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Assessing the acute hazards of zinc oxide nanomaterials
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Abstract These studies were undertaken in order to propose and test new methods for the assessment of the acute hazard of ZnO nanoparticles (NPs) to the sediment dwelling oligochaete worm Lumbriculus variegatus. In order to support the developing nanotechnology sector, comprehensive studies must be conducted to assess the toxicity of nanomaterials (NMs) using environmentally relevant organisms. An important part of such studies will entail characterising and understanding the physicochemical properties of these NMs. In this study NMs were characterised using a range of techniques, in order to assess agglomeration/aggregation and dissolution. Toxicology studies included a behavioural assay and the measurement of oxidative stress. When considering the toxicology results from all experiments using L. variegatus within this paper ZnO NPs (0–10 mg/l) were found to cause acute toxicity in terms of behavioural response, but not to cause acute oxidative stress in terms of glutathione (GSH) depletion. It was also concluded that the behavioural assay and the GSH assay were both suitable techniques for assessing the acute hazard of NMs to L. variegatus.

Keywords Nanoparticles · Lumbriculus variegatus · Zinc oxide · Ecotoxicology

Introduction

Since the 1990s there has been a rapid increase in both the use of engineered nanomaterials (NMs) and the implementation of nanotechnologies. Nanomaterials are particulate matter with at least one dimension on the nanoscale (1–100 nm) whereas nanoparticles (NPs) have 3 dimensions in the nanoscale. Terrestrial and aquatic environments are thought to be where releases of NMs may end up since ultimately they both receive run off and wastewater inputs from both domestic and industrial sources and so the investigation into the effects of NMs on the environment (water column, sediment and soils) is of utmost importance. NMs can enter the environment through a number of routes including both intentional and unintentional releases. Unintentional releases can result from atmospheric emissions, solid or liquid waste streams from industrial facilities or transport accidents and spillages. NMs will also enter the environment through the manufacture and use of cosmetics, paints, sunscreens, fabrics, food packaging and medical products, in between others. Deliberate releases of NMs include those of environmental remediation activities (e.g. Fe NPs—Zhang and Elliot 2006) and the use of potentially NM-contaminated sewage sludge derived from wastewater treatment (Tourinho et al. 2012). The physicochemical properties of NMs will determine their bioavailability and toxicity (Powers et al. 2006). The physical and chemical properties of the receiving environment play a critical role in determining the fate of the NMs in the environment and this will affect their behaviour and toxicity (USEPA 2007). NMs are also prone to aggregation/agglomeration and sorption onto organic and inorganic material and so this will also change their fate in the environment (Holsapple et al. 2005).

Zinc oxide NMs have a variety of applications, such as optoelectronics, cosmetics, catalysts, ceramics, pigments...
and personal care products (Blinova et al. 2010) due to their unique properties and diverse nanostructures. The properties of ZnO, e.g. UV absorption and specific surface area, are improved at the nanoscale. Natural zinc NPs exist in ecosystems and play an important role in biogeochemical processes as zinc is an essential micronutrient (Wigginton et al. 2007), however the potential impact of engineered ZnO NMs on the environment has yet to be fully evaluated. The findings of a literature review for ZnO NMs suggest they can be both toxic and non-toxic in vitro and in many environmental studies. The range of ZnO NM concentrations that are considered toxic vary across these studies and the results are species specific. Some environmental studies found no or little difference between the toxicity of micro- and nanoscale ZnO particles (Aruoja et al. 2009; Zhu et al. 2008), some found that ZnO NMs had less of a detrimental effect than zinc ions (Hooper et al. 2011), some found no effect (Mortimer et al. 2008; Adams et al. 2006) whereas others found that development, reproduction and survival were affected after exposure to ZnO NMs (Blinova et al. 2010; Heinlaan et al. 2008; Huang et al. 2008). Some of the ZnO NM studies which have used crustaceans (Blinova et al. 2010), protozoa (Blinova et al. 2010) and algae (Franklin et al. 2007; Aruoja et al. 2009) as test species, have stated that the toxicity observed in their studies was down to ions rather than NMs. Some studies, however, in human toxicology (cell lines; Xia et al. 2008), mammalian toxicology (adult mice; Wang et al. 2008) and ecotoxicology (crustaceans and bacteria; Heinlaan et al. 2008) have also shown nano-specific toxicity. Generalisations cannot be made to state that ZnO NM toxicity is ultimately solely down to zinc ion release (Wong et al. 2010) and the dissolution of NMs may result in an additional layer of complexity when examining their potential toxicity (Xia et al. 2008).

