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Nasser, Fatima; Lynch, Iseult

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Secreted protein eco-corona mediates uptake and impacts of polystyrene nanoparticles on *Daphnia magna*

Fatima Nasser *, Iseult Lynch

University of Birmingham, School of Geography, Earth and Environmental Science, Edgbaston, Birmingham B15 2TT, United Kingdom

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ABSTRACT

Nanoparticles (NPs) are defined as having at least one external dimension between 1 and 100 nm. Due to their small size, NPs have a large surface area to volume ratio giving them unique characteristics that differ from bulk material of the same chemical composition. As a result these novel materials have found numerous applications in medical and industrial fields with the result that environmental exposure to NPs is increasingly likely. Similarly, increased reliance on plastic, which degrades extremely slowly in the environment, is resulting in increased accumulation of micro-/nano-plastics in fresh and marine waters, whose ecotoxicological impacts are as yet poorly understood. Although NPs are well known to adsorb macromolecules from their environment, forming a biomolecule corona which changes the NP identity and how it interacts with organisms, significantly less research has been performed on the ecological corona (eco-corona). Secretion of biomolecules is a well established predator–prey response in aquatic food chains, raising the question of whether NPs interact with secreted proteins, and the impact of such interaction on NP uptake and ecotoxicity. We report here initial studies, including optimisation of protocols using carboxylic-acid and amino modified spherical polystyrene NPs, to assess interaction of NPs with biomolecules secreted by *Daphnia magna* and the impact of these interactions on NP uptake, retention and toxicity towards *Daphnia magna*.

Biological Significance: Daphnia magna are an important environmental indicator species who may be especially sensitive to nanoparticles (NPs) as a result of being filter-feeders. This paper demonstrates for the first time that proteins released by *Daphnia magna* create an eco-corona around polystyrene NPs which causes heightened uptake of the NPs and consequently increases toxicity. The secreted protein eco-corona also causes the NPs to be less efficiently removed from the gut of *D. magna* and NPs remaining in the gut of *D. magna* affected the rate of subsequent feeding. Thus, fate of NPs in the environment should be evaluated and monitored under more realistic exposure scenarios.

1. Introduction

There has been a significant increase in the incorporation of NPs into industrial products due to their unique characteristics, provided by their large surface area compared to larger bulk materials of the same chemical composition [1–3]. Next-generation nanoparticles provide a wide variety of morphologies and shapes which give the NPs heightened characteristics that are not exhibited by spherical legacy nanoparticles [4–6]. A substantial amount of research has been conducted on various legacy NPs and the benefits they provide to industrial applications, although there is still insufficient information on the implications of these NPs on environmental health to make informed risk assessments. Even less information is available on future or next-generation NPs and their impact on biological organisms, leaving a major knowledge-gap for regulators [7].

In parallel to the growth of applications of nanomaterials, there has been a significant increase in use of microplastics, either manufactured to be microscopic (<5 mm) in size, or derived from the degradation of larger plastic debris and whose further weathering will likely result in nanoscale entities [8]. Microplastic litter has been identified in marine habitats across the globe [9], and more recently has been sampled in freshwater systems [10]. The destination of ingested microplastics within aquatic organisms, in addition to potential adverse effects, remains unknown, emphasising a need for laboratory studies focussing on the physical impacts of microplastics [3]. Polystyrene NPs, as model systems for both NPs and microplastics, can be used to answer important questions regarding the impacts of advanced materials on aquatic species, as a result of their ready availability in a range of sizes and surface functionalisations, including fluorescently labelled variants to allow tracking of uptake and localisation.

Daphnia magna (*D. magna*), a small aquatic crustacean, is considered a "keystone" species in ecological food webs and is an indicator species for toxicant exposure. Their sensitivity to changes in water quality and

* Corresponding author.
E-mail address: FXN138@bham.ac.uk (F. Nasser).

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their transparent exoskeleton make them an ideal indicator species. *D. magna* is abundant in fresh water systems, consuming phytoplankton such as algae, which are responsible for creating organic compounds from dissolved carbon dioxide which maintain the aquatic ecosystem [11]. *D. magna* are also consumed by many invertebrates and therefore occupy a unique and important role in the aquatic food web and act as an ideal candidate for fresh-water toxicity testing [12–14]. *D. magna* abundance in fresh water environments may cause multi-trophic level changes by fluctuating the bioavailability of organisms that it preys upon [15–17].

D. magna are filter feeders, filtering small particles out of the water column, and are thus exposed to everything present in the water, and show dramatic responses in terms of feeding rate, body size, reproduction rate and other traits in response to changes in water quality or in response to the presence of signalling molecules from their predators (kairomones) [18]. Suspended particles, for example, are known to reduce the filtering rate of *Daphnia* species leading to reduced body size [19], while filamentous cyanobacterium effectiveness at reducing filtration by *Daphnia* depended on the organism size [20].

Standardised toxicity tests have been developed by the Organisation for Economic Cooperation and Development (OECD) using *D. magna* immobilisation (OECD 202) [21] and reproduction (OECD 211) [22] which have also been applied for assessment of the toxicity of NPs. However, several studies recently have suggested that these tests are not suitable (without significant adaption) for NPs for a number of reasons, including: (1) the lack of feeding and consideration of depuration in the short term tests which means that assumptions behind the traditional way of quantifying bioconcentration are not fulfilled when NPs are studied [23]; and (2) the fact that the test waters do not take account of the complexity of natural waters which contain dissolved organic matter that influences NP stability and bioavailability [24].

