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Particle size determines the accumulation of platinum nanoparticles in the estuarine amphipod, *Leptocheirus plumulosus*†

Mithun Sikder,[ab] Emily Eudy,[b] Bo Cai,[c] G. Thomas Chandler[b] and Mohammed Baalousha *[ab]

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Nanoparticles (NPs) typically display a wide distribution of different sizes in aquatic environments, yet little information is available on the impact of particle size dispersity on organismal uptake and elimination. This study investigated uptake and elimination of polyvinylpyrrolidone-coated platinum nanoparticles (PVP-PtNPs) of different sizes (*e.g.*, 20.0 ± 4.8 nm, 40.5 ± 4.1 nm, and 70.8 ± 4.2 nm) by the estuarine amphipod *Leptocheirus plumulosus*. Accumulation and elimination were determined by measuring total Pt body burden in amphipods exposed to PtNPs using inductively coupled plasma-mass spectroscopy, as well as the mass and number PtNP body burden using single particle-ICP-MS (sp-ICP-MS). *L. plumulosus* accumulated Pt from PtNP suspensions of different sizes from water exposure, mostly (*e.g.*, >90%) as PtNPs rather than as dissolved Pt. Mass- and number-based uptake increased with decreases in PtNP size whereas mass- and number-based elimination increased with increasing PtNP size. The residual whole-animal body burden of PtNPs after 48 h elimination increased with decreases in PtNP size, with residual body burdens approximately two-fold higher for amphipods exposed to 20 nm PtNPs than amphipods exposed to 70 nm PtNPs. PtNP influx rate (k_{uw}) increased with decreasing NP size, with k_{uw}s of 1.07 ± 0.31, 0.82 ± 0.22, and 0.67 ± 0.10 μg g^{-1} d^{-1} for 20 nm, 40 nm, and 70 nm PtNPs, respectively. PtNP efflux rate (k_e) increased with increasing PtNP size, with k_es of 0.31 ± 0.08, 0.66 ± 0.04, and 0.83 ± 0.07 d^{-1} for 20 nm, 40 nm, and 70 nm PtNP, respectively. When exposed to mixtures of 40 and 70 nm PtNPs with equal masses, surface areas, or number concentrations of 40 nm and 70 nm PtNPs, *L. plumulosus* accumulated higher numbers of the 40 nm PtNPs than 70 nm PtNPs from all mixtures. The increased exposure concentration of 70 nm PtNPs in the mixture did not affect the uptake of 40 nm PtNPs, suggesting that in a polydispersed NP suspension the uptake of a given size fraction is independent of other size fractions in the mixture.

Environmental significance

Many nanoparticle uptake and toxicity studies have been performed with polydispersed suspensions, that is suspensions with broad size distributions. However, the impact of nanoparticle size dispersity on uptake, elimination, and toxicity have not been investigated experimentally. By tracking nanoparticles at the single particle level, this study provides insights on how nanoparticle size and size dispersity affect the uptake and elimination of polyvinylpyrrolidone coated platinum nanoparticle (PVP-PtNPs) in *Leptocheirus plumulosus*. Uptake of PtNPs increased with decreases in PtNP size whereas elimination of PtNPs increased with increases in PtNP size. When exposed to mixtures of 40 nm and 70 nm PtNPs ($PtNP_{40}$ and $PtNP_{70}$, respectively) with equal masses, surface areas, or number concentrations of $PtNP_{40}$ and $PtNP_{70}$, *L. plumulosus* accumulated higher numbers of $PtNP_{40}$ than $PtNP_{70}$. Moreover, increases in $PtNP_{70}$ concentration did not affect the uptake of $PtNP_{40}$ from the mixture. These results reveal that in a polydispersed nanoparticle suspension the uptake of a given fraction is independent of other size fractions.

[a] *South Carolina SmartState Center for Environmental Nanoscience and Risk (CENR), Arnold School of Public Health, University of South Carolina, Columbia, SC, 29208, USA*
[b] *Department of Environmental Health Sciences, Arnold School of Public Health, University of South Carolina, 921 Assembly Street, Columbia, SC, 29208, USA. E-mail: mbaalous@mailbox.sc.edu; Tel: +1 803 777 7177*
[c] *Department of Epidemiology and Biostatistics, Arnold School of Public Health, University of South Carolina, Columbia, SC, 29208, USA*

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Introduction

Although it can be assumed that nanoparticles (NPs) in an aquatic environment display a wide distribution of different sizes, little information is available on the impact of particle size polydispersity on their environmental behaviors. Numerous studies demonstrated negative impacts of NPs to aquatic organisms, but divergent results have been reported on the

importance of NP size on their uptake and toxicity. For instance, several studies revealed that NPs might have size-dependent toxic effects;[1–4] yet it is common to find studies attributing toxicity to a certain size fraction[4] while others report no size-dependent toxicity.[5] Furthermore, the relationship between NP uptake and size remains uncertain. Several studies have shown decreases in NP uptake with increases in NP size,[6–8] with decreases in NP size,[9–11] and even a lack of NP size-dependent uptake.[12–14] Such contradictory inferences might be due to the use of different analytical techniques for NP characterization in the different studies and/or to the heterogeneity and polydispersity of the NPs most widely studied and reported in the literature.[15] Size polydispersity may result in incorrect measurements of NP average size, underestimation of NP dose, masking of size-dependent properties and effects.[15,16]

Many early metal-based NP uptake and toxicity studies suffered from a lack of robust NP quantification and characterization during exposures, including concentration measurements and size distributions in test media, in organisms, and the relative impacts of the nanoparticulate *versus* dissolved metals.[17] Most early metal-based NP uptake studies have inferred NP uptake by measuring the total NP-metal burden within organisms exposed to NPs.[18,19] This approach can provide accurate body burden measurements for insoluble metal NPs, but it falls short of differentiating between dissolved metals and intact NP uptake from soluble NP suspensions. Additionally, this approach does not yield any information on the size distribution of the accumulated NPs.[15,16] The inability to differentiate the form of measured body burden (*e.g.*, dissolved metal *vs.* NPs), in addition to the lack of concurrent characterization techniques to determine NP aggregation state in media and tissues, limits a robust understanding of NP toxicology. Single-particle inductively-coupled plasma mass spectrometry (sp-ICP-MS) can bridge this gap as it concurrently measures NP size distributions, and NP number and mass concentrations[20] at environmentally- and toxicologically-relevant particle concentrations (*e.g.*, ng L^{-1} range).[21] Recent studies demonstrated the uptake of AuNPs in environmental organisms such as *Daphnia magna* and zebra fish[22] and *Caenorhabditis elegans* (*C. elegans*)[23,24] using sp-ICP-MS. The latter studies investigated the uptake by *C. elegans* of individual AuNP size suspensions (*e.g.*, 30 nm, 60 nm, 80 nm, and 100 nm) and did not consider the uptake from mixtures of AuNPs of different sizes.[23,24] Other studies demonstrated the uptake of AuNPs in the alga *Pseudokirchneriella subcapitata* using single cell (sc)-ICP-MS.[22,25]