There appears to be a significant gap in the literature dealing with the effects of ZnO NMs on sediment dwelling organisms such as Lumbriculus variegatus. L. variegatus have been widely used to test potentially harmful substances since the early 1970s (e.g. Alekseev and Uspenskaya 1974). Since then, L. variegatus have been the most widely used oligochaete to test the toxicity of a number of chemical substances, particularly its status as standard OECD test species. This work has included metals (e.g. Schubauer-Berigan et al. 1993; Chapman et al. 1999), metal oxides (e.g. Stanley et al. 2010), various chemicals (e.g. ammonia: Schubauer-Berigan et al. 1995; PCB: Fisher et al. 1999; PAHs: Monson et al. 1995; chlorophenols: Nikkilä et al. 2003) and more recently NMs (e.g. Petersen et al. 2008; Stanley et al. 2010; Pakarinen et al. 2011). L. variegatus is an OECD test species particularly selected for their specific characteristics, such as being widely available, being easily cultured and having a known chemical exposure history. Advantages of their use include their widespread distribution, availability of adequate amount of tissue for chemical analysis, high tolerance for a wide variety of exposure scenarios, can be exposed via all important routes of concern, and are suitable for both long- and short-term exposures (Brunson et al. 1998). L. variegatus feeding and locomotion behaviour are relevant in potential exposures to NMs given that they are deposit feeders which do not selectively ingest specific particles and swim/burrow within sediments or live at the interface water–sediment (Ding et al. 2001). In addition, they have been used in the testing of a wide range of chemicals substances which means that there is a large data set that can be used for comparison purposes.

In this study two endpoints were selected to test the potential toxicity of ZnO NPs, a behavioural endpoint and an oxidative stress endpoint. The behavioural endpoint was chosen as the locomotive behaviours of L. variegatus are significant indicators of the physical, chemical and biological properties of sediments and water bodies (Drewes 1997). The behaviours of L. variegatus are well documented and so are very useful as a biomarker for toxicity. Drewes (1999) hypothesised that since L. variegatus tend to feed en masse, when a negative stimulus occurs the worms will execute body reversal behaviours which rapidly disaggregate the assembled mass and this can confuse or startle a predator. The body reversal behaviour positions the head of the worm away from the predator or negative stimulus and swimming behaviour allows for rapid, short distance retreats from threats, where the worm does not have the benefit of protection or traction (Drewes 1999). A number of papers have used this behaviour of L. variegatus. Some authors have employed electro-physical techniques to test the conductivity of neural pathways (Rogge and Drewes 1993; Lesiuk and Drewes 2001; Sardo and Soares 2010), while others have used touch-evoked response techniques (Lesiuk and Drewes 2001; Ding et al. 2001; O’Gara et al. 2004, 2006). This behavioural endpoint has not been used yet to assess toxicity of NMs and therefore it is important to evaluate its use in this context. The oxidative endpoint was chosen as oxidative stress is known to play a major role in the toxicity mechanism of ZnO NMs (e.g. Johnston et al. 2012). The method employed in this study was adapted from a cell line protocol and has not been used with L. variegatus previously.

This research was conducted in order to address a gap in the literature on the ecotoxicity of ZnO NPs, particularly in bentic systems and using a novel method. Hazard information on the toxic effects of ZnO NPs on L. variegatus is limited. The aim of this study was to investigate the effects of ZnO NPs and larger (‘bulk’) particles on the behaviour of L. variegatus and whether these particles would induce oxidative stress during acute 96 h toxicity tests, with and
without the addition of dissolved organic matter. This aim was achieved using a well-documented behavioural assay and the measurement of glutathione (GSH) fluctuations in the tissues of the worms, both of which are sublethal assays that have not been used with this species before in the investigation of NM toxicity.

Materials and methods

Characterisation

The United States Environmental Protection Agency Hard Water (EPA HW) medium was prepared according to the published method (USEPA 2002). The ZnO NPs (“Zinc oxide Nanogard”) and bulk (“Zinc oxide ACS 99 % min.”) were sourced from Alfa Aesar, Germany.

Dynamic light scattering

Dynamic light scattering (DLS) was used to examine the hydrodynamic diameter (HD) and zeta potential (ZP) of the particles immediately after suspensions were made, using Malvern recommended minimum concentrations (Malvern User Manual 2008), and also across a 96 h period using a wide range of concentrations considered to incorporate environmentally relevant concentrations. From a sonicated stock suspension (30 min at 20 °C) of 0.5 mg/ml NPs in filtered EPA HW suspensions of 0.1 and 0.01 mg/ml ZnO NP (immediate HD/ZP measurement) and from a sonicated stock suspension (30 min at 20 °C) of 0.5 mg/ml NPs in unfiltered EPA HW, 0–10 mg/l ZnO NP (96 h study) suspensions of different concentrations were made. Suwannee River humic acid (HA) (International Humic Substances Society, USA) was added to the stock suspensions as required, after sonication. Stock suspensions of humic acid at 5 mg/l were prepared by adding 5 mg humic acid to 1 l of EPA HW medium and stirring (with a magnetic stirrer) for 15 min. The humic acid was added after sonication as sonication can cause the acid to change via oxidation, pyrolysis or mechanical degradation (Naddeo et al. 2007). For the initial size study the HD and ZP was read immediately and for the 96 h study both HD and ZP readings were taken at 0, 24, 48, 72 and 96 h. Samples were taken from the top 1–2 cm of suspensions at each time point using a 2 ml syringe, with care not to disturb the suspension to prevent the re-suspension of any settled particles. This method was repeated with the bulk particles of ZnO.