Another area where the standard tests may not be environmentally realistic is in relation to the role of secreted proteins and biomolecules from aquatic organisms, as reference media lack any macromolecular components. Thus, aquatic organisms condition their aquatic surroundings by releasing proteins and other biomolecules, which is the basis of predator–prey relationships along the food chain [18,25]. The presence of proteins is well-known to have stabilizing or destabilizing effects on NPs which in turn, influences their uptake and toxicity [26]. A fundamental point to consider in terms of the fate and transport of NPs through the environment is how different biomolecules may adsorb onto the NP surfaces creating an 'eco-corona' around the NP which may alter NP identity and subsequently its toxicity and interaction with organisms [25–27]. It has also been widely recorded that proteins adsorbed to the surface of nanoparticles have an influence on biodistribution within cells [28], and an initial report on impacts of NP eco-coronas showed that the earthworm *Eisenia fetida* preferentially recognised NPs coated in *E. fetida* coelomic proteins compared to the same NPs coated in foetal bovine serum, as reported by Hayashi et al (2013).

D. magna secrete proteins as signalling factors, which can potentially coat NPs and therefore change their identity and size, allowing them to be more compatible to interact with recognition receptors on cell surfaces. Currently there is limited research on the effect of the eco-corona of NPs and their impact on aquatic organisms. The purpose of this study was to assess the effect of secreted biomolecules from *D. magna* or from its food source (the algae *Chollera vulgaris*) on uptake of carboxylic acid ($-COOH$) and amino functionalized ($-NH_2$) polystyrene NPs by *D. magna.* Consequently we also assessed the impact of bare and protein-exposed NPs on survival and evaluated the rates of elimination of the NPs from the organism. Although earlier studies have shown impact of NPs such as titanium dioxide (TiO_2) and fullerenes (nano-C_{60}) on factors such as heart rate and hopping rate of *D. magna* [15], these studies do not assess the interactions of the proteins released by the *D. magna* with NPs and how this influences uptake and interaction. This data will further understanding of the eco-corona and how this influences NP identity and subsequent interactions with, and uptake by, environmental organisms under more realistic exposure conditions.

2. Materials and methods

2.1. Daphnia magna culturing

Genetically identical *D. magna* (Bham 2 strain) [29] were cultured in High-Hardness (HH) Combo media (pH 7.6–7.8). Each culturing vessel maintained 15 adults in 900 mL of media and organisms were sustained at 20°C in a culturing chamber with maintained humidity in accordance with our standard protocol. *D. magna* cultures were fed *Chollera vulgaris* algae (1 mL days 1–2, 1.5 mL days 3 and more; Note 1 mL algae = 0.5 mg carbon at OD = 0.8) daily and their media refreshed weekly in order to maintain healthy broods of neonates.

2.2. Media conditioning

To condition media by *D. magna*, 10 neonates (1–3 days old) were placed in 5 mL of HH Combo medium and were allowed to condition the medium for a specified conditioning time. Conditioning time starts when neonates are put into HH Combo medium and allowed to secrete proteins for a given length of 'conditioning time' (1, 3 or 6 h) whereby they are then removed from the medium leaving behind only medium with the secreted proteins (conditioning time for medium). Neonates were not fed in order to ensure the proteins accumulated in the medium were only proteins released by the neonates. At the end of the conditioning time neonates were carefully pipetted out of the medium leaving only the remaining conditioned medium. A sample of the 6-h conditioned media was sent for mass spectrometry analysis to identity the proteins secreted.

To condition medium by algae, 1.5 mL of algae was pipetted into 5 mL of HH Combo medium for a specified conditioning time. At the end of the conditioning time the medium was filtered with coarse filter paper, allowing smaller proteins to be filtered through with the medium. Visual confirmation of the filtered HH Combo medium confirms that the algae was removed leaving only conditioned medium (supplementary Fig. 1).

2.3. Nanoparticles and dispersions

Carboxylic and amino polystyrene NPs were obtained from a previous FP7 project (QualityNano, who developed them as negative and positive controls, respectively, for apoptosis in cells) with a stock concentration of 20 mg/mL. The stock concentrations were diluted to various concentrations ranging between 0.0001–1 mg/mL using HH Combo medium and exposed to *D. magna* neonates in order to determine dose–response behaviour and determine the EC_{50}. A concentration of 10 μg/mL was chosen for all subsequent experiments as the optimal concentration as it allowed a high survival of neonates yet showed some effect from the NPs. Fluoresbrite carboxylate yellow/green fluorescent polystyrene nanoparticles (Polysciences: em 486 nm/ex 441 nm) were used for uptake studies with an original stock concentration of 250 mg/mL which was diluted with HH Combo to obtain the optimal concentration of 10 μg/mL.