Many studies demonstrated the toxicity of PtNPs toward environmental organisms such as *Pseudokirchneriella subcapitata* (EC_{50} of 15 to 200 mg Pt per L);[26,27] duckweed (*Lemna minor*), water fleas (*Daphnia magna* – EC_{50} of 406 to 514 μg Pt per L), bacteria (*Vibrio fischeri* – EC_{50} of 135 to 255 μg Pt per L);[28] brine shrimp (*Artemia salina* – 96 EC_{50} of 117 μg Pt per L),[29] and zebra fish.[30] These EC_{50} values are much higher than Pt concentrations in surface waters (*ca.* ng L^{-1} to μg L^{-1}).[31] Furthermore, to date, many studies investigated the uptake of metal and metal oxide (*e.g.*, Au, Ag, TiO_2, ZnO, CeO_2) NPs in aquatic organisms.[32,33] However, despite the multiple sources of PtNPs in the environment,[34–39] only a few studies demonstrated the uptake and translocation of PtNPs in environmental organisms such as zebrafish,[30] the freshwater snail *Lymnaea stagnalis*,[6] aurgula and escarole plants,[40] and *Sinapis alba* and *Lepidium stavium* plants.[41,42]

Therefore, the objectives of this study are to (1) evaluate the size-dependent bioaccumulation (*i.e.*, uptake and elimination) of PtNPs in the absence of food by the common estuarine amphipod *Leptocheirus plumulosus* using sp-ICP-MS, and (2) examine the effect of NP size dispersity on the accumulation of PtNPs from polydispersed suspensions (*e.g.*, mixtures of 40 nm and 70 nm PtNPs with equal number, surface area, and mass concentrations). Throughout this report the term 'uptake' is used to describe particles entering and measured within the test organism and does not necessarily imply that membrane passage or tissue translocation of NPs has occurred. The term elimination is used to describe the proportion of particles excreted/eliminated *vs.* retained within the organism over time. The term 'bioaccumulation' represents the net outcome of all uptake and elimination processes.[43,44] The study was carried out using the USEPA-approved[45] estuarine amphipod test species *L. plumulosus* as a model particle-ingesting benthic species exposed to PtNPs of three different controlled sizes. PtNPs were chosen as a model study particle for a number of reasons: (1) PtNPs exhibit very low toxicity which if present might interfere with NP uptake and elimination,[46] (2) the low solubility of PtNPs minimizes differential uptake of dissolved *versus* particulate Pt,[6] (3) there are usually undetectable background concentrations of PtNPs in aquatic environments and the test organism,[47] (4) sp-ICP-MS provides a low size-detection limit for PtNPs (*i.e.*, ~17 nm),[48] and (5) the release of PtNPs from photocatalytic convertors in vehicles is growing should not be considered irrelevant in the environment without further study,[34] and (6) the limited number of existing studies on the uptake of PtNPs.

Methodology

Platinum nanoparticles (PtNPs)

Polyvinylpyrrolidone-coated platinum nanoparticles (PVP-PtNPs) with nominal size of 20 nm, 40 nm, and 70 nm ($PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$, respectively) were synthesized using a seed-mediated growth approach[49] and briefly described in ESI.† The synthesized PtNPs were thoroughly characterized using dynamic light scattering (DLS), atomic force microscopy (AFM), energy dispersive X-ray (EDX) coupled transmission electron microscopy (TEM), and single particle inductively coupled mass spectroscopy (sp-ICP-MS) as reported elsewhere.[49] The concentrations of $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$ stock suspensions were 24.5 ± 0.6, 23.4 ± 1.2, and 24.8 ± 0.7 mg-Pt per L, respectively.

Test organism

Leptocheirus plumulosus is a euryhaline, infaunal amphipod that reaches densities of $>2.5 \times 10^4$ individuals m^{-2} in muddy estuaries along the east coast of North America.[50] *L. plumulosus* is a facultative suspension and surface-deposit feeder[51] that ingests a wide range of particle types and sizes. *L. plumulosus* also serves as an important prey organism for fish and shellfish,[52] and is a sensitive species commonly used as a USEPA approved standard test species in acute[53] and chronic[45,54] sediment toxicity tests in the USA.

Leptocheirus plumulosus were cultured in muddy sediments in the laboratory following USEPA testing standard protocols.[45] All cultures and treatments used 15 parts per thousand (ppt) synthetic seawater (SW) prepared with Crystal Seas® bioassay grade sea salts (Marine Enterprises International, Baltimore, MD, USA) followed by aeration of >90% O_2 saturation and then filtered twice using 0.45 μm filter. The pH of these waters was 8.1 ± 0.1 throughout the study. For each uptake experiment, adult amphipods were harvested by passing mixed-aged cultures through a 2 mm sieve and sorting adults retained on a 500 μm sieve. Similar size/age adults were transferred onto a 600 μm sieve partially submerged in a 2 L crystallizing dish filled with 15 ppt SW agitated continuously by an X-shaped stir bar placed underneath the sieve. The amphipods were allowed to depurate/defecate through the sieve for 2 h in darkness before being collected and allocated to 50 mL exposure chambers. *L. plumulosus* used in this study were sized as closely as possible to one another using stereomicroscopy, with a mean dry weight of 1.68 ± 0.32 mg (n = 225).

Waterborne PtNP uptake experiments

Two sets of uptake experiments were performed in this study without food additions. In the first uptake experiment, *Leptocheirus plumulosus* (n = 3 amphipods in 5 replicates of each PtNP suspension) were exposed to 45 mL of 25 μg L^{-1} individual $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$ suspensions in 50 mL crystallizing dishes for 72 h (Table S1†). These exposure concentrations were selected as the lowest concentrations that allow detection by sp-ICP-MS of the accumulated PtNPs following extraction five fold higher than the background PtNP number concentrations detected by sp-ICP-MS (Table S2†). In the second uptake experiment, *L. plumulosus* similarly were exposed to mixtures of equal mass-, surface area-, number-concentrations of $PtNP_{40}$ and $PtNP_{70}$ (Table S1†). The equal mass, surface area, and number concentration mixtures were prepared by mixing 25 μg L^{-1} NP_{40} with 25, 64.3, and 98.3 μg L^{-1} NP_{70}, respectively. Each treatment consisted of 5 replicates (15 amphipods per treatment), and each test included an equivalent zero-dose control (*i.e.*, no PtNP). Zero amphipod mortality occurred in any of the treatments or controls. Each glass chamber was covered and placed in a temperature regulated incubator at 25 °C with 12 : 12 h light:dark photoperiod according to EPA protocols.[45] Amphipods were collected at 6, 24, 48, and 72 h for total (*e.g.*, sum of dissolved and PtNP) Pt concentration and PtNP number and mass concentration determinations and size distribution analyses as described below. Amphipods were removed from the test chambers, rinsed in 3 successive baths of 15 ppt seawater (SW), and transferred to glass chambers containing 45 mL fresh 15 ppt SW for a 2 h elimination period to purge gut contents before being washed thoroughly once in ethylene diamine tetra-acetic acid (EDTA) and twice in ultrapure water (UPW). Amphipods were then blotted dry and immediately weighed (exactly at 60 s post-removal) and suspended in 5 mL UPW for particle extraction for sp-ICP-MS analysis. Dry weights of amphipods were calculated from measured wet weights using a simple regression between wet and dry weights (Fig. S1†). Water samples were collected at the middle point of the water column from each exposure chamber at 0 and 72 h to monitor potential changes (*e.g.*, size distribution, dispersity, aggregation, and number concentration) in PtNP exposure during uptake experiments. The test water and amphipods were frozen at −80 °C and later thawed to room temperature before analysis using ICP-MS and sp-ICP-MS.