Transmission electron microscopy

Dispersions of 0.1 and 1 mg/l ZnO NPs were prepared in milli-Q water, EPA HW medium and EPA HW medium with 5 mg/l humic acid. Freshly cleaved sheets of muscovite (around 50 × 50 mm) and 300 mesh Formvar/carbon coated Cu TEM grids were placed in flat bottomed ultracentrifuge tubes, 10 ml of the ZnO-particle suspensions were added to the tubes, and the NPs were deposited onto the muscovite and TEM-grids by 30,000 rpm ultracentrifugation (Beckman L-75) during 60 min. The muscovite and TEM-grids were cleaned from salt and unadsorbed particles by gentle immersion in milli-Q water, and were thereafter dried under cover overnight. The same method was used to prepare samples after worm exposures, but 2.5 and 10 mg/l ZnO particle suspensions and 300 mesh carbon coated Cu grids were used.

Dissolution

The production of soluble Zn (<1 kDa species e.g. Zn²⁺) from dissolution of ZnO particles was assessed using dialysis. ZnO particle suspensions (10 mg/l, 2 l each) were prepared in EPA HW medium and EPA HW medium with 5 mg/l humic acid. Dialysis bags of regenerated cellulose (SnakeSkin, 1 kDa molecular weight cut off) were filled with 100 ml of medium without ZnO particles, closed with specially designed clips (SnakeSkin, 50 mm) and immersed in the suspensions with ZnO particles. Two replicate bags were used for each ZnO particle suspension. Samples of 5 ml were withdrawn from the bags on 17 occasions over a period of 33 days and were immediately acidified by adding 100 μl of concentrated ultrapure HNO₃ to each 5 ml sample. The samples were diluted 100 times with milli-Q water and again acidified to 1 % with ultrapure HNO₃, before analysis of Zn concentrations using ICP-MS (Agilent 7500ce with collision cell).

Surface area

The total surface area of ZnO NPs and bulk particles was assessed using the Brunner–Emmett–Teller (BET) gas adsorption method. Amounts of 0.1–0.3 g of ZnO NPs and bulk particles were outgassed under vacuum at 200 °C in BET-vials for 12 h. Following this the samples were weighed precisely (±1 mg precision) and analysed using a SA 3100 Surface area analyzer (Beckman Coulter). The temperature was controlled by immersing the vials in liquid nitrogen. The gas was used to determine the free space in the vials and adsorption isotherm of N₂ gas to determine the total surface area of the sample (0.05–0.2 relative pressure range). The correlation coefficients of the adsorption isotherms were always ≥0.999. The BET surface area was calculated by dividing the total surface area by the sample mass.
Toxicology

Toxicology studies were undertaken in several steps; firstly a pilot study investigated the behavioural impact of feeding the worms prior to exposing them to ZnO NPs, secondly a full study investigated the behavioural impact of ZnO NPs and bulk particles on *L. variegatus* and finally an oxidative stress study. The cultures of *L. variegatus* were held in EPA HW medium (USEPA 2002). *L. variegatus* cultures were maintained according to a protocol that was adapted from the OECD Guideline 225 (2007). Twelve days prior to testing, worms were artificially fragmented according to the OECD Guideline 225 (2007). Posterior parts of the worms were transferred to a 1 l beaker containing EPA HW medium and shredded, unbleached, prewashed and autoclaved paper towels. The beakers were then placed in an incubator (20 ± 1 °C) covered with parafilm and with aeration provided via a pump that circulated filtered air. For all studies, except the pilot study, at day 7 of regeneration the beaker of worms were fed 0.35 g pre-powdered aquaria tropical flake food (“TetraMin”, Tetra, Germany). After regeneration, worms that were actively swimming or crawling upon gentle stimulus were used in testing. Full details of these tests are provided below.