2.4. Assessing nanoparticle impact on D. magna – EC_{50} determination

Ten *D. magna* neonates (1–3 days old) were exposed to a range of concentrations (0.1 μg/mL-1 mg/mL) of either carboxylic-acid or amino functionalized polystyrene NPs, diluted with HH Combo medium, with a final volume of 5 mL, and exposed for 24 h, after which time the % survival was determined by counting the living organisms which can be observed visually. The EC_{50} values of the carboxylic-acid or amino functionalized polystyrene NPs were determined to be

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0.0363 mg/mL and 0.0258 mg/mL respectively (n=3). The same study was also performed using the particles incubated for 6 or 24 h in *D. magna* conditioned medium (medium exposed to *D. magna* neonates for 6 or 24 h followed by removal of the neonates as described above) prior to presentation of the NPs to *D. magna* in order to assess the impact of eco-corona formation and rearrangement on the particle toxicity.

2.5. Nanoparticle uptake and removal by D. magna

Based on the EC_{50} values determined, a concentration of 10 μg/mL was chosen so that the majority of the neonates were viable yet the NPs showed some effect on stress and survival. Fluorescently labelled carboxylic-acid functionalized polystyrene (COOH-PS) NPs (Polysciences) were used for assessment of NP uptake by *D. magna* neonates. NPs were exposed to n=10 neonates per exposure scenario for different exposure lengths (1, 2 and 3 h). At the end of the exposure, neonates were removed and washed with fresh medium three times to remove NPs bound to the exoskeleton in order to only assess particles that had been ingested. Each sample consisting of ten neonates was put into a well of a 96-well plate in three replicates and analysed using the fluorescent function on a plate-reader (FLUOstar Omega, BMG Labtech). For experiments that had a conditioning step, *D. magna* neonates conditioned the medium for a specified duration of time (typically 6 h) whereupon the neonates were removed leaving only conditioned medium. NPs were then be added for various exposure times following the aforementioned protocol and uptake was again assessed. Statistical significance was calculated using a standard 2-tailed student's *t*-test with p-values of 0.05, 0.01 and 0.001 with *, ** and *** indicating the respective significances in the data.

To assess removal of NPs by *D. magna* neonates, after neonates had been exposed to fluorescently labelled COOH-PS NPs for various exposure times (1, 2, and 3 h as stated above), neonates were placed into fresh HH Combo medium to terminate the exposure. Neonates were then placed into fresh media every 15 min in order to recover any NPs expelled by the neonates and to ensure they would not be taken up again by the neonates. Neonates were assessed at 1, 3 and 6 h post-exposure with the aforementioned method using a 96-well plate in order to assess the amount of NPs (fluorescence) remaining in the neonates.

To assess if NPs remaining in the GI tract of *D. magna* neonates affect the rate of subsequent feeding on algae, *D. magna* neonates were exposed to fluorescent COOH-PS NPs (which had been previously exposed to *D. magna* conditioned media for 6 h) for 2 h and then moved to fresh HH medium for 6 h post exposure. *D. magna* neonates were then exposed to 1.5 mL of algae and 10 neonates each were collected at different time points (0, 1, 2, 3 and 6 h) and assessed for total fluorescence using a plate reader. In parallel, a control was done to assess normal feeding behaviour of *D. magna* neonates on algae (where they had not been exposed to NPs but had been treated to the same exposure and recovery times, i.e. 2 + 6 h in medium followed by exposure to 1.5 mL of algae and assessment over 6 h).

2.6. Assessment of NP size (in media and conditioned media)

NPs were measured at 0, 1, 3 and 6 h at 25 °C from their initial dispersion (HH medium) or exposure (conditioned medium) using a nano zetasizer ZS (Malvern Instruments) which has a red laser (633 nm). The autocorrelation function calculates the diffusion coefficient (D), which can then be converted to hydrodynamic radius (R_H) using the Stokes-Einstein equation $R_H = K_BT/(6\pi\eta D)$ where η is viscosity and K_BT is thermal energy. The R_H gives the size of the particle which includes a thin layer of solvent that has surrounded the NP and therefore is always larger than the size of the core NP. Samples were run in replicates of three to ensure reliability (see supplementary Fig. 2 for an example of size graphs by DLS plotted by intensity and indicating the z-average size and the polydispersity index; and supplementary Fig. 3 for stability data for COOH-PS NPs in D. magna conditioned media).

2.7. Determining protein concentration released by organisms

A BCA assay was conducted to quantify the concentration of protein released by *D. magna* neonates as a function of conditioning time using a Peirce BCA assay protein kit. Ten *D. magna* neonates were used to condition 5 mL of HH Combo medium for a fixed duration of conditioning time and were then removed using a Pasteur pipette from the media. A range of dilutions (0–2 mg/mL) of bovine serum albumin (BSA) of known concentration in three replicates were made using DI water in order to create a standard curve using a 96-well plate (costar). Samples (100 μL each) of the conditioned medium were also put into separate wells in the 96-well plate in replicates of three. A 50:1 mixture of reagent A to reagent B was made ensuring enough volume to add 200 μL to each reaction well (each reaction well holds 300 μL). The 96-well plate was microwaved for 30 s alongside a beaker of water to act as a heat sink. The absorbance function set to 560 nm was used to obtain the absorbance of the different reaction wells. A standard curve was created using the known BSA concentrations against absorbance. Samples of unknown concentration could then be found by inserting the absorbance provided by the plate reader for each of the conditioned medium samples to calculate concentration.