Elimination experiments

A separate set of experiments was performed to determine the elimination (efflux rates) of PtNPs from *L. plumulosus* following a 24 h exposure to 100 μg L^{-1} PtNPs. An intentionally higher exposure concentration was used in the elimination experiment *versus* the uptake experiment to ensure that *L. plumulosus* accumulated a sufficient amount of PtNPs within their bodies to be detectable by sp-ICP-MS over the full 48 h elimination period. As previously, *L. plumulosus* of similar sizes (retained on a 500 μm sieve) were collected from lab cultures, followed by gut purging in freshly prepared 15 ppt SW for 2 h to empty the contents of the digestive tract. Amphipods were then transferred for 24 h to 15 new glass chambers containing 45 mL each of 15 ppt SW spiked with 100 μg L^{-1} $PtNP_{20}$, $PtNP_{40}$, or $PtNP_{70}$ without food. Each glass chamber was covered and incubated in a temperature-regulated incubator at 25 °C with 12:12 h light:dark photoperiod. Each of 3 treatments consisted of 5 replicates and each replicate consisted of 3 *L. plumulosus* (n = 45 total). No mortality was observed in any of the experiments. After 24 h, amphipods were removed from the test chambers, rinsed in 3 successive baths of 15 ppt SW, followed by 2 h gut purging. The amphipods were then transferred to glass chambers containing 45 mL freshly-prepared 15 ppt SW without food for a 48 h dissolved Pt and PtNP elimination period. The water was renewed every 12 h during the elimination experiment to minimize the reuptake of depurated Pt or PtNPs from the water column. The amphipods were collected at 0, 6, 12, 24, 36, and 48 h, and washed thoroughly once in EDTA and twice in UPW. The amphipods were immediately weighed as before, and finally suspended in 5 mL UPW. The dry weight of amphipods was calculated from regressed wet weight (Fig. S1†). Water

samples were also collected during the elimination experiment at the middle point of the water column from each elimination chamber at 0, 6, 12, 24, 36, and 48 h to monitor potential changes in NP size distribution, aggregation, and number concentration. The water and amphipods were frozen to −80 °C upon collection and thawed to room temperature before analysis in ICP-MS and sp-ICP-MS.

PtNP extraction from *L. plumulosus*

PtNPs were extracted by probe sonication of amphipods in UPW using a Q700 probe-sonicator (QSonica, Newtown, CT). The PtNP extraction procedure was optimized for PtNP recovery by varying the number of amphipods (*e.g.*, 1, 3, 5, and 10, Table S3a†) in each extraction replicate, sonication times (*e.g.*, 1, 5, and 10 minutes, Table S3b†), and centrifugation forces (*e.g.*, 100 × *g* and 2000 × *g* using Eppendorf 5810R, Table S3c†) needed to isolate amphipod tissues. Total Pt concentration in the supernatant and the pellet were determined by ICP-MS. The overall performance of the extraction procedure was determined by comparing the Pt concentrations in the supernatant and the pellet (consisting mainly of the exposed amphipods' tissue). Equal Pt concentrations in the pellet and the supernatant indicates that PtNPs were fully released from the organisms. Higher concentrations of PtNPs in the pellet indicates that PtNPs remained attached to the organism tissues and ended up being more concentrated in the pellet. The optimal extraction conditions (*i.e.*, Pt concentrations were the same in the supernatant and pellet) were determined as 3 *L. plumulosus* per replicate, 5 min pulse sonication with 2 seconds on and 1 second off pulsing at room temperature, and low gravity centrifugation at 100 *g*. Under these extraction conditions, the Pt concentration in the supernatant and the tissue pellet (50.0 ± 1.4 and 51.9 ± 2.1 μg L^{-1}, respectively) were not statistically different (*t*-test, $p > 0.8$; Table S3c†), inferring the successful extraction of PtNPs from the amphipod tissue into the suspension and the lack of PtNP sedimentation during the centrifugation step.

PtNP concentration measurement by single particle ICP-MS

Prior to sp-ICP-MS analysis, all extracted tissue samples were bath sonicated (Branson, Model 2800, 40 kHz, USA) for 30 min to breakdown NP aggregates, followed by 100-fold dilution to minimize the introduction of organic matter to the ICP-MS. Aqueous samples were diluted 100 fold without sonication to prevent aggregate breakup. The number and mass concentrations of the extracted PtNPs and the aqueous samples were measured using sp-ICP-MS following methods described elsewhere.[55–57] All samples were analyzed using a PerkinElmer NexION™ 350D ICP-MS (Waltham, MA, USA) equipped with a Meinhard glass concentric nebulizer, a PerkinElmer glass cyclonic spray chamber, and 2 mm ID quartz injector. Instrument calibration was performed using a blank and at least four Au and Pt standards ranging from 0.5 to 20 μg L^{-1}. All data were collected in single particle mode, with signals averaged for the entire analysis period (60 s). ICP-MS nebulization efficiency was determined daily using the particle size method[56] with 60 nm NIST™ AuNP standard reference material (actual TEM size of 56 nm; reference material 8013 manufactured by the National Institute of Standard and Technology, MD, USA) and varied between 4% and 6% for all experiments described herein. Data were acquired at an RF power of 1600 W, a 50 μs dwell time, a 0 μs settling time, and a 60 s acquisition time. 1.2×10^6 readings were collected for each sample during 60 s acquisition time per sample. Particle number concentration was determined directly by counting all PtNPs measured by sp-ICP-MS in a given sample, while PtNP mass concentration was determined by integrating the masses of all the particles measured by sp-ICP-MS following the procedure described by Pace *et al.*[55,56] Recoveries, total mass determinations, and body burdens were reported as the mean and standard error of 5 replicates.

