Not all that glitters is gold

Electron microscopy study on uptake of gold nanoparticles in Daphnia magna and related artifacts

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NOT ALL THAT GLITTERS IS GOLD – ELECTRON MICROSCOPY STUDY ON UPTAKE OF GOLD NANOPARTICLES IN *DAPHNIA MAGNA* AND RELATED ARTEFACTS

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NOT ALL THAT GLITTERS IS GOLD – ELECTRON MICROSCOPY STUDY ON UPTAKE OF GOLD NANOPARTICLES IN *DAPHNIA MAGNA* AND RELATED ARTEFACTS

Running title: Analysis of gold nanoparticles in *Daphnia magna* gut

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Abstract: Increasing use of engineered nanoparticles has led to extensive research into their potential hazards to the environment and human health. Cellular uptake from the gut is sparsely investigated and microscopy techniques applied for uptake studies can result in misinterpretations. Various microscopy techniques are used to investigate internalization of 10 nm gold nanoparticles in Daphnia magna gut lumen and gut epithelial cells upon 24h exposure and outline potential artefacts, i.e. high contract precipitates from sample preparation related to these techniques. Light sheet microscopy confirmed accumulation of gold nanoparticles in the gut lumen. Scanning transmission electron microscopy and elemental analysis revealed gold nanoparticles attached to the microvilli of gut cells. Interestingly, the peritrophic membrane appeared to act as a semipermeable barrier between the lumen and the gut epithelium, permitting only single particles through. Structures resembling nanoparticles were also observed inside gut cells. As elemental analysis could not verify these to be gold they were likely artifacts from the preparation, such as osmium and iron. Importantly, gold nanoparticles were in fact found inside holocrine cells with disrupted membranes. Thus, false positive observations of nanoparticle internalization may result from either preparation artefacts or by mistaking disrupted cells for intact. These findings emphasize the importance of cell integrity and combining elemental analysis with the localization of internalized nanoparticles using transmission electron microscopy.

Keywords : Nanotoxicology, Nanoparticles, Freshwater toxicology, Aquatic invertebrates

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INTRODUCTION

Use of engineered nanoparticles (NPs) has increased rapidly over the past decade and consequently it is important to evaluate their environmental fate and potential effects. Most studies have focused on assessing toxicity of NPs, while less is known about bioaccumulation of these particles [1]. The uptake of NPs in aquatic organisms and in particular the cellular internalization following uptake are matters of crucial importance for understanding the potential effects of NPs.

Electron Microscopy (EM) is a well-established technique for imaging of biological samples, including cellular ultra-structures. High resolution images of biological structures can be achieved by EM, with chemically or cryo fixed biological specimens [2]. In addition EM has become a vastly used technique in the field of nanotechnology, for characterizing the primary particle size and morphology of NPs [3-5]. More recently, EM techniques are increasingly being applied for detection of NPs in biological samples, to examine uptake and internalization of NPs in cells of whole aquatic organisms [6-10]. While Garcia-Alonso et al. [7] observed cellular uptake of NPs from the gut in the estuarine polychaete *Nereis diversicolor* and Santo et al. in *D. magna* [10] most studies using EM imaging are inconclusive and/or report no or limited detection of NP internalization [6, 8, 9] albeit disturbed gut cells [8]. The use of EM techniques such as transmission electron microscopy (TEM) also involves the risk of misinterpretation of results obtained from microscopy of NP internalization. Edgington et al. [11] found structures similar to single-walled carbon nanotubes in *D. magna*. However, additional analyses using high resolution TEM, Energy Dispersive X-ray Spectroscopy (EDX) and selected area diffraction revealed that the observed structures were in fact either staining artefacts or amorphous carbon. Brandenberger et al. [12] studied the cellular uptake of Quantum Dots (QD) in murine macrophage-like cells and found electron dense structures inside the cells. With Electron Energy Loss Spectroscopy (EELS) analysis they showed that only one area out of the six that were originally

believed to contain quantum dots, did in fact contain them. The particle-like structures found in the other areas were probably either precipitates from the post staining or osmophilic structures within the sample [12].

This type of study underline that we are still in the infancy of imaging and characterizing the uptake of NPs in organisms. Furthermore, it shows that the understanding of the mechanisms and artefacts associated with assessment of *in vivo* distribution of NPs can be improved.