2.8. Confocal microscopy imaging of retention of NPs in D. magna gut

Confocal microscopy was used to visualise uptake of fluorescently labelled COOH-PS into *D. magna* neonates. Laser scanning confocal microscopy (LSCM) was conducted using a Zeiss LSM 710 ConfoCor using the 458 nm laser. Neonates were exposed to NPs for a given exposure time and then transferred to fresh HH Combo medium and subsequently washed twice with fresh HH Combo medium to remove any NPs bound to the carapace. Fluorescent images of *D. magna* neonates were taken by placing a single neonate in a 35 mm glass bottom dish and reducing the surrounding liquid to a minimum to reduce movement and using the 10× objective lens to capture images (n=3). Both fluorescent and transmitted light images were recorded and overlayed to show both parameters in a single image.

2.9. Eco-corona isolation and analysis of proteins by PAGE

Protein coronas surrounding NPs were isolated using a well-established method documented in detail by Docter et al. [33]. In brief, nanoparticle-protein complexes have been isolated by sedimenting complexes through a 0.7 M sucrose cushion to end exposure of NPs to conditioned medium and therefore stop any subsequent binding. Nanoparticle–protein complexes then underwent a series of centrifugation and washing steps to remove unbound or loosely bound proteins by centrifugation in phosphate buffered saline (PBS) at (15,200×g/4 °C/20 min). Nanoparticle–protein complexes then underwent elution of the NPs by incubation (95°/5 min) and centrifugation (15,200×g/20 °C/15 min) in 100 μL of sodium dodecyl sulphate (SDS) leaving only those proteins existing in the so-called 'hard corona' in the supernatant which were then gently transferred to a fresh tube and could be stored at −20°C for future analysis. Isolated proteins were run on a 12.5% polyacrylamide gel electrophoresis (PAGE) using Coomassie blue (0.25%) and silver stain using 20 μL of eluted protein per well and were run at 100 V for approximately 3 h. Bands were observed confirming the presence of proteins which formed the hard corona.

3. Results and discussion

3.1. Effect of media conditioning and NP incubation time on NP stability

Understanding the interactions between proteins released by *D. magna* neonates and NPs is crucial to understanding how the secreted eco-corona around NPs influences their stabilization (or destabilization) in suspension. Fig. 1(a) shows that as conditioning time of medium by

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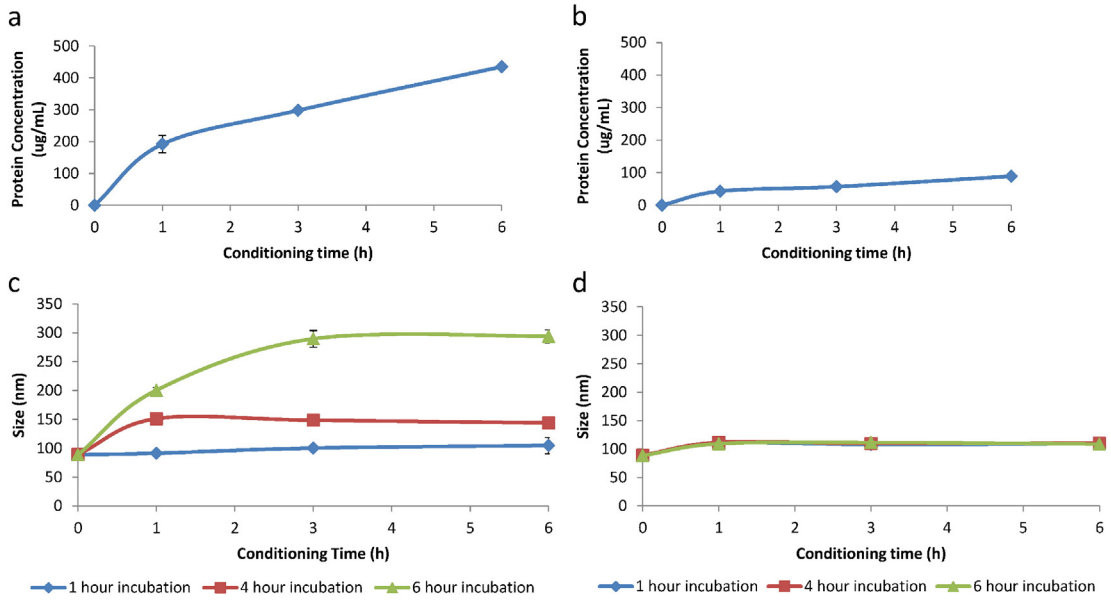

Fig. 1. Conditioning of media and effect on NP stability. a) Media conditioned by *D. magna* neonates over six hours quantified by BCA assay; b) Stability of amino-functionalised polystyrene NPs incubated for different durations (1, 4 and 6 h) in media conditioned by *D. magna* neonates for 6 h; c) Media conditioned by *algae vulgaris* over six hours quantified by BCA assay; d) Stability of amino-functionalised polystyrene NPs incubated for different durations (1, 4 and 6 h) in media conditioned by *algae vulgaris* for 6 h.