Total Pt concentration measurement by ICP-MS

Total Pt concentration was measured by ICP-MS following digestion of the extracted PtNPs in a concentrated *aqua regia* (HNO_3 : HCL = 1 : 3 M) one day before analysis. Briefly, 500 μL aliquots of the extracted PtNPs were transferred to Eppendorf tubes followed by addition of 1 mL of a freshly prepared concentrated *aqua regia*. The tubes were incubated under the hood at room temperature for 24 h. Samples were diluted at least 5 times prior to ICP-MS analysis.

Biodynamic model

Biodynamic modeling (BDM) is a popular approach for deconstructing metal accumulation as it quantifies its mechanistic components.[58] BDM describes bioaccumulation as the net outcome of uptake (*i.e.,* influx), physiological elimination (*i.e.*, efflux), and body growth dilution (if any). eqn (1) describes the changes in Pt concentration in an organism ($[Pt]_{org}$) overtime after a waterborne-only exposure:

$$\frac{[Pt]_{org}}{dt} = k_{uw} \times [Pt]_{water} - k_e \times [Pt]_{org} - k_g \times [Pt]_{org} \quad (1)$$

where, k_{uw} (L g^{-1} d^{-1}) is the unidirectional Pt influx rate constant from solution/suspension, $[Pt]_{water}$ (μg L^{-1}) is the dispersed PtNP concentration in the solution/suspension, and k_e (d^{-1}) and k_g (d^{-1}) are the rate constants for physiological elimination and body growth dilution, respectively. k_g is negligible for *Leptocheirus plumulosus* for the short experimental period (Fig. S2†). Therefore, growth dilution was not considered in determining influx rate constants. k_{uw} was determined by dividing uptake rate by exposure concentration, assuming the uptake rate follows first-order kinetics for the exposure concentration used[59] and k_e is determined using a nonlinear regression that includes compartments representing fast and slow exchanging pools

(eqn (S1)†). Further details and the parameterization of these rate constants is described in the ESI.†

Statistical analysis

Statistical analyses were performed with SAS® version 9.4 software (SAS institute, Cary, NC, United States). Correlation coefficients between $PtNP_{70}$ concentrations in the exposure medium and those accumulated in the amphipods after 48 h exposure were calculated using Pearson's correlation method. Fisher's *Z*-transformation was used to compute confidence intervals for each correlation, followed by *z*-tests to calculate *p*-values for correlation comparisons between every two mixture combinations. The particle size distribution (PSD) of PtNPs in the exposure medium and those accumulated in the amphipods were analyzed using Kolmogorov–Smirnov (K–S) tests with Bonferroni correction to compare size distributions of NPs in exposure and concurrent accumulation. In all cases, statistical significance was set at p-value < 0.05.

Results and discussions

PtNP characterization and behavior in SW

The physiochemical properties of PVP-PtNPs were determined using DLS, TEM, and sp-ICP-MS[49] (Fig. S3 and Table S4†). Briefly, the mean core diameters of $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$ measured by TEM were 18.5 ± 5.0 nm, 44.5 ± 5.0 nm, and 72.5 ± 3.9 nm, respectively. The energy dispersive X-ray (EDX) spectra exhibited three peaks corresponding to platinum M-shell (2.05 keV), Lα-shell (9.44 keV), and Lβ-shell (11 keV, Fig. S3f–h†), confirming the chemical composition of PtNPs. The core diameters of $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$ measured by sp-ICP-MS were 24.7 ± 1.6 nm, 42.9 ± 0.8 nm, and 77.1 ± 0.8 nm, in close agreement with those measured by TEM. The *z*-average hydrodynamic diameter of $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$ were 51.0 ± 0.7 nm, 74.7 ± 0.2 nm, and 93.4 ± 1 nm, respectively. The magnitude of the zeta potential of PtNPs increased from 22.9 ± 1.4 to 27.2 ± 1.7 mV with the increase in PtNP size.

DLS is the most commonly used analytical technique to measure NP aggregation. However, the low PtNP concentration (*i.e.*, 25 μg L^{-1}) used in this study hampers the use of DLS to measure PtNP aggregation because it is not possible to reliably measure NP hydrodynamic diameter by DLS at such low concentrations. Therefore, the aggregation behavior of PtNPs (*i.e.*, $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$) in SW was monitored by measuring their number concentration and number size distribution at 0 h and 72 h following mixing with SW using sp-ICP-MS (Fig. 1). PtNPs remained dispersed as single particles immediately (0 h) after mixing with SW. After 72 h of mixing PtNPs with SW, the number concentration of primary PtNPs decreased (Fig. 1, Table S5†) due to aggregate formation with aggregate sizes of 30 to 130 nm, 70 to 200 nm, and 90 to 250 nm for $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$, respectively. The PVP-PtNPs used in this study have

