c) day 3 F1 control to compare with d) day 3, two $F1_{rec}$ individuals from the (pristine TiO_2 PVP NMs in salt-only culturing 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 $F1_{rec}$ (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 $F1_{exp}$ aged uncoated TiO_2 NMs (culturing medium) showing evidence of lipid accumulation. m) A matched time point for day 12 $F1_{rec}$ aged uncoated TiO_2 NMs (culturing medium) exposure, showing no lipid accumulations. Scale bars are 500 µm.

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.1A, Supporting Information). Daphnids were also significantly (23%, $p = <0.0002$, Table SI.1, Supporting Information) smaller than the control populations until they were 9 days old. However, the daphnids had a rapid increase in growth between days 10 and 21 (Figure 1b). Despite this grown spurt, the rate of change coefficient for the overall growth over time was 0.006 (Table SI.2, Supporting Information), showing that relative to the controls, they grew more slowly overall. The reproductive cycle was also altered as their fifth were broods not observed until day 33 (Figure SI.3B, Supporting Information) with only an average of two neonates per daphnid (Table SI.4, Supporting Information) 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.

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 SI.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 SI.3D and Table SI.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_{exp}) and recovery (F_{rec}) set of paired generations (Figure SI.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 ($F1_{rec}$) or continued exposure ($F1_{exp}$), with 100% lethality observed by day 16 (Figure SI.1, Supporting Information; Figure 2a). Thereafter, there were no subsequent generations to monitor. Sensitivity was also inherent in the $F1–3_{exp}$ generations exposed to the pristine PVP TiO_2 NMs in salt-only culturing medium, with reduced survival (Figure 2a). The $F1\text{-}3_{exp}$ generations were also significantly ($p = <0.02$) smaller than the controls (Table SI.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).[36] The pristine uncoated TiO_2 NMs parent-exposed recovery generations ($F1–3_{rec}$) each had comparable sizes and growth relative to the unexposed controls (Table SI.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.[9] 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 SI.5, Supporting Information), leading to reduced longevity in the exposed *Daphnia*.

Further toxicological effects were apparent in $F2_{exp}$ generations exposed to the pristine PVP TiO_2 NMs in the salt-only culturing media, with reproductive delays (Figure SI.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 $F3_{exp}$ 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 $F1\text{-}3_{exp}$ and $F1_{rec}$ generations were always smaller than the controls, and $F2/3_{rec}$ 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 SI.7, and Table SI.7, Supporting Information). Finally, the toxicological effects of the pristine NMs in the artificial river water were further evidenced by reproductive issues, as the $F1_{exp}$ and $F3_{exp}$ generations had large variances in brood numbers and timings (mainly delays), whereas $F2_{exp}$ were early in comparison with the control populations. For the $F2–3_{rec}$ 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 $F2_{rec}$ and $F3_{rec}$ 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 $F1_{exp}$ and $F1_{rec}$ generations had 97% and 93% survival, whereas none of the $F2_{exp}$ generations survived past day 19. Furthermore, the $F2_{rec}$ generation did not produce sufficient neonate numbers in their third broods, for successive $F3_{rec}$ generations. The results neither correlated with increased TiO_2 uptake when compared with the salt-only culturing medium exposures (Table SI.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 SI.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 TiO_2 NMs in the realistic water may have reduced the available food source due to TiO_2-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 TiO_2 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 TiO_2 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 TiO_2 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 SI.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 daphnid 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 TiO_2 NMs in the artificial river water were all significantly larger (Table SI.1, Supporting Information) than the controls, and had deviations in tail lengths (Figure 4) and morphology, with negative effects on reproductive succession (Figure SI.3D and Table SI.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 TiO_2 NMs in the artificial river water were, on average, between 2% and 7% larger than the controls but smaller than the former pristine PVP TiO_2 exposures under the same conditions (Figure 2d). There were also morphological effects to the aged TiO_2 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 TiO_2 NMs in the gut (**Figure 5**b). Maternal feeding rates have also been documented to affect offspring growth and reproduction.[39] A decline in fecundity is also linked with a deterioration in fitness and is linked with ageing.[38] 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 TiO_2 NMs (Tables SI.5-6, Supporting Information) leads to excessive oxidative stress in the *Daphnia*. Prolonged oxidative stress is further linked to ageing,[15,30] reduced fitness, reduced survival, and the inability to produce offspring,[38] 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[32] and lipid deposition.[40]