D. magna neonates increases, the concentration of proteins in the medium steadily increases to 435 μg/mL in six hours as quantified via a BCA assay. Considering that *D. magna* use algae as a food source, it is important to determine if any change in NP stability is caused by proteins released by algae as a result of binding to the NPs. The concentration of proteins released by algae into high-hardness Combo medium was also quantified by a BCA assay and was shown to have a concentration of only 140 μg/mL over a 6 h conditioning time (Fig. 1(b)), which was significantly lower than the amount of proteins released by *D. magna*.

When amino-functionalized polystyrene (NH_2-PS) NPs are incubated in conditioned media, in all cases the particle size and polydispersity index (PDI) increased slightly, as shown in Fig. 1(c). The increase in size is suggestive of formation of a layer of bound proteins at the particle surfaces rather than significant agglomeration in the case of 1 h condition time, irrespective of how long the NPs were incubated in the conditioned media. However, the incubation time effects particle size and stability over longer incubation times in the conditioned media, as longer exposure time showed evidence of some agglomeration of particles. It is unlikely that the adsorbed protein layer would account for a size increase of 150 nm, and indeed the polydispersity index also increased during the longer incubation times, which is suggestive of some agglomeration. Clearly some exchange or rearrangement of the proteins adsorbed at the NP surface is occurring during the longer (4 and especially 6 h) incubation times, contributing to the increased agglomeration propensity, and ongoing work will investigate this in greater detail. Results indicate that as conditioning time (and incubation time) increases, agglomeration of NPs also increases and that the proteins released by *D. magna* act as a potential destabilizer which can be seen in an increase in hydrodynamic size of amino-functionalized polystyrene NPs.

The results shown in Fig. 1(d) indicate that the low concentration of proteins released by algae cause no significant changes to NP size for the NH_2-PS NPs, and confirms that any instability in particle size is primarily caused by *D. magna* secreted proteins. Algae also may be releasing other macromolecules such as polysaccharides or lipids into the media although these also are not affecting the NP stability, while the BCA assay confirms there are indeed proteins present in the media as well. These findings are important as organisms naturally condition the environment and have the potential to cause changes to NP stability and, surface characteristics and thereby affect further NP uptake. A table of NH_2-PS NP size and corresponding PDI incubated in *D. magna* and *algae vulgaris* conditioned media can be seen in Table 1(a) and (b) respectively.

3.2. Effect of secreted eco-corona on EC_{50} of Daphnia magna

As determined above, conditioning of medium by *D. magna* neonates and incubation of NPs in conditioned medium causes changes in NP size as a result of formation of a secreted eco-corona and gradual agglomeration of particles. We quantitatively determined the effect of the secreted eco-corona around the COOH-PS NPs on their EC_{50} to *D. magna*. As seen in Fig. 2a, COOH-PS NPs that were incubated in conditioned media for 6h showed a decrease in EC_{50} from 0.0363 mg/mL to 0.0337 mg/mL compared to those that had no conditioning step. NPs that were incubated in conditioned media for 24h showed an even greater decrease in EC_{50} to 0.0095 mg/mL (n=3 in all cases where fluctuation is within 10% of the mean). These results are notable as it shows that a conditioning step causes the proteins released by *D. magna* to coat the NPs which has a negative effect on the survival of *D. magna*. Longer incubation times lead to a greater amount of proteins adsorbing to the surface of the NPs and/or a rearrangement of the adsorbed proteins which causes an increase in size. It has been documented that in

Table 1
Table of amino-functionalised polystyrene NP size and PDI with various incubation time and conditioning time by (a) *Daphnia magna* and (b) algae *Cholera vulgaris* in HH Combo medium.

	Incubation time (h)					
	1		4		6	
Conditioning time (h)	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI
(a)						
0	88.83	0.018	89.09	0.025	85.51	0.030
1	91.26	0.169	151.2	0.318	200.5	0.034
3	100.2	0.186	148.5	0.366	289.7	0.477
6	104.7	0.12	143.8	0.323	293.8	0.510
(b)						
0	88.83	0.018	89.09	0.025	88.51	0.030
1	110.4	0.193	111.6	0.196	109.4	0.206
3	108.1	0.215	109.9	0.175	111.5	0.156
6	109.3	0.184	110.5	0.153	108.8	0.200

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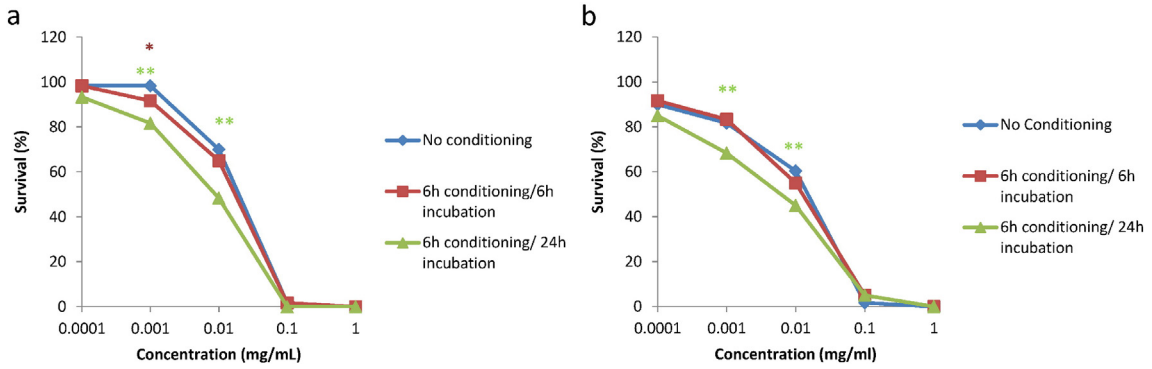