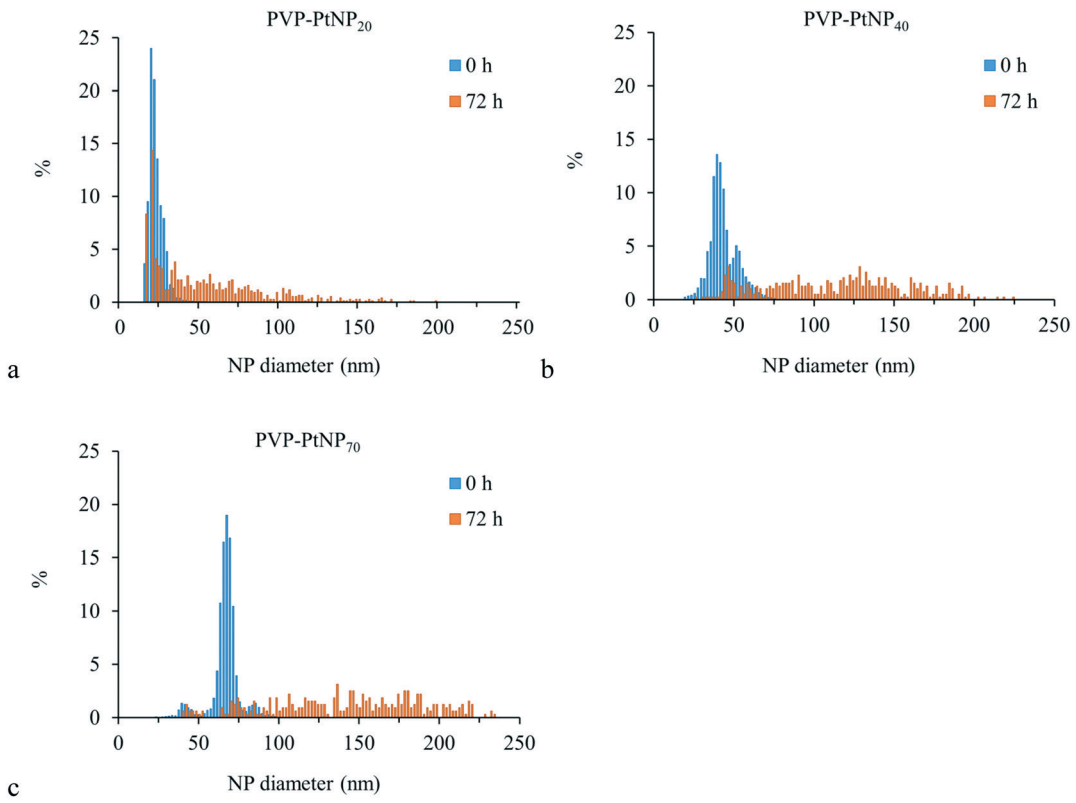

Fig. 1 Number particle size distribution of PVP-PtNPs at 0 and 72 h post mixing of 25 μg L^{-1} PtNPs with 15 ppt synthetic seawater without amphipods for (a) $PtNP_{20}$, (b) $PtNP_{40}$, and (c) $PtNP_{70}$. All measurement were performed by SP-ICP-MS.

been shown (using DLS and high PtNP concentrations) to aggregate in SW due to surface charge screening by high counterions (mainly Na^+) concentrations in SW.[60]

Uptake of PtNPs

Following exposure to individual PtNP size groupings, all PtNPs accumulated in *L. plumulosus* generating body burdens that were significantly greater than controls (ANOVA, $p < 0.001$). The whole-animal body burden of $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$ in *L. plumulosus* from waterborne exposure of 25 μg-Pt per L increased exponentially as a function of exposure time (Fig. 2a). The $PtNP_{20}$ showed a steeper exponential uptake than $PtNP_{40}$, which in turn showed steeper exponential uptake than $PtNP_{70}$. The whole animal body burden reached 47.4 ± 7.4, 15.8 ± 4.5 and 8.3 ± 1.8 μg Pt per g dry wt organism after 72 h exposures to $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$, respectively (Table S6†). The whole-animal Pt body burden from the three PtNP exposures after 72 h was three orders of magnitude greater than the amphipod's background Pt body burden of 11.31 ± 0.01 ng g^{-1} dry weight. *L. plumulosus* did not gain weight during the 72 h uptake period (Fig. S2†). Therefore, growth dilution was not considered in determining influx rate constants. The influx rate constants (k_{uw}s, mean ± SE) from waterborne exposures were 0.042 ± 0.006, 0.040 ± 0.009, and 0.026 ± 0.003 μg g^{-1} d^{-1}, respectively for $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$ (Table S6†), indicating decreases in PtNP uptake with increase in PtNP size. The decrease in PtNP influx rate constant with increases in NP size might be attributed to the formation of larger PtNP aggregates (Fig. S4a–c†) and correspondingly faster sedimentation from the water column, consistent with previous studies.[6,61] The measured size distribution of PtNPs in the exposure seawater shifted toward larger sizes and the number concentration decreased over time indicating particle aggregation (Fig. S4a–c†). Additionally, the mass concentration of $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$ in the exposure seawater decreased by 15.4 ± 3.2, 51.3 ± 3.5, and 72.5 ± 6.8%, respectively after 72 h indicating increased PtNP losses with increased PtNP size due to particle uptake and/or sedimentation (Fig. S4d–f†). However, the mass balance of the test system revealed that only a small fraction (*e.g.*, 3.8 ± 0.2, 6.8 ± 0.2, and 3.6 ± 0.2%) of total $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$, respectively, accumulated in *L. plumulosus* after 72 h, implying very low depletion of PtNPs from the exposure suspensions *via* organismal uptake (Table S7†). Since PtNP concentrations in exposure chambers were not depleted by amphipod uptake, the decline in PtNP mass concentration can be attributed to particle sedimentation which in turn increases with increased NP size.

Pt accumulation in *L. plumulosus* was mainly due to the nano-form of Pt rather than the dissolved Pt fraction as the PtNP body burden, measured by sp-ICP-MS after 72 h exposure, and representing >70% of the total Pt body burden for $PtNP_{20}$ and >90% of the total Pt body burden for both $PtNP_{40}$ and $PtNP_{70}$ (Tables S8–S10†). Additionally, the dissolved Pt concentration represented <5% of the Pt exposure concentration[49] which is very unlikely to produce a residual sorption of Pt ions onto the surfaces of the organism sufficient to result in their detection as single particle events. The lower PtNP body burden relative to the total Pt body burden for $PtNP_{20}$ compared to $PtNP_{40}$ and $PtNP_{70}$ likely is due to the size-detection limitations of sp-ICP-MS. Only 46% of the primary PtNPs in the $PtNP_{20}$ stock suspension were larger than the sp-ICP-MS size detection limit (*e.g.*, 17 nm for PtNPs),[48] thus 53% of primary PtNPs in the $PtNP_{20}$ stock suspension were not detected by sp-ICP-MS. However, all primary PtNPs in $PtNP_{40}$ and $PtNP_{70}$ stock suspensions were larger than the sp-ICP-MS size detection limit (Table S4†).[62] The higher than expected recovery of $PtNP_{20}$ after NP accumulation in *L. plumulosus* was due to the formation of PtNP aggregates larger than the sp-ICP-MS size detection limit in the test seawater (Fig. S5a and b†).[62] Overall, these results indicate that the extraction procedure, which was validated for $PtNP_{70}$ only, provides accurate results for all PtNPs used in this study. The lower recovery of $PtNP_{20}$ was most likely due to the sp-ICP-MS size detection limit rather than due to the extraction procedure.

Based on mass concentration, the accumulation of $PtNP_{20}$ exceeded the accumulation of $PtNP_{40}$ and $PtNP_{70}$ by 3 and

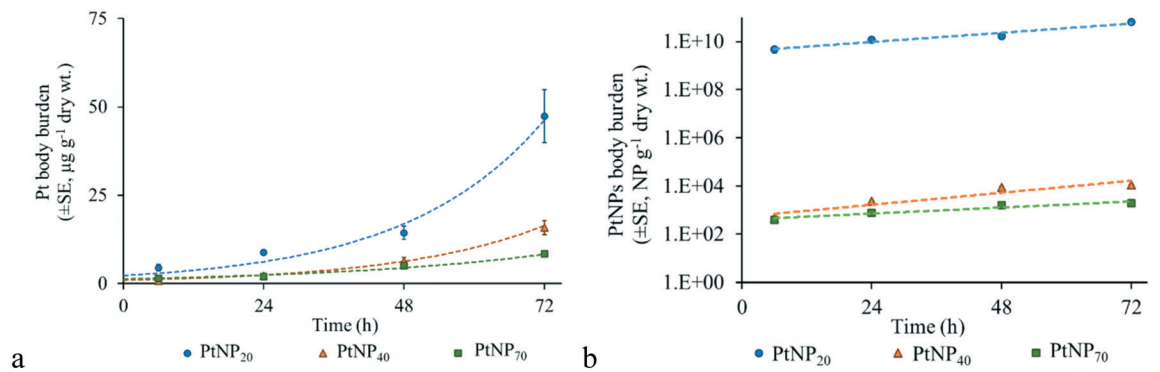

Fig. 2 Platinum influx into *Leptocerious plumulosus* soft tissues after waterborne exposure to 25 μg L^{-1} $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$ for 72 h. PtNPs are presented as (a) platinum mass influx (μg g^{-1}) and (b) PtNPs number influx (NP g^{-1}). All measurement were performed by SP-ICP-MS. Data points represent mean and standard errors.