Daphnia magna is a widely used model organism in ecotoxicological studies due to their ecological relevance and limited maintenance requirements in the laboratory. Furthermore, they are ideal for bioaccumulation studies with NPs as they are transparent which enables visualization of accumulated material, including agglomerations of NPs, in the gut. In addition, their filter feeding behavior results in the filtration of large volumes of water, including suspended NPs. *D. magna* can filter particles down to around the size of 200 nm out of their feeding current. However, smaller particles are also available for uptake through either direct interception [13, 14] or by drinking the surrounding media [15, 16]. Previous studies have shown uptake and accumulation of Au NPs in the gut of *D. magna* either by measurements of accumulated Au [17] or based on observations of a darkly-colored gut [18-20]. In addition, Au NPs have been shown to be taken into the gut of *D. magna* by trophic transfer with *E. gracilis* and *C. reinhardtii* [21]. Hence, uptake into the gut is a likely route of exposure for NPs in *D. magna*.

Au NPs have several properties that make them ideal for bioaccumulation studies, including relatively low toxicity and high stability in suspension, *e.g.* limited dissolution and aggregation. In addition, their high mass density increases contrast and detection with EM.

The aim of the present study was to examine uptake of Au NPs into *D. magna* gut lumen and potential internalization into gut cells using multiple microscopy techniques: Light Sheet Microscopy (LSM), Focused Ion Beam Scanning EM (FIB-SEM), TEM, High-Angle Annular Dark-Field Scanning

Transmission EM (HAADF-STEM) and elemental analysis with EDX. In addition, the aim was to examine the potential artefacts related to microscopy which may lead to misinterpretation regarding NP internalization into cells.

MATERIALS AND METHODS

Nanoparticles

Citrate stabilized Au NPs in suspension were synthesized at the University of Alberta, Canada by Dr. Jonathan GC Veinot and Guibin Ma as described by Skjolding et al. [17]. The manufacturer of the Au NPs reported a primary size of 10 nm. The particles were non-aggregated as imaged by cryo-TEM of a prepared stock solution containing 40 mg Au/L in MilliQ water (Figure 1) and the size was validated by TEM and DLS before ingestion (Supplemental Data, Figure S1 and Table S2 [17]). Fluorescein isothiocyanate (FITC) labeled Au NPs were synthesized according to Jølck et al. [22] and used to allow imaging of NPs in whole organisms using LSM.

Exposure of Daphnia magna *to Au NPs*

The test suspensions for *D. magna* exposures were prepared immediately prior to exposure by adding the required amount of stock suspension into Elendt M7 medium [23] in a volumetric flask to a concentration of 0.4 mg Au/L. This concentration was used for all exposures and chosen from previous studies showing marked uptake at this concentration [17].

No stirring or ultra-sonication was applied. The *D. magna* was cultured at DTU Environment in Elendt M7 medium in a temperature-controlled room at 20 (±1) °C with a 12h:12h light: dark cycle.

D. magna neonates (< 24h old) were exposed to 0.4 mg Au/L for 24h in 100 ml beakers containing 25 mL test solution. In addition, controls without added Au NPs were included in each test series. Beakers with five *D. magna* neonates in each were incubated in the dark at 20 (±1) °C without feeding. After 24h exposure the animals were removed from the test beakers and gently rinsed in Elendt M7 medium to remove potential Au NPs adhered to the exoskeleton.

For LSM additional *D. magna* (n=3) was exposure to fluorescently labelled Au NPs in 1.5L aquariums filled with 1 L exposure solution. Exposure suspensions of 1 mg Au/L were prepared immediately prior to exposure, without the use of sonication or prolonged stirring. Exposed daphnia were sampled at different time points and analyzed in the LSM. Similarly, unexposed controls were also analyzed with LSM. Time points used for uptake phase were 1, 2, 4 and 24h. No feeding was carried out during the uptake phase.

Specimen preparation for EM

D. magna (both exposed animals and controls) were washed in sodium cacodylate buffer (0.15M), fixed in 2 % glutaraldehyde in 0.05 M cacodylate buffer for 1h at room temperature. Specimens were washed twice in sodium cacodylate buffer (0.15M), post-fixed in 1 % OsO_4 with 0.02 M KFeCN in 0.12 M cacodylate buffer, rinsed three times in MilliQ water, submerged for 1 hour in 1% w:vol tannic acid and washed three more times in MilliQ water and *en bloc* stained with 1% (w/vol) uranyl acetate for 2h. Specimens were dehydrated in a series of 2x10 min steps using ethanol of 70%, 96%, 100%. After 2x10 min dehydration in propylene oxide specimens were infiltrated by gradually increasing Epon (embed 812, standard recipe) to propylene oxide ratio in a series of steps: 1:3 for 30 min, 1:1 overnight, 3:1 for 1h and pure Epon for 2h. Finally specimens were embedded and cured at 60 °C for 72h.