Fig. 2. EC_{50} of *D. magna* neonates in response to exposure of different concentrations of (a) carboxylic-acid functionalised polystyrene NPs with no conditioning; 6 h conditioning/6 h incubation or 6 h conditioning/24 h incubation; b) EC_{50} of *D. magna* neonates in response to exposure to different concentrations of amino-functionalised polystyrene NPs under the same conditions.

biological fluid such as plasma, proteins which are high in abundance and have a low affinity bind to NP surfaces first, and are then displaced by proteins lower in abundance that have a higher affinity for the NP surface, [29] which may also occur for environmental coronas. Preliminary assessment by 12.5% PAGE was done using Coomassie blue (0.25%) and silver stain indicates the presence of proteins associated with the NPs (Ps-COOH and PS-NH_2) hard corona. Significant bands appear between 50–80 kDa indicating the presence of proteins such as Type VI secretion system (74.8 kDa), a stress response protein, and QseC sensor protein (50.4 kDa) used in cell-to-cell signalling, both of which were present in mass spectrometry results from the conditioned media. Table S1 in the supplementary materials lists the proteins identified by mass spectrometry as having been secreted into the conditioned media, and therefore available for interaction with the NPs to form the eco-corona.

It is well established that although *D. magna* are filter feeders they are able to selectively take up food sources based on size, texture and consistency. The larger size of NPs caused by the destabilization of the proteins in the eco-corona could make the NPs a more attractive size as a food source and therefore cause *D. magna* to take up protein-coated NPs more readily than bare and monodisperse NPs. Indeed literature suggests that uptake rates by filter feeders are affected by particle size as a result differences in particle encounter rates and flow control regimes [30]. Similar results of conditioning also occur with the NH_2-PS NPs and can be seen in Fig. 2b where conditioning was shown to influence the EC_{50} as well as longer incubation times of NPs in conditioned media (unconditioned 0.0258 mg/mL, 6h conditioning and 6h incubation 0.0189 mg/mL, 6h conditioning and 24h incubation 0.0081 mg/mL). It was also determined that NH_2-PS NPs were generally more toxic compared to the COOH-PS which can be attributed to the positive charge more strongly interacting with the negatively charged cell membrane of *D. magna*. Such enhanced toxicity of amine versus carboxyl modifications of PS NPs has also been demonstrated in a variety of cells [31], and NH_2-PS NPs have also been shown to cause developmental toxicity in sea urchins [32], however, no study has yet reported a secreted corona-mediated effect on EC_{50}.

3.3. Rates of uptake and rate of removal of PS NPs after exposure to *D. magna*

As incubation time of unconditioned NPs with *D. magna* neonates increases, the uptake of these particles into *D. magna* neonates contingently increases as shown in Fig. 3a where COOH-PS NPs are being removed from the media and taken up by *D. magna* neonates. Equally important as assessing uptake, is the need to monitor rates of removals of NPs in order to determine the actual body-burden and correlate this with observed effects. Inability to efficiently remove NPs after uptake can lead to accumulation and potential long-term effects. Fig. 4a–c demonstrate that as exposure time of NPs to *D. magna* neonates increase, so does the uptake of NPs, as expected. We determined that NPs are consistently removed during post-exposure regardless of having being exposed for either 1, 2 or 3 h. It appears that by 6 h post exposure, approximately 15% of NPs still remain within the neonates and that the rate of removal starts to plateau at 3 h post-exposure whereupon *D. magna* neonates are less effectively able to remove the remaining NPs. Bioaccumulation of NPs that are removed can potentially lead to future consequences where those same NPs may be re-taken up by daphnia continuously, the consequences of which are unknown as yet.

We also monitored rates of removal of NPs that had been incubated in conditioned media (6 h conditioning, 2 h exposure) as shown in Fig. 4d. NPs were removed from *D. magna* neonates but less efficiently than those without a conditioning step. At six hours post exposure it appeared that approximately 20% of NPs remained in the neonates. This could potentially be the reason for the lower EC_{50} in the conditioned media case, as a result of a higher retained dose of NPs in the presence of the eco-corona. NPs which have a corona are taken up slightly less by *D. magna* neonates though are retained longer within the gut and are not excreted with the same rate of efficiency compared to NPs without a corona. Confocal microscopy imaging has been used to confirm that the retained COOH-PS particles remain in the gut both in the absence and presence of the eco-corona, as there is no evidence of fluorescence signal being internalised beyond the gut as shown in Fig. 5(a) and (b).