**Pilot study: assessing the effect of particle exposure on fed versus unfed worms**

Twelve days prior to testing worms were artificially fragmented according to a protocol adapted from the OECD Guideline 225 (2007). Large worms, that did not show signs of natural fragmentation, were chosen and transferred from the culture aquarium to a glass petri dish which contained a small amount of EPA HW medium. The worms were dissected along the median region using a scalpel. Care was taken that the posterior ends were of similar size. Posterior parts of the worms were transferred to a 1 l beaker containing EPA HW medium and shredded, unbleached, prewashed and autoclaved paper towels. The beakers were then placed in an incubator (at 20 ± 1 °C) covered with parafilm and with aeration. At day 7 of regeneration one beaker of worms were fed 0.35 g pre-powdered aquaria tropical flake food (“TetraMin”, Tetra, Germany). After regeneration, worms that were actively swimming or crawling upon gentle stimulus were used in testing. A further beaker of synchronised worms were kept under the same incubation conditions but were not fed during the synchronisation. Stock suspensions of NPs (0.5 mg/ml) in EPA HW medium were sonicated in a sonication bath (Ultrawave Q series, 400 W) for 30 min at 20 °C. Suspensions of 1.25, 2.5, 5, and 10 mg/l ZnO NP were made up in EPA HW medium from the sonicated stock. Following this, 20 ml of the required concentration was added to each glass vial followed by one worm per vial. There were a total of ten worms per concentration. The vials were then moved to the 20 ± 2 °C controlled temperature room and arranged at random under a light regime of 16:8 h light:dark for 96 h. Upon completion of the 96 h period organisms were checked for mortality. Following this, the behavioural assay was performed (Drewes 1999). Worms were gently removed from the vials using a pastette and transferred to a glass petri dish along with some liquid from the vial to allow the free movement of the worm. Touch stimuli were delivered via the tip of a pastette. The anterior portion of the worm was gently touched to evoke a behavioural response. The flexibility of the pastette tip ensured the worm was not injured during stimulation. The worm was touch stimulated a total of ten times and the behavioural response was recorded in terms of the total number of body reversal movements that occurred. To avoid observer bias only completed 180° turns were accepted to be counted. The results of this study were used to determine whether worms in subsequent tests should be fed during regeneration.

**Nanoparticle and bulk particle studies**

Twelve days prior to testing, worms were artificially fragmented according to the protocol, as described Sect. Pilot study: assessing the effect of particle exposure on fed versus unfed worms. The same concentrations of bulk particles and NPs were used as described for the feeding pilot study, both with and without the addition of 5 mg/l humic acid. Humic acid was added to the dilutions after sonication. There were a total of ten worms per concentration and the study was replicated three times. Upon completion of the 96 h period the vials were removed from incubation and assessed for mortality and behavioural response according to Drewes (1999). Positive controls were set up using CuSO₄ at 0.2 and 0.4 μM and ZnSO₄ at 2.5 and 10 mg/l, in the presence and absence of 5 mg/l humic acid. Again the experiment was conducted with ten worms per concentration and the study was performed three times.

**Oxidative stress study**

Oxidative stress was assessed using the “GSH-Glo Glutathione Assay” kit from Promega, U.K. Worms were removed from culture 12 days prior to testing and were physiologically synchronised as described in Sect. Pilot study: assessing the effect of particle exposure on fed versus unfed worms. Worms were then exposed in 20 ml to 0, 2.5 and 10 mg/l ZnO NPs and bulk particles (±5 mg/l humic acid) as described above in the previous studies. At
0, 4, 8, 24, 48, 72 and 96 h 5 worms were removed from each concentration. Worms were transferred using a metal pick to minimise the transfer of any liquid into plastic vials containing 1 ml of PBS + 2 mM EDTA and weighed. After weighing, the worms and liquid were transferred into a round bottom tube using a plastic pastette. They were homogenised using a hand held homogeniser (PowerGen 125, Fisher Scientific) until no visible remains were left in the tube. Following homogenisation the suspension was transferred into a 15 ml falcon tubes using a pipette. The tubes were centrifuged for 10 min at 800 rpm (136 g) and 4 °C. The Promega “GSH-Glo Glutathione Assay” kit protocol was followed. This study was replicated three times.

Data analyses

All results were analysed statistically using PASW software 17.0. For the DLS studies the significance value \( P \) was set at 0.05 for all studies. A Kolmogorov–Smirnov test (KS) was performed to check for normality. Data were found to be noncompliant with requirements of parametric tests and so were transformed via log transformation. After transformation data were found to be compliant and a general linear model (GLM) was performed to analyse the data. Tukey tests were performed in order to ascertain where these differences lay. For the pilot study data were normalised to the control behavioural response by dividing the percent behavioural response by the mean of each test and multiplying by 100. Data were then tested using a KS test, and a Scheirer-Ray-Hare test (SRH), followed by Mann–Whitney tests, with a Bonferroni correction. For the NP/bulk behavioural study data were again normalised to the control behavioural response. Data were then tested using a KS test, a 2 way ANOVA with post hoc Tukey tests (parametric) or a SRH test with Mann–Whitney tests and a Bonferroni correction (non-parametric), depending on the data distribution. Finally, in the oxidative stress study, data were normalised to the control to show % GSH per mg of tissue with respect to the control worms. Data were tested for normality using a KS Test and found not to comply with parametric requirements. Three SRH tests were then performed.