Semi-thin sections (1µm) were obtained with a Leica Ultracut UCT ultramicrotome using glass knifes made on a knife maker (LKB instrument group 7800). Ultra-thin sections were obtained using a RMC MT-7 ultramicrotome and a diamond knife (Ultra 45°, Diatome). Semi-thin sections were placed on a glass slide and stained with toluidine blue for light microscopy (Olympus BX51 microscope) to localize the gut for EM.

To rule out the possibility of Au NP transfer into *D. magna* gut resulting from the preparation steps, two methodological controls were included. Au NPs were added at two different steps of the

sample preparation (referred to as controls C1 and C2). For C1 Au NPs were added with the uranyl acetate during *en bloc* staining. For C2, Au NPs in acetone was used instead of propylene oxide during infiltration. Au NP suspension in acetone was obtained by gentle heating and evaporation of water from stock suspension and the subsequent suspension of Au NPs in acetone.

Specimen preparation for LSM

Before LSM imaging the daphnia were briefly transferred to clean media to rinse off exterior bound NPs. The daphnia were anesthetized to avoid movements during microscopy. For anesthesia 17% ethanol and 0.55 mg/L phenoxyethanol solutions in VH US EPA media (0.24 g/L $CaSO_4*2H_2O$, 0.24 g/L $MgSO_4*7H_2O$, 0.016 g/L KCl and 0.384 g/L $NaHCO_3$ in MilliQ water) was used. After anesthesia the test organisms were embedded in freshly prepared 1% low temperature melting agarose in an Eppendorf tube. The agarose solution was kept in a heating block at 38°C to keep it liquefied. To avoid thermal damage of the daphnia the Eppendorf tube containing agarose was briefly taken off the heating block before embedding. Appropriate glass capillary and plunger was used to suck the test organism from the agarose solution. Care was taken to keep the daphnia vertically aligned with the capillary to receive the best penetration of light from all angles when imaged. When solidified in the agarose (approximately 1 min) the test organism was imaged in the LSM.

EM and elemental analysis

FIB-SEM (Quanta FEG 3D, FEI) was conducted on entire epoxy embedded specimens mounted on a holder and gold coated (High Resolution Sputter Coater, Cressington). A cross section of the gut was made by milling a trench into the sample. Imaging was done with a low-kV high contrast backscatter detector at 5 kV. Ultra-thin sections of 80-100 nm were placed on copper grids (Cu, 3 mm, 250 mesh square, SPI-grids) for TEM (CM 100 Phillips, operating voltage 80 kV). Ultra-thin sections (70 nm) were placed on carbon coated grids (Cu, 3 mm, 200, Agar scientific) and carbon coated (208C, Cressington) prior to HAADF STEM & EDX (Tecnai G2 T20, FEI, operating voltage 200 kV).

Elemental analysis of single NPs was conducted in STEM mode with EDX (80 mm^2 X-Max SDD detector, Oxford Instruments). Cryo-TEM (Tecnai G2 T20, FEI, operating voltage 200 kV) was performed using a single tilt liquid nitrogen cryo-transfer holder (626, Gatan) with Au NP stock suspension which had been placed on a lacey carbon grid (Agar scientific) and plunge-frozen in liquid ethane using a Vitrobot (FEI).

LSM

The LSM was performed using a Lightsheet Z.1 (Carl Zeiss Microscopy) equipped with a 5x objective, using the standard exposure chamber filled with VH US EPA media for imaging of the daphnia. Imaging of the samples was carried out using two lasers, 566 nm (laser 1) and 488 nm (laser 2). Broad pass filters with a range of 505-545 nm and 575-615 nm were used for laser 1 and 2, respectively. A laser intensity of 3 and 10 % was used for laser 1 and 2, respectively, during the imaging of the uptake of Au NPs in daphnia. The exposure time used for all the samples was set to 154.8 ms. The imaging was performed with two-sided illumination to obtain maximum illumination of the sample and avoid shaded areas. All imaging was performed as a series of slides referred to as the "z-stack" using an optimal thickness of the slides determined by the ZEN software (Carl Zeiss Microscopy). Furthermore, a 3D-multiview of the sample was made by rotation of the samples at 6 different angles (0°, 60°, 120°, 180°, 240°, 300° and 360°). When complete, the image were fused and averaged with a pixel average of 3 in the x and y direction. A maximum intensity projection was created to evaluate the overall uptake of the Au NPs.