3.4. Ability of *D. magna* to effectively feed on Algae after NP accumulation

It is widely recorded that *D. magna* prey on algae in their natural fresh water habitat and there is evidence to propose that feeding rate is affected by the presence of consumed substances already in the gut [17]. We have determined that a small fraction of NPs remain in the

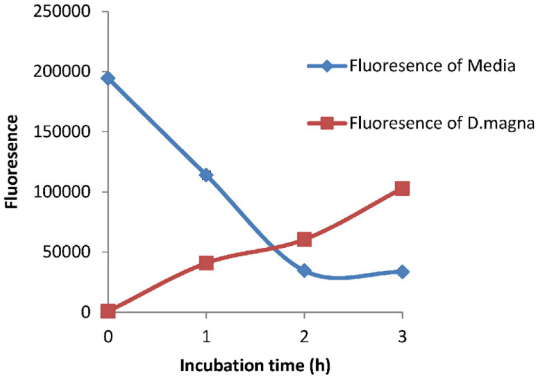

Fig. 3. Uptake of carboxylic-acid functionalised polystyrene NPs by *D. magna* neonates and their corresponding removal from HH Combo media determined by fluorescence.

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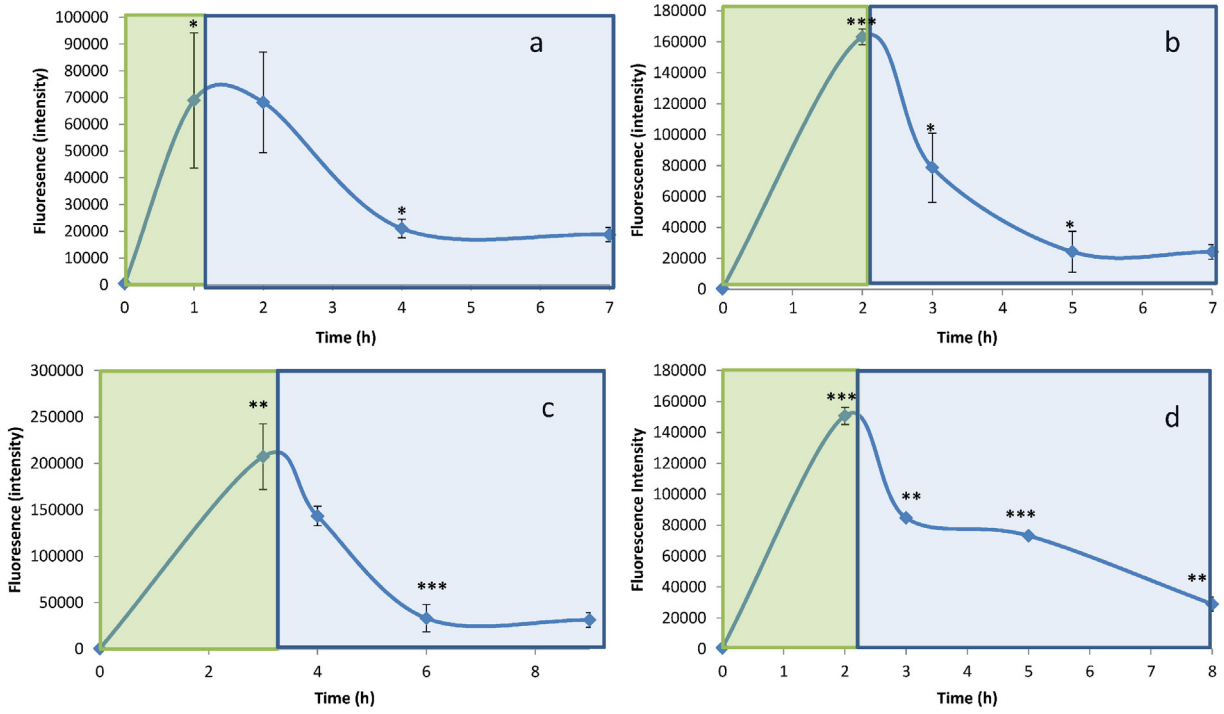

Fig. 4. Rates of uptake and removal of carboxylic-acid functionalised polystyrene NPs under defined conditions. a) with 1 h exposure/6 h post-exposure; b) 2 h exposure/6 h post-exposure; c) 3 h exposure/6 h post exposure; d) 6 h conditioning of the medium using *D. magna* neonates/2 h exposure to corona-modified carboxylic-acid functionalised polystyrene NPs/6 h post-exposure.

gut 6 h post- exposure and it is imperative to assess if this affects the subsequent feeding rate of *D. magna* on algae. Our data, as shown in Fig. 6, suggests that feeding rate does in fact decrease in neonates that had been exposed to conditioned NPs. Though there appears to be a decrease in feeding rate, rates are not statistically significant over 6 h, though could potentially become significant over a longer duration. Longer-term studies would be required to assess whether this effect persists and could potentially lead to development delays.

The feeding rate of *D. magna* is extremely important as it occupies an important position in the aquatic food web. *D. magna* are the primary consumers of algae and therefore act as a regulator of algae levels, who in turn provide chlorophyll vital for the photosynthesis that is a key step in maintaining aquatic dissolved oxygen levels. Alterations in *D. magna* feeding rate could potentially lead to multi-trophic level changes in aquatic systems, and *D. magna* are also prey for different types of invertebrates, thus changes to their survival can cause an inadequate food source for many higher level organisms. As a result of being consumed by other organisms, NPs have the potential to move up the food chain and accumulate in other organisms as a result of secondary transfer through consumption. Overall, it is important to understand how proteins released by organisms influence the eco-corona around NPs and how this influences uptake and rate of export from organisms, and the consequent total body burdens and impacts of these.