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).[20,41] The first instance of the tail abnormalities was seen in the $F1_{rec}$ and $F2_{rec}$ generations of those daphnids exposed to pristine PVP TiO_2 NMs in the salt-only culturing medium. Complete loss of tails was also observed in both the pristine PVP and uncoated TiO_2 NMs in the exposed and recovery generations in culturing medium. Notably, these abnormalities were only found in the pristine TiO_2 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[32] 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

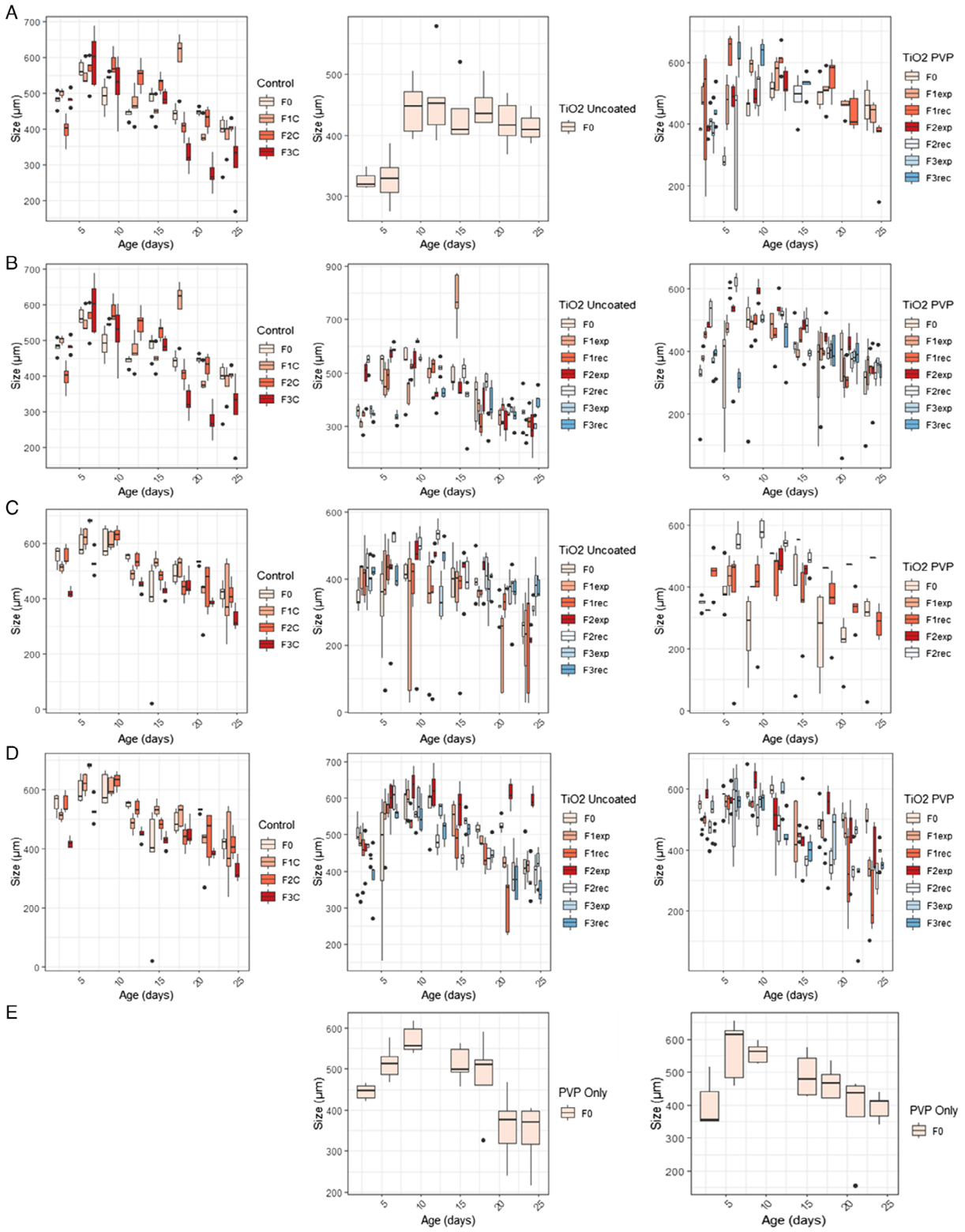

Figure 4. Box plots of the tail lengths compared with age over time for daphnids exposed to a) pristine TiO_2 NMs in the salt-only culturing media, b) aged TiO_2 NMs in the salt-only culturing