5.7 fold after 72 h of exposure (Fig. 2a). However, based on particle number concentration, the accumulation of $PtNP_{20}$ exceeded the accumulation of $PtNP_{40}$ and $PtNP_{70}$ by 6 and 7 orders of magnitude after 72 h of exposure. The whole-animal body burden of PtNPs (based on number concentration) also increased exponentially with time (Fig. 2b) and the number of PtNPs accumulated in *L. plumulosus* followed the trend of $PtNP_{20}$ (6.5×10^{10} $PtNPs_{20}$ per g dry weight) > $PtNP_{40}$ (1.1×10^{4} PtN_{40} per g dry weight) > $PtNP_{70}$ (1.9×10^{3} $PtNP_{70}$ per g dry weight). For a fixed mass concentration (25 μg L^{-1}), the number of NPs in a $PtNP_{20}$ suspension is much higher than that in a $PtNP_{70}$ suspension (Table S5†). Therefore, NPs were more abundantly available for uptake from $PtNP_{20}$ suspensions than from $PtNP_{40}$ and $PtNP_{70}$ suspensions, resulting in the observed higher $PtNP_{20}$ influx into *L. plumulosus* relative to $PtNP_{40}$ and $PtNP_{70}$ (Fig. 2b). This relationship produced a higher body burden for the smaller PtNPs. Collectively, mass- and number-concentration based PtNP body burdens decreased with increases in PtNP size. A similar decrease in NP uptake with increases in particle size was observed for other filter feeders and deposit feeders with silver oxide, copper oxide, and gold NPs.[63,64] Similarly, lower body burdens were observed for smaller (30 nm and 60 nm) *versus* larger (80 nm and 100 nm) AuNPs in *C. elegans*.[23,24] The lower body burden of $PtNP_{70}$ compared to smaller PtNPs (*i.e.,* $PtNP_{40}$ and $PtNP_{20}$) might be due to the formation of larger aggregates in SW (Fig. 1 and S4a–c†). The faster sedimentation of these larger aggregates from the water column (Fig. S4d–f†) and/or inherently greater difficulties passing larger aggregates through cell membranes likely render larger PtNPs less bioavailable to *L. plumulosus*.

Our previous study revealed that the majority of PtNPs remained in particulate form as shown by the low concentration of the dissolved Pt in the water relative to the amount of PtNPs added.[49] Therefore, particle ingestion is likely a major route of uptake of all PtNP sizes. Uptake of metal oxide NPs and quantum dots *via* ingestion was shown previously for *L. plumulosus*.[65,66] CdSe/ZnS quantum dots accumulated to a greater extent than their respective dissolved ions in *L. plumulosus*.[65] Estuarine benthic invertebrates accumulate metals from their surrounding environment through two pathways: dissolved metals are accumulated *via* passage across permeable membranes,[43] whereas particle-associated metals are accumulated mostly by ingestion.[67] *L. plumulosus* is capable of both suspension feeding (by ingestion of planktonic and suspended benthic microalgae) and surface deposit feeding (by ingestion of sediment, detritus, phytodetritus, and benthic microalgae).[51] Without sediment, *L. plumulosus* feeds on suspended particles; when burrowed, it selects food particles from the water column and from surface sediments. *L. plumulosus* can ingest up to 3 times its body weight in sediment per day.[68] *L. plumulosus* are filter feeders, enabling them to ingest particles from the size of 70 μm down to around 200 nm.[69] In most cases, larger particles (*e.g.*, microalgae) are consumed most efficiently. The ingestion of PtNPs by *L. plumulosus* is not surprising as all PtNP treatments formed aggregates in 15S seawater with equivalent spherical core diameters of up to 130 to 250 nm (Fig. 1). Aggregate hydrodynamic diameters may actually be much larger as NPs form porous aggregates. These aggregate sizes are within the lower size range of the food particles filtered/ingested by *L. plumulosus*, thus promoting their passive incidental uptake and leading to measurable tissue accumulation. The possibility that additions of algal food might influence NP dispersivity and create unknown and variable aggregate NP: food diameters led to an *a priori* decision to eliminate suspended food as a factor in these experiments. Future studies should manipulate food quality and quantity as test variables in the matrix. In the present study, this additional manipulation increased the experimental matrix considerably, and was beyond the scope of present resources.

Elimination of PtNPs

The whole-animal Pt body burden following 24 h exposure to 100 μg L^{-1} of $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$ in seawater reached 18.3 ± 4.5, 22.2 ± 3.8, and 28.1 ± 3.8 μg Pt per g dry wt organism, respectively. After this 24 h exposure, the whole animal Pt body burden decreased with time (Fig. 3a). Generally, *L. plumulosus* displayed two elimination phases for PtNPs: an initial, rapid loss (0–6 h), and a second, slower loss (6 to 48 h) (Fig. 3a). After 6 h of depuration, the whole animal mean body burdens dropped to 11.4 ± 3.4, 18.7 ± 8.2, and 14.3 ± 6.2 μg Pt g^{-1} dry wt organism for $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$, respectively – a decrease of 38 ± 2 to 49 ± 3% of the accumulated PtNPs. Between 6 and 48 h, the whole-animal body burden of PtNPs decreased linearly and reached 8.5 ± 3.6, 6.1 ± 1.9, and 4.5 ± 4.6 μg Pt per g dry wt organism for $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$, a decrease of 54%, 72% and 84% of the initially accumulated PtNPs over 48 h of depuration, respectively. A similar depuration behavior has been observed for Cd release from *L. plumulosus* after ingestion/accumulation of Cd from pelagic and benthic microalgae.[70] These results indicate that *L. plumulosus* depurated larger NPs more efficiently than smaller NPs. This behavior is in good agreement with the observed increased elimination with increasing particle size of citrate-coated AuNPs and polystyrene NPs by *D. magna*.[9,64] The residual (*i.e.*, non-depurated) animal body burdens after 48 h of depuration represent 47 ± 2%, 28 ± 1%, and 16 ± 2% of the accumulated $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$ after 24 h exposures to 100 μg L^{-1} of PtNP, respectively (Fig. 3a). It is worth noting that after 48 h of depuration, the residual body burden was approximately two orders of magnitude higher than the measured background body burden in non-exposed animals. The efflux rates (k_e) were 0.31 ± 0.08, 0.66 ± 0.04, and 0.83 ± 0.07 d^{-1}, respectively, for $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$, indicating increased depuration with increases in NP size (Fig. 3a).