Preliminary analysis of the proteins released by *D. magna* neonates after exposure to 10 μg/mL of COOH-PS NPs identified Type VI secretion system protein. This acts as a defence mechanism in times of stress by releasing effector proteins which puncture target cells and cause them to lyse. It appears that NP accumulation within *D. magna* induces stress which was effectively shown by their decreased survival rate (lower EC_{50}) and exporting out Type VI secretion system as a defence mechanism under stressful conditions. Further work is being continued in order to assess binding rates of proteins released by *D. magna* to NPs

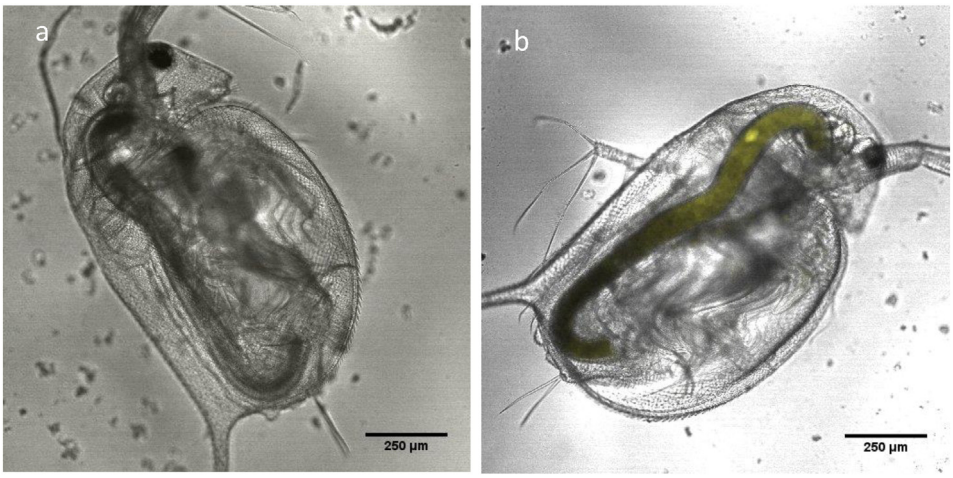

Fig. 5. Confocal microscopy images of *D. magna* in (a) nanoparticle-free HH Combo medium as a control and (b) exposed to fluorescent COOH-PS NPs after 24 h.

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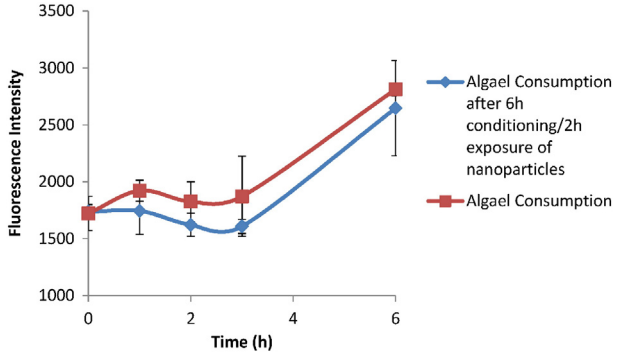

Fig. 6. Rate of consumption of *algae vulgaris* by *D. magna* after 6 h conditioning step/2 h exposure of carboxylic-functionalised polystyrene nanoparticles/6 h post-exposure (identical conditions to Fig. 4d) and subsequently presented with 1.5 mL *algae vulgaris* for 6 h.

and how these proteins can change the identity NPs and impact their interaction with organisms.

4. Conclusions

As a well-established model for toxicity assessment, with a central position in the aquatic food chain, *D. magna* is an important indicator species known to engage in numerous predator–prey interactions via secreted macromolecules. Coupling this with the propensity of NPs to bind proteins and other molecules from their surroundings to form a corona, there is an urgent need to understand the role of secreted proteins in mediating NPs toxicity to *D. magna* and the potential for food-chain effects. The work presented here is a first step towards addressing this important issue.

Conditioning the dispersion medium by exposing it to *D. magna* neonates resulted in significant release of proteins, which quickly coated the COOH- or NH_2-PS NPs forming an eco-corona and caused destabilization of the dispersions over the subsequent 6 h. Interestingly, the eco-corona coated NPs result in a lower EC_{50} than equivalent unconditioned ones, and were less effectively removed from *D. magna* compared to uncoated NPs (20% remaining after 6 h for corona coated NPs compared to 15% for the uncoated). The NPs remaining in the gut of *D. magna* affected its ability to feed on algae over the subsequent 6 h, which could be indicative of a secondary effect from the presence of NPs, although this would need to be investigated over longer time periods coupled with assessment of any consequential developmental delays. Not surprisingly, the NH_2-PS NPs were more toxic than the COOH-PS NPs, both in the absence and presence of the eco-corona, as had also been observed in cells and in developmental studies using sea urchins.

The data presented confirms the important role of secreted proteins in modulating NP toxicity, as well as confirming that removal of NPs from the gut, via excretion and/or uptake into the organism, needs to be considered as part of determination of bioaccumulated dose for toxicity assessment. This is not currently considered as part of OECD standard tests, and represents a significant modification of the tests that is needed to enhance their appropriateness for assessment of NP toxicity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.09.005.

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