media, c) pristine TiO_2 NMs in the artificial river water containing NOM, d) aged TiO_2 NMs in the artificial 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.

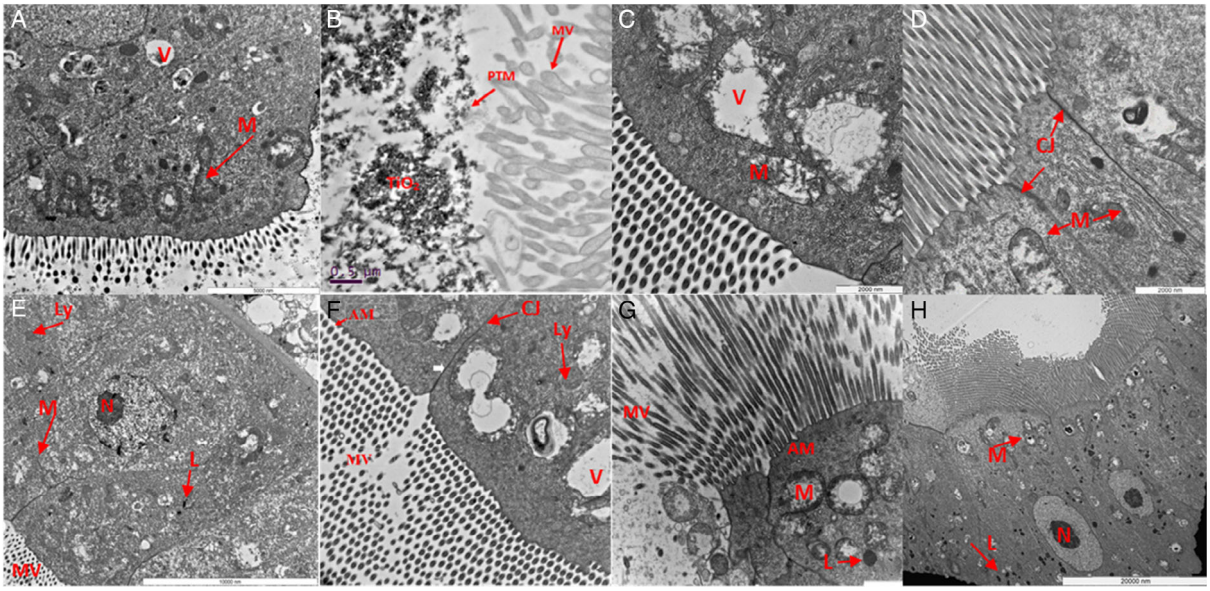

Figure 5. TEM images of daphnid gut cells: a) control daphnid gut, b) pristine uncoated TiO_2 NMs in culturing medium (evidence of lipofuscin and autophagy vacuoles), c) TiO_2 uncoated NMs aged in culturing medium, d) uncoated TiO_2 NMs aged in artificial river water, e) control daphnid gut, f) TiO_2 PVP NMs aged in culturing media, g) pristine PVP TiO_2 NMs in artificial river water, and h) aged PVP TiO_2 NMs in artificial river water. Key: mitochondria (M), cell junctions (CJ), nucleus (N), apical membrane (AM), microvilli (MV), peritrophic membrane (PTM), vacuole (V), lysosome (L), and secondary lysosomes (ly).