Results

Characterisation

In the 96 h DLS study both the NP and bulk ZnO PdI (polydispersity index) results obtained for a large proportion of the samples tested were not reliable. At 0 h the most stable suspension was milli-Q water with humic acid, all other suspensions were unstable. The largest HD was seen in the EPA HW with humic acid and milli-Q water suspensions at 10 mg/l. At 24 h a high level of agglomeration was seen in the EPA HW suspension across all concentrations and this was reflected in the ZP results which were all greater than −30 mV (i.e. between 0 and −30 mV). At 48 h the EPA HW suspension had the highest level of agglomeration. At 72 h the EPA HW suspensions were all unstable. At 96 h no suspension appeared different from the previous time point of 72 h. In the 96 h bulk ZnO study, at 0 h the EPA HW suspension had a HD that was larger than that of the milli-Q water and milli-Q water with HA suspensions. The ZP of the milli-Q water with humic acid suspensions indicated that they were stable since they were less than −40 mV. At 24 h milli-Q water with humic acid were seen to be the most stable across the concentrations (ZP less than −30 mV). At 48 h all suspensions were unstable. At 72 h EPA HW with humic acid suspension was stable at 10 mg/l. The milli-Q water suspensions were stable at 72 h at 1.25, 2.5 and 5 mg/l. At 96 h only 5 mg/l was seen to be stable. The milli-Q water with humic acid suspensions were stable at 2.5 and 5 mg/l.

The second DLS study examined the HD and ZP of ZnO particles at the minimum recommended concentration. Figure 1 shows the HD and ZP of ZnO NPs and bulk particles in filtered EPA HW medium and filtered milli-Q water, both in the presence and absence of 5 mg/l humic acid. Not all of the results obtained for the samples tested were reliable, i.e. did not meet the standards as laid out in the Malvern Zetasizer user manual (2008). The media type (GLM; \( P = 0.011 \)) and the particle type (GLM; \( P = 0.008 \)) were found to lead to significantly different results for HD. Within the media factor, the EPA HW samples were found to have significantly higher HD than milli-Q water samples. The bulk ZnO HD was significantly greater than NP ZnO HD. Interactions factors were also tested for significance in this model. It was found that the interaction between concentration, particle and media (GLM; \( P = 0.048 \)) was found to be significant for HD measurements. This indicates that the effects of the factors were not additive, i.e. the groups observations assigned to one factor do not respond in the same way as those assigned to another factor (Dytham 2011). No factor or interaction was found to be significantly different in the examination of ZP data.

The TEM micrographs of ZnO suspensions in EPA HW medium (Fig. 2) showed agglomerated or aggregated particles that in total were several hundred nm in diameter, appearing to be composed of smaller nanoparticles. The form factor and roundness of the agglomerates of both nano and bulk particles in EPA HW medium were low (average form factor around 0.2, average roundness around 0.4–0.5) showing that the 2-dimensional shapes of the
agglomerates were far from circular. The convexity and solidity of the agglomerates of both nano- and bulk-particles were also rather low (average convexity around 0.6, average solidity around 0.7), showing that the agglomerates were highly ‘branched’ and concave. The TEM micrographs from samples of EPA HW medium with humic acid showed particles with varying structure and morphology that looked rather different from the particles in EPA HW medium without humic acid. However, no significant differences in size, form factor, roundness, convexity or solidity could be shown between agglomerates in EPA HW medium with and without humic acid.

**Fig. 1** The hydrodynamic diameter and zeta potential of ZnO nanoparticles in filtered Environmental Protection Agency Hard Water (EPA HW) medium and filtered deionised water, both in the presence and absence of 5 mg/l Suwannee river humic acid (Data represent mean ± SE; n = 3) (**a**), the hydrodynamic diameter and zeta potential of ZnO bulk particles in filtered EPA HW medium and filtered deionised water, both in the presence and absence of 5 mg/l Suwannee river humic acid (Data represent mean ± SE; n = 3) (**b**).

**Fig. 2** TEM micrographs of ZnO nanoparticles and ZnO bulk particles in Environmental Protection Agency Hard Water (EPA HW) medium and EPA HW medium with humic acid, before and after a 96 h exposure to worms.
Assessing the acute hazards of zinc oxide nanomaterials to *Lumbricus variegatus*