Based on mass concentration, the retention of $PtNP_{20}$ exceeds the retention of $PtNP_{40}$ and $PtNP_{70}$ by 1.4 and 1.9 fold after 72 h of depuration (Fig. 3a). However, based on

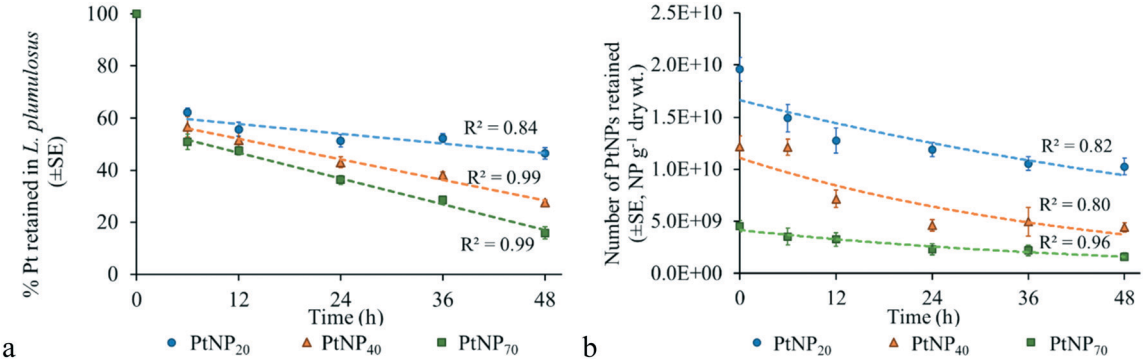

Fig. 3 Retention of accumulated Pt in *L. plumulosus* after 24 h waterborne exposure to 100 μg L^{-1} $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$ presented as (a) mass based retention of Pt, and (b) NP number based retention of Pt (NP g^{-1}). All measurement were performed by SP-ICP-MS. Data points represent mean and standard errors.

particle number concentration, the retention of $PtNP_{20}$ exceeds the retention of $PtNP_{40}$ and $PtNP_{70}$ by 2.3 and 6.4 fold after 48 h of depuration (Fig. 3b). Similar depuration trends were observed based on number concentration analysis as were observed for mass concentration analysis. Initially, *L. plumulosus* accumulated $2 \times 10^{10} \pm 2.5 \times 10^{9}$ $PtNP_{20}$ per g dry wt, $1.2 \times 10^{10} \pm 2.4 \times 10^{9}$ $PtNP_{40}$ per g dry wt, and $4.5 \times 10^{9} \pm 1.2 \times 10^{9}$ $PtNP_{70}$ per g dry wt after 24 h exposure to 100 μg L^{-1} PtNPs. *L. plumulosus* retained 1×10^{10} $PtNP_{20}$, 4.4×10^{9} $PtNP_{40}$, and 1.6×10^{9} $PtNP_{70}$ per g dry wt after 48 h depuration (Fig. 3b). *L. plumulosus* gained weight during the 48 h depuration period (Fig. S6†). However, the growth rate constants (k_g) were minimal *i.e.*, 0.002 ± 0.001, 0.005 ± 0.002, and 0.007 ± 0.002 d^{-1} for $PtNP_{20}$, $PtNP_{40}$, $PtNP_{70}$ treatments, respectively. Therefore, no adjustment was made in k_e due to growth dilution.

The decrease in PtNP elimination with decreases in size might be attributed to increased NP internalization and tissue/cell translocation with decreases in NP size[71] Therefore, increased accumulation of PtNPs with decreases in size appears to be a result of a combination of increased influx and decreased efflux, which may in turn contribute to increased toxicity for smaller NPs if size-dependent cell and tissue translocation is real.

Depurated PtNPs were measured in the water column at the end of each water renewal period during the depuration experiment. The number particle size distribution of each PtNP (Fig. S7a–c†) was similar to that in the corresponding original PtNP suspension (K–S test, $p > 0.22$). The number and mass concentrations at the end of each water renewal period decreased over time (Fig. S7d–f†). The total mass of the PtNPs detected in the water column during the elimination experiment was 0.024 ± 0.008, 0.031 ± 0.001, and 0.103 ± 0.02 μg for $PtNP_{20}$, $PtNP_{40}$, and $PtNP_{70}$, respectively, and accounted for 41 ± 1.9, 40.2 ± 0.3, and 74.9 ± 1.0% of the total depurated PtNPs (Table S11†). The remaining fractions might have been lost from the suspensions by sedimentation or organism re-uptake. In the absence of food, the depurated PtNPs may be available for re-uptake, which may contribute to slower observed depuration over time.

NP uptake from mixture of PtNPs: effect of size dispersity

When exposed to a mixture of $PtNP_{40}$ and $PtNP_{70}$ of equal mass, surface area, and number concentrations (Fig. 4a–c), *L. plumulosus* accumulated both PtNP size distributions, and the number of accumulated PtNPs increased with time (Fig. 4d–f). *L. plumulosus*, accumulated significantly higher numbers of $PtNP_{40}$ than $PtNP_{70}$ (K–S test, $p < 0.05$) from all mixtures (equal mass, surface area, or number concentrations). This is consistent with the higher accumulation of $PtNP_{40}$ than $PtNP_{70}$ observed in the exposures to individual NPs (Fig. 2). The higher accumulation of $PtNP_{40}$ than $PtNP_{70}$ from equal mass (Fig. 4d) and surface area (Fig. 4e) mixtures might be attributed to the higher number concentration of $PtNP_{40}$ than for $PtNP_{70}$ (Fig. 4a and b) and/or higher accumulation of $PtNP_{40}$ than $PtNP_{70}$ in the amphipod exposures. The higher accumulation of $PtNP_{40}$ than $PtNP_{70}$ from equal number concentration mixtures (Fig. 4f) is attributed to higher influx (Fig. 2) and lower depuration (Fig. 3) of $PtNP_{40}$ compared to the $PtNP_{70}$. These results clearly indicate that in a mixture of PtNPs, smaller PtNPs accumulate to a higher extent than larger PtNPs, even if the number concentration of both sizes are the same in each exposure.