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.[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[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.[40] The accumulation of lipid deposits has been previously linked with ageing in multiple species,[43–46] as a response to exposure from environmental toxicants.[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.[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.[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[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.[51] These genes were significantly increased when exposed to the pristine uncoated and PVP TiO_2 NMs in both media conditions (Figures SI.4–6, Supporting Information).

Increased ROS is directly linked to reproductive inability due energy diversion to fight oxidative stress[30] in response to the uptake and bioaccumulation of TiO_2 NMs (Table SI.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.[6]

Prolonged oxidative stress and disruption of the melatonin may also be linked with ageing as a stress response[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.[52] Barata et al.[15] 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,[51] 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.[40] Studies by Bundschuh et al.[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.[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[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 SI.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.[55]

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[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.[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.[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.[26,59,60] Research by Colbourne et al.[26] and others[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.[62] By searching a gene orthologs database,[63] 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 (*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.[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 *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 *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.[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 *Daphnia* as part of a larger published study,[66] to identify the biological interactions of the NMs, including uptake and toxicity. Ellis and Lynch[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.[66] Proteins bound only to the aged NMs were indicative of significantly lower toxic responses, thus showing that the NM ecocorona 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 *Daphnia* culturing media results in very dramatic multigenerational 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 *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 *Daphnia* and humans suggests that *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.[72] Statistical significance of changes in gene expression were computed in RStudio, as follows: models were fit using lmfit, eBayes 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

Table 1. Orthologs: mapping genomics to functional data.

Gene	Group at metazoan level	*Homo sapiens*			*Daphnia magna*			Phyletic Profile	Link
		Ortholog	Aminoacids	inter pro	Ortholog	Amino acids	inter pro		
Glutathione s-transferase (GST)	510902 at 33208	GSTA4 (A0A024RD5 8) Glutathione S- transferase A4, isoform CRA_b	222	IPR004045 36249 03080 36282 10987 04046	A0A0N8A9D6_9 CRUS (A0A0N8 A9D6)	232	IPR036249 0 4045 36282 10987 0404 6	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_0,9796_0,9798_0,186990_0,9974_0,291302_0,109478_0,225400_0,59463_0,9402_0,132908_0,89399_0,9407_0,9606_0,35525_0&query=510902at33208
Dehydrogenase (NADH)	565519 at 33208	NDUFB9 (Q9Y6M9)	179	IPR033034 08011	A0A0P5EAV7_9 CRUS (A0A0P5 EAV7)	195	IPR033034	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_0,132908_0,89399_0,9407_0,9606_0,35525_0&query=565519at33208
β-Actin (act)	No matches	No matches	No matches	No matches	No matches	No matches	No matches	No matches	No matches
Catalase (cat)	172396 at 33208	CAT (P04040) Catalase	527	IPR020835 24711 1802 8 37060 11 614 24708 02226 1058 2	A0A0P5UME9_9 CRUS (A0A0P 5UME9)	529	IPR020835 2 4711 18028 37060 1161 4 24708 022 26 10582	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_0,132908_0,89399_0,9407_0,9606_0,35525_0&query=172396at33208
Metallothionein	No matches	No matches	No matches	No matches	No matches	No matches	No matches	No matches	No matches
DNA Polymerase	48271at33208	POLD1; Q308M6_HU MAN (M0R2B7)	1133	IPR012337 06133 0617 2 36397 06 134 23211 17964 2568	A0A0P5YTY3_9 CRUS (A0A0P5 YTY3) DNA polymerase	1122	IPR012337 0 6133 06172 36397 0613 4 23211 179 64 25687	942 genes in 448 species (out of 450) single copy in 62 species, multi-copy in 386 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_0,132908_0,89399_0,9407_0,9606_0,35525_0&query=48271at33208
18S ribosomal RNA	No matches	No matches	No matches	No matches	No matches	No matches	No matches	No matches	No matches
Hem-oxygenase-1	459843at33208	HMOX2 (A0A087WT 44) Heme oxygenase 2	370	IPR002051 16084 1605 3 18207	A0A0P6ICX8_9 CRUS (A0A0P6I CX8)	373	IPR002051 1 6053 16084	738 genes in 386 species (out of 450) single copy in 142 species, multi-copy in 244 species	https://www.orthodb.org/?query=HO1&level=33208&species=73337_0%2C29078_0%2C9793_0%2C9796_0%2C9798_0%2C186990_0%2C9974_0%2C291302_0%2C109478_0%2C225400_0%2C59463_0%2C9402_0%2C132908_0%2C89399_0%2C9407_0%2C9606_0%2C35525_0
		HMOX1 (P09601) Heme oxygenase 1	288	IPR002051 16084 1605 3 18207					

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^{-1} was used throughout the experiments.