The numbers of agglomerates used for the $T$ tests (6–42) were probably too low for the tests to be meaningful. The TEM micrographs from the EPA HW medium (where worms had been cultured during 10 days without ZnO particles) showed large agglomerates of small elongated (up to about 50 nm in length) particles. It is possible that this particulate matter was produced by the worms, or that they were formed by precipitation from the salts composing the EPA HW medium. Some micrographs showed a diffuse ‘matrix’ surrounding the particles, probably organic matter produced by the worms or by bacteria in the exposures. The micrographs of control samples of EPA HW with humic acid sometimes showed small (about 10 nm) particles, probably representing the macromolecules of humic acid. Some of the micrographs from the exposures with ZnO particles showed the same type of small elongated particles that were found in the control samples. However, the micrographs from the exposures of both ZnO NPs and bulk particles were dominated by larger (about 10 nm to a few 100 nm) particles with oval, rounded or angular shapes that occurred both as discrete particles and as part of larger agglomerates. Some of those particles were surrounded by the diffuse ‘matrix’, presumably organic matter. The agglomerates of ZnO-particles in the exposures looked rather different from the agglomerates of ‘raw’ ZnO particles, as they did not appear to be ‘fused’ or linked together in the same way, but appeared to be composed of distinct particles. However, in most cases the size and shape factors of the agglomerates in the different samples could not be shown to be significantly different (based on $T$ test with 5 % confidence level), neither when the samples of ‘raw’ particles were compared with the exposure media, nor when the different exposure media with different concentrations of ZnO NPs (2.5 and 10 mg/l) and exposure media with and without humic acid were compared. As previously stated, the number of agglomerates found on the TEM-micrographs was probably too low to carry out a meaningful $T$ test.

The containers outside the dialysis bags contained 10 mg/l ZnO particles, equivalent to 8.03 mg/l Zn. The concentrations of Zn inside all the dialysis bags increased rapidly immediately after the start of the experiments (Fig. 3). Although the results indicate some variability, it is clear that the increase in Zn concentrations tended to plateau and the concentrations usually stabilised after around 200 h. The average Zn concentrations (Fig. 3) were 0.9 ± 0.15 mg/l for NPs in EPA HW medium, 0.8 ± 0.12 mg/l for NPs in EPA HW medium with humic acid, 0.6 ± 0.13 mg/l for bulk particles in EPA HW medium and 0.6 ± 0.23 mg/l for bulk particles in EPA HW medium with humic acid. The NPs showed a greater solubility than bulk particles in EPA HW medium (based on $T$ test at 5 % significance level). There was no significant difference between the solubility of NPs compared with NPs with humic acid, between bulk particles and bulk particles with humic acid and between NP with humic acid and bulk particles with humic acid.

The BET surface area was 12.06 ± 0.023 m$^2$/g for ZnO NPs, and 5.8 ± 0.37 m$^2$/g for the ZnO-bulk particles. The average values and standard deviations are based on 3 replicate measurements each.

**Toxicology**

The toxicology studies were carried out in several steps; a pilot study investigating the behavioural impact of feeding during synchronisation, followed by an investigation of the effects of ZnO NP and bulk particles on behaviour, oxidative stress study and tissue histology.

Figure 4 depicts the data obtained from the pilot study which shows the behavioural response of fed or unfed worms that were exposed to suspensions of varying concentrations of ZnO NPs (0–10 mg/l). These worms were either fed at day 7 of their 12 day synchronisation period or not fed at all during the synchronisation period. When comparing fed and not fed worms across each concentration, the behaviour of the control (EPA HW medium alone) worms and worms exposed to 2.5 and 10 mg/l ZnO NPs were not found to be significantly different from each other. The behaviour of fed worms exposed to 1.25 and 5 mg/l ZnO NPs and starved worms exposed to 1.25 and 5 mg/l ZnO NPs were found to be significantly different from each other ($P < 0.001$) with the fed worms having a less inhibited behavioural response than worms that were not fed. This study suggested that the fed worms were more likely to turn in response to a stimulus than worms that were not fed. It was also noted that worms in the fed study had stronger colouring and were more active than non-fed worms. In the subsequent studies fed worms were therefore used.

The behavioural response data of worms exposed to ZnO NPs and NPs with humic acid are shown in Fig. 5. Data were normalised to the control behavioural response by dividing the percent behavioural response by the mean of each test and multiplying by 100 and this is why the behavioural response is greater than 100 % in some instances. The concentration of NPs was found to have a significant negative effect on the behaviour of the worms as the concentration increased (SRH; $F = 6.786$, d.f. = 4, $P < 0.001$). This was further confirmed in that the control (EPA HW medium alone) was found to be significantly different from all other ZnO NP concentrations ($P < 0.001$) indicating that the NPs inhibited the behavioural response at 96 h. In addition, worms exposed to 1.25 and 2.5 mg/l ZnO NPs had a significantly less
inhibited behavioural response than worms exposed to 10 mg/l ZnO NPs (P < 0.01).

The addition of 5 mg/l humic acid had a significant positive effect on the behaviour of the worms compared to the worms that were treated with particles only (SRH; F = 72.347, d.f. = 1, P < 0.001) in that humic acid reduced the NP impact on behaviour. There was no significant difference observed in the behaviour of worms exposed to the control with humic acid and 1.25, 2.5, 5 and 10 mg/l ZnO NP with 5 mg/l humic acid suggesting that the dose dependent inhibition of the behavioural response was lost in the presence of humic acid. This was confirmed in that all worms exposed to concentrations of NPs without humic acid were found to have significantly greater inhibition of behaviour than worms which were exposed to NPs mixed with 5 mg/l humic acid (P < 0.001).