The accumulation of $PtNP_{70}$ from equal surface area- and number-concentration mixtures (Fig. 4e and f) increased compared to the accumulation of $PtNP_{70}$ from equal mass concentration mixtures (Fig. 4d). This can be attributed to the increased number of $PtNP_{70}$ in equal surface area- and number-concentration mixture exposures (Fig. 4b and c) compared to equal mass concentration mixture exposures (Fig. 4a). Conversely, $PtNP_{40}$ accumulation from these three mixtures remained constant despite the increase in $PtNP_{70}$ number concentrations. Therefore, the increased exposure concentration and accumulation of larger PtNPs (*i.e.*, $PtNP_{70}$) did not affect the accumulation of smaller PtNPs (*i.e.*, $PtNP_{40}$), and the presence of larger NPs does not impact the accumulation of smaller NPs in a polydipsersed mixture. It is worth noting that all PtNP mixtures in our experiments formed aggregates in 15S seawater as indicated by the decrease in particle number concentration (Fig. S8–S10†).

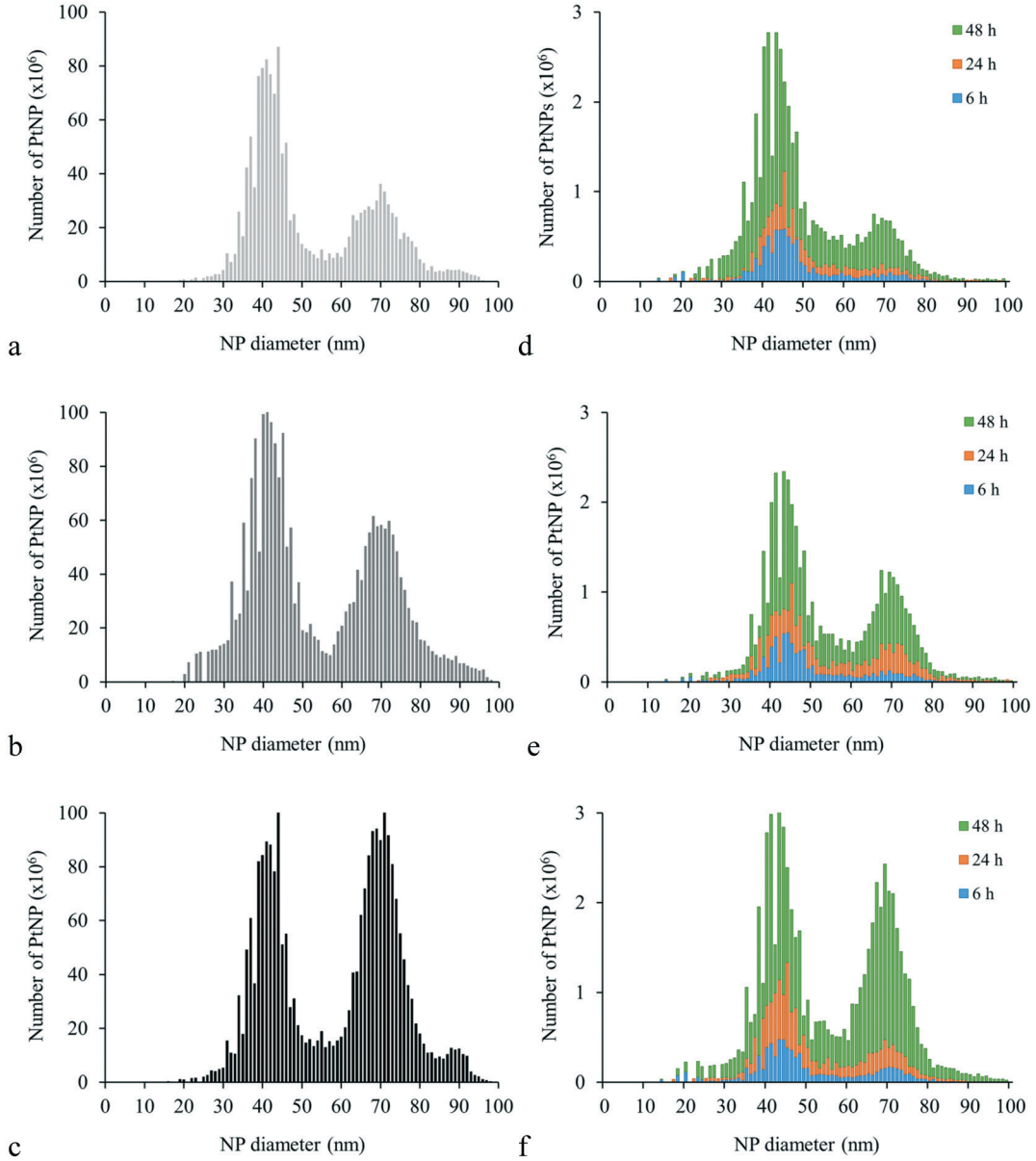

Fig. 4 (a–c) Number particle size distribution of and (d–f) the corresponding PtNP accumulation from mixtures of $PtNP_{40}$ and $PtNP_{70}$ containing: (a and d) the same mass of $PtNP_{40}$ and $PtNP_{70}$ (25 μg L^{-1} $PtNP_{40}$ + 25 μg L^{-1} $PtNP_{70}$), (b and e) the same total surface area of $PtNP_{40}$ and $PtNP_{70}$ (25 μg L^{-1} $PtNP_{40}$ + 64.25 μg L^{-1} $PtNP_{70}$), and (c and f) the same total number of $PtNP_{40}$ and $PtNP_{70}$ (25 μg L^{-1} $PtNP_{40}$ + 98.25 μg L^{-1} $PtNP_{70}$). All measurement were performed by SP-ICP-MS.

The present study demonstrated that the common estuarine amphipod *L. plumulosus* will accumulate PtNPs of different sizes (*e.g.*, 20 nm, 40 nm, and 70 nm) in seawater-only exposures without food. It also demonstrated that mass and number uptake increases with decreases in NP size. All PtNPs were depurated to varying degrees, with depuration increasing with increases in NP mean size. *L. plumulosus* retained/accumulated higher amounts of small PtNPs than larger PtNPs as a result of a combination of increased influx and decreased efflux of smaller PtNPs. The residual whole-animal body burden of PtNPs after 48 h depuration was higher for treatments of reduced NP size, with residual body burdens about two-fold larger for animals exposed to $PtNP_{20}$ than for animals exposed to $PtNP_{70}$. When exposed to mixtures of small and large PtNPs of equal masses, surface areas, or number concentrations, *L. plumulosus* accumulated higher numbers of the small PtNPs than large PtNPs from all mixture combinations. Additionally, an increased exposure concentration of larger PtNPs did not affect the uptake of smaller PtNPs from a mixture of NPs of different sizes. The increased retention of small NPs compared to larger ones might partially explain the higher toxicity of small NPs compared to larger ones observed in other studies using NPs of more toxic metals. Filter and suspension-feeding amphipods are an important food source for many marine and freshwater birds, fish and macro-invertebrates and thus

may contribute to metal-NPs accumulation by predatory organisms that otherwise may not be exposed. In addition to the inherent and largely well-studied toxic effects of most metals used in NP manufacture today, this study further suggests that NP size spectra in aquatic environments is an important additional parameter to consider in NP ecological risk assessment.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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