RESULTS AND DISCUSSION

Distribution of Au NPs at organism level

The *in vivo* distribution of fluorescently labelled Au NPs in *D. magna* after 0, 2, 4 and 24h exposure to fluorescein (FITC)-labelled Au NPs imaged by LSM shows a continuous increase in fluorescent signal from the gut with time in Figure 2.

After 24 hours the gut was filled with fluorescent Au NPs. No fluorescent signal could be observed outside the gut of the daphnia. It is observed that initially the Au NPs accumulates in the hindgut as expected for inorganic particles with no nutritional value [24]. The fluorescent tag is covalently bound to the nanoparticles thus digestion of the fluorescent label is unlikely. The pH in *Daphnia magna* varies from pH 6-7.2 with increasing pH from the midgut to the anus. Consequently, no harsh environment in relation to pH is encountered after ingestion of the Au NPs and at physiological pH there was no change in the emission peak of the fluorescent tag.

Preliminary embedding of FITC-labelled Au NPs dispersed in agarose showed no signal, thus only larger clusters of fluorescent Au NPs would be observed (data not shown). Similar observations were made by Rothen-Rutishauser et al. [25] using laser scanning microscopy, in a study demonstrating that clusters of approximately 150 Au NPs would yield a fluorescent event. Thus, localization of dispersed NPs is not possible with this method which underline the need for reliable EM procedures.

Distribution of Au NPs in the gut with EM

FIB-SEM images (back scattered electrons, BSE) of *D. magna* exposed to Au NPs showed the gut lumen and surrounding cellular structures (Figure 3). A strong BSE signal was registered from the gut lumen, indicating regions with high concentration of Au NPs and thus uptake into the gut (Figure 3A, arrow), which was also expected based on images from LSM. The peritrophic membrane (PTM) appeared to retain the Au NPs in the gut lumen, except for single bright spots, which could possibly be Au NPs, observed near the microvilli (Figure 3B). However, elemental analysis of small particles outside the aggregates was not possible because of the relatively small volume of a 10 nm gold particle compared to the interaction volume of a 5 kV electron beam.

TEM images of gut cross section from *D. magna* exposed to Au NPs exhibited regions of electron dense material within the gut lumen indicating the presence of Au NPs (Figure 4), asterisk). Mostly single electron dense particles and a few small aggregates were observed near the microvilli of

the epithelial gut cells (Figure 4, arrows), again indicating that the PTM acts as a low permeability membrane between the microvilli and the lumen. The PTM is generally assumed to consist of chitin and proteins, and creates a barrier with a pore size of approximately 10 nm [26]. It has previously been suggested to act as a barrier for NP access to *D. magna* gut cells [9]. Therefore, we find it likely that agglomerates of Au NPs are retained in the gut lumen by the PTM. However, as single Au NPs are of similar size as the PTM pores, single Au NPs may possibly cross the PTM and reach the microvilli of the gut cells. Au-NP like objects were indeed observed across the PTM associated with the microvilli of gut cells (Figure 5A). Furthermore, structures with high contrast and similar size as the Au NPs were also observed inside cells, suggesting cellular internalization of Au NPs (Figure 5B).

Elemental analysis with HAADF-STEM and EDX

To further investigate the occurrence of Au NPs in *D. magna* subsequent imaging and analysis were conducted using HAADF-STEM imaging and EDX to facilitate elemental analysis of single particles. The results revealed that the identification of NPs solely on observations (*i.e.* contrast, size and morphology) can be deceiving, especially for NPs inside the cellular matrix where both nano-sized cellular structures and precipitates can exhibit similar contrast and size ranges. The large Au NP aggregate-like structures retained by the PTM inside the gut lumen (Figure 4, asterisk) were confirmed to be Au by STEM EDX (Supplemental Data, Figure S3). In all samples of exposed *D. magna*, objects were observed both across the PTM and inside cells exhibiting size, shape and contrast similar to that of the Au NPs used in the study. Elemental analysis of single particles with STEM EDX was conducted for a large number of high contrast Au NP-like objects found associated with or inside cells. Examples are shown in Figure 6.

Particles attached to the surface (Figure 6A) and at the base of microvilli (Figure 6B) were clearly distinguishable, due