In the absence of humic acid, no concentration dependent impact of bulk particles on the behaviour of the worms was observed. However, worms exposed to 5 mg/l bulk particles with 5 mg/l humic acid were found to have a

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**Fig. 3** Variations in Zn concentration with time inside the different dialysis bags immersed in 10 mg/l suspensions of ZnO particles in Environmental Protection Agency Hard Water (EPA HW) medium, with and without humic acid (HA); a ZnO NPs suspended in EPA HW medium; b ZnO NPs suspended in EPA HW medium with HA; c ZnO bulk particles in EPA HW medium; d ZnO bulk particles in EPA HW medium with HA.

**Fig. 4** Behavioural response (i.e. the number of turns of the worm in response to a tactile stimulus) of worms exposed to various concentrations of ZnO nanoparticles (NPs). Worms were fed at day 7 of their synchronisation period and not fed for the 12 day synchronisation period. (Data represent medians (with interquartile range), n = 3; comparisons made on graph are between each concentration in fed versus non fed worms—a shared letter between 2 columns indicates no significant difference)
significantly inhibited behavioural response when compared to humic acid alone (SRH; P < 0.01). In addition, only 5 mg/l ZnO bulk particle in the absence of humic acid was significantly different from the bulk ZnO/humic acid counterpart (SRH; P < 0.001).

The NP and bulk data (without humic acid) were compared to each other to investigate whether particle size influenced the behavioural response of the worms. Worms exposed to 1.25, 2.5, 5 and 10 mg/l NP ZnO were found to have a significantly inhibited behavioural response when compared to their bulk counterparts (P < 0.001), suggesting that smaller ZnO particles were more potent. In addition, worms exposed to 5 mg/l (P < 0.001) NP with humic acid were found to have a significantly less inhibited response than worms exposed 5 mg/l bulk with humic acid. This suggests that at higher concentrations and in the presence of humic acid, the bulk particles are potentially more effective at inhibiting the behavioural response than the smaller NPs.

Positive controls were employed using CuSO₄ and ZnSO₄. The behaviour of the worms in CuSO₄ with and without humic acid data were compared (Fig. 5). As the concentration increased the behaviour of the worms was increasingly inhibited (SRH test; F = 31.921, d.f. = 2, P < 0.001). The addition of humic acid and the interaction between humic acid and concentration were not found to be significant factors. The ZnSO₄ and ZnSO₄ with humic acid data were compared to each other to investigate whether there were any significant differences in the behaviour of the worms. The behaviour of the worms exposed to 10 mg/l ZnSO₄ was significantly inhibited when compared to the control group (2 way ANOVA; P < 0.001). The behaviour...
of worms exposed to 10 mg/l ZnSO₄ was also significantly more inhibited than those exposed to 2.5 mg/l ZnSO₄ (2 way ANOVA; P < 0.05). The addition of humic acid improved the behaviour of the worms but only at the highest concentration of 10 mg/l ZnSO₄ (2 way ANOVA; P < 0.01).

**Discussion**

The present study aimed to investigate the potential impact of ZnO NPs on *L. variegatus*. The ZnO NPs were found to induce acute detrimental impacts on the behaviour of *L. variegatus* after 96 h; however they did not induce an acute oxidative response in the worms according to the GSH assay. This suggests that oxidative stress was not involved in mediating the behavioural response.

After a 96 h exposure ZnO NPs induced a negative behavioural response in the worms between 1.25 and 10 mg/l, in contrast, bulk ZnO particle did not. The addition of humic acid to the ZnO NPs prevented the impact of the particles on the behaviour of the worms, while the addition of humic acid to the bulk particles enhanced the effect on behaviour at 5 mg/l. An impairment of the escape behaviour in these worms would significantly impact their survival in the wild. Locomotor behaviours are integrally linked with the worm’s ability to forage, sexually reproduce, avoid predators, disperse quickly and react to general environmental cues (Drewes 1997).

The difference between the NP and bulk results may be attributed to a number of the physicochemical characteristics of the particles, such as surface area, solubility, size and stability. The BET study found that the surface area of the NPs was significantly greater than that of the ZnO bulk particles. Many studies indicate that the greater the surface area of a particle, the greater the reactivity of that particle and in turn the greater the potential for that particle to have an increased toxicity (e.g. Brown et al. 2001; Duffin et al. 2002; Li et al. 2006; Pal et al. 2007; Navarro et al. 2008). The solubility of ZnO may have also played a role. The solubility of the NPs was found to be significantly greater than that of the bulk particles during the dissolution experiments. This would mean that the worms were exposed to more Zn²⁺ ions in the NP exposures, compared to the bulk particle exposures, giving rise to potential ion mediated toxicity. However, they may also have been exposed to insoluble NP/aggglomerates as the solubility equilibrium was reached at 0.9 ± 0.15 (SE) mg/l and the exposure concentrations in this experiment ranged from 1.25 to 10 mg/l ZnO. This is something that is supported by a number of other studies (Heinlaan et al. 2008; Xia et al. 2008; Blinova et al. 2010; Wong et al. 2010). The dissolution of NPs may result in an additional layer of complexity when examining their potential toxicity (Xia et al. 2008). The potential for NMs and divalent metal cations to interact synergistically has also been demonstrated (Wilson et al. 2002, 2007).