Media and Representative Waters: Experiments were conducted in *Daphnia* high hardness combo medium (HH combo)[67] and in an artificial river water representative (Class V artificial water).[68] 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, France, the Balearic countries, and the Iberian Peninsula.[68] A description of the water combinations is shown in Table SI.11/Table SI1.11A–C, Supporting Information.

NM Characterization and Ageing: The NMs used in this study include anatase uncoated TiO_2 (9 ± 2 nm) and PVP-coated TiO_2 (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) TiO_2 NMs hydrodynamic diameters using a Malvern Nanosizer 5000 (Table SI.10, Supporting Information). Transmission electron microscopy (TEM) analysis of the TiO_2 NMs (Figure 5) was carried out using JEOL 1200EX 80 kV and JEOL 1400EX 80 kV microscopes. TiO_2 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 SI.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 pools of third brood Bham2 strain (genetically identical), which originated from the University of Reading[69] 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[67] and the artificial river water representative[68] 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 (20×3) 60 neonates (<24 h) were exposed to a range of concentrations of pristine uncoated TiO_2 and pristine TiO_2 PVP NMs over 24 and 48 h to assess their immobilization and survivability (Figure SI.8, Supporting Information). The *Daphnia* were not fed during these observations in accordance with the standard OECD test for acute immobilization 202.[70] An EC_{30} value was established for pilot studies at 45 mg L^{-1} (TiO_2 PVP) and 30 mg L^{-1} (TIO_2 uncoated). The pilot studies were conducted to identify any issues with study design and/or the NM concentrations used. The EC_{30} also showed high mortality in pilot studies with almost 100% mortality after 6 days exposure. For this reason, the EC value for the main studies was reduced further to the EC_5 values of 5 mg L^{-1} which also matched other reported concentration values used in *Daphnia* studies.[9,71] We understand there was a difference between using environmental concentrations and ECs, our justification for using ECs in this study was because regulation and environmental risks were assessed by characterizing the effects in biological receptors.

NM Exposure and Study Design: For the multigenerational studies (Figure SI.9, Supporting Information), each NM was exposed to 10 daphnids/250 mL in three replicates (total of 30 daphnids per exposure) to the F0 parent generation using the EC_5 concentration. The third broods (F1) from the F0 generation were split to produce a continuously paired exposure (F_{exp}) over four successive generations (F0, $F1_{exp}$, $F2_{exp}$, and $F3_{exp}$) using the EC_5 concentration and recovery (F_{rec}) generation for three generations (F_{rec}, $F2_{rec}$, and $F3_{rec}$). Daphnids, referred to as controls, were the unexposed Daphnia in each of the respective media that were run alongside the exposure experiments, and which were used as the reference life history measurements of healthy daphnids. The media (with or without NMs for the exposed and recovery experiments, respectively) were refreshed once a week, measurements of body size were taken every 3 days (in accordance with carapace shedding), and neonates were counted for the first five consecutive broods. The F1–3 generations were always made from the third brood of the previous generation (unless otherwise stated), with the neonates removed from the experiments within 24 hours of birth for the next generation to be set up.

Survival, Growth, and Reproduction: *Daphnia* were checked daily for survival, egg production, and neonate release. Measurements of body size were taken every 3 days in accordance with molting of the carapace (between days 3 and 24),[35] and neonate numbers were counted for the first five consecutive broods. Third brood neonates were used to set up the following generation and/or subsequently harvested for gene expression analysis. Body lengths were measured (days 3–24) measuring from the apex of the helmet to the base of the apical spine using a Nikon (Japan) stereomicroscope, model SMZ800 Digital Sight fitted with a D5-Fi2 camera using NIS Elements software.

Gut Tissue Sample Preparation for TEM: TEM cross sections of F0 generations after 7 days exposure were prepared by the Centre for Electron Microscopy at the University of Birmingham (UK). Briefly, whole *Daphnia* were euthanized 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 an ultramicrotome to cut 0.1 µm sections with a diamond knife. Images were visualized using JEOL 1200EX 80 kV and JEOL 1400EX 80 kV microscopes.

Total Body Burden and Metal Concentrations: For each experimental condition (i.e., exposure or recovery, pristine or aged NMs and for all water conditions), 10 *Daphna* juveniles (7 days old) were euthanized (using liquid nitrogen) and mechanically homogenized in 2% nitric acid (HNO_3) using a Precellys 24 instrument (Bertin Technologies) with 2 cycles of a 30s pulse at a 6000 pulse speed. Samples were then analyzed for their total Ti metal concentration (NM and ionic) using inductively coupled plasma mass spectrometry (ICP-MS) (Nexion 300X instrument, Perkin Elmer). Quantification of the dissolved Ti and TiO_2 NM concentrations in solutions was carried out after the first 7 days of exposure only, when the media were refreshed. Samples of the old media containing NMs were analyzed by single particle-ICP-MS (NexION 300D, Perkin Elmer).

Gene Expression: A total of 8 genes were selected for target-specific amplification using a mix of previously published primer sequences (Table SI.12, Supporting Information). Primer sequences were also checked using NCBI primer blast software (https://www.ncbi.nlm.nih.gov/gene) for the probability of amplifying nonspecific products.

RNA Extraction: RNA extraction was performed using 20 neonates (≤24 h old) which were euthanized using liquid nitrogen and stored in precellys tubes (containing ≈30 beads) at −80 °C until extraction. An Agencourt RNAdvance Tissue Kit (Beckman Coulter A47943) using paramagnetic bead-based technology was used for total RNA isolation and purification steps. First, purification of whole *Daphnia* samples (as per the manufacture's recommended protocol), required 20 µL of proteinase

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-GL). 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 10µL buffer, 2µL dNTP mix, and 2 µL enzyme mix. Water was added to bring the final volume to 20µL. 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 SI.13, Supporting Information).

Gene assay mixtures were created by mixing 1 µL of a 100 µM stock of each primer. Set A consisted of GST, 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 SI.13, Supporting Information. Following preamplification, the reaction mixtures (Table SI.14, Supporting Information) were cleaned up using Exonuclease I (Table SI.15, Supporting Information). To each sample, the following was added; 1.4 µL DNAse-free water, 0.2 µL ExoI reaction buffer, and 0.4 µL ExoI (NEB M0293L). Samples were mixed, and thermal cycled at the following conditions: (1) 37 °C for 30 min. (2) 80 °C for 15 mins. (3) Held 4 °C (until sample removed from cycler).

After ExoI 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 Preamp Gene Assay Master Mix was combined to produce the preamplification premix (Table SI.13, Supporting Information), which was individually separated into inlets of a 96-well sample plate. Each individual sample (measured in triplicate) containing cDNA was added into one-well inlet (1 inlet per sample). The preamplification was conducted on an Eppendorf Mastercycler 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 SI.15, Supporting Information).

qPCR: Gene expression was conducted using Flex Six Integrated Fluidic Circuit (IFC) Delta Gene Assay (72 × 72) in combination with a HX Prime (153×) system and a Fluidigm BioMark (Standard) Real-time PCR instrument, as per the manufactures recommended protocol. The purified Exo I-treated samples were mixed with the EvaGreen supermix and 2× loading assay (Table SI.16, Supporting Information) to produce the sample premix. The samples were then utilized for high-throughput 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 HX instrument (BioMark, Fluidigm). Samples and gene assays were loaded into the IFC and the "run script" on the HX 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 SI.17, Supporting Information.

Supporting Information

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

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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.

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ecotoxicology, epigenetic effects, nanoparticle transformations, reproductive effects

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