The species being used for testing is also an important consideration as the feeding behaviour of *L. variegatus* may have also played a role in the observed difference in toxicity between NP and bulk particles. The agglomerates that the worms may have come in contact with within the bulk study may potentially have been too large for them to ingest. Within the DLS study the HD of bulk ZnO particles were found to be significantly larger than ZnO NPs. Finally, the introduction of organic matter had an impact on the effects of the ZnO particles.

The addition of humic acid mitigated the negative effect of the ZnO NPs on the behavioural response. TEM images taken of ZnO NPs dispersed with 5 mg/l humic acid indicated that the particles agglomerated and were also surrounded by what appeared to be an organic matrix. This matrix may have increased the stability of the dispersion (indicated by DLS data) and kept the agglomerates in suspension throughout the exposure. As the worms sat at the base of the vial during exposure there was therefore less potential for them to have come into contact with the humic acid dispersed particles compared to NP exposures alone. Other studies have also noted that humic acids increase the stability of NP suspensions (Chen and Elim-enech 2007; Handy et al. 2008; Zhang et al. 2009). When examining the DLS data, humic acid did not appear to affect the HD of the bulk particles, however their stability was increased. It would be expected that since humic acid should have increased dispersion and kept the bulk particles in suspension, the worms would also have come into less contact with the particles. However, at the highest particle concentration with humic acid, a decrease in the behavioural response was observed. It is possible that large agglomerates may have formed and deposited onto the area where the worm was placed, or that decreased agglomerate size in the presence of humic acid allowed sufficient particles to fall into a size range that could be ingested by the worms.

Both ZnO NPs and bulk particles did not cause oxidative stress as measured by GSH depletion in *L. variegatus* after.
a 96 h exposure to ZnO NPs and bulk particles. A number of reasons may be put forward in order to potentially explain why an oxidative stress reaction was not observed. It has been suggested that pre-exposure to low intensity oxidative stress may enhance an organism’s tolerance to a subsequent higher intensity oxidative stress (Lushchak 2011). The culture conditions used for the worms may have induced a low intensity oxidative stress. It is also possible that the ZnO exposure concentrations were too low to produce enough ROS to induce lasting oxidative stress and the worms were able to adapt sufficiently quickly to prevent detection of a response. This is supported by the observation in studies using aquatic organisms that changes in antioxidant mechanisms are transient and variable for different species and chemicals (Livingstone 2001; Barata et al. 2005; Cochón et al. 2007). Finally, it has been suggested that a number of biomarkers for oxidative stress should be used when investigating oxidative stress in aquatic organisms (Lushchak 2011); therefore measurement of GSH alone is not sufficient to conclude a lack of oxidative stress. The results of this study are not in line with other ZnO NP toxicity studies using several human cell lines (e.g. De Berardis et al. 2010; Huang et al. 2010; Fukui et al. 2012; Sharma et al. 2012) or environmental toxicity studies (e.g. Xiong et al. 2011; Ali et al. 2012; Hao and Chen 2012) where oxidative stress has been measured. However, in Hao et al. (2009) it was observed that after exposure to ZnO NPs the enzymatic and non-enzymatic antioxidant defences of carp (Cyprinus carpio) acted in different ways in different organs. They suggested that this was proof that in order to fully evaluate oxidative stress, biomarkers it is necessary to assess a number of markers in the organism using different tissues. For _L. variegatus_ measurement of oxidative stress markers in different tissues will be difficult due to the small size of the organism.

**Concluding remarks**

Oligochaetes are important constituents of freshwater ecosystems as they feed on subsurface sediments, processing and recycling deposited material (Leppänen and Kukkonen 1998). The sediment reworking and recycling by oligochaetes have been documented to cause significant changes to the biological, chemical and physical characteristics of sediments and overlying waters (McCcall and Tevesz 1982) and so if the behaviour of the worms is inhibited by a toxic substance it can have a profound effect on ecosystem functioning. These studies were able to demonstrate that ZnO NPs can have toxic effects on _L. variegatus_, however this toxicity is endpoint specific and considering toxicity in environmentally relevant dispersions is key to understanding their potential hazard and risk. The behavioural endpoint was proven to be useful in assessing the toxicity of NPs in this species as a NP specific effect was observed; however, in order to assess oxidative stress in this worm, further endpoints need to be investigated to examine multiple indicators of oxidative stress.

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**Compliance with Ethical Standards**

**Conflicts of interest** The authors declare there is no conflict of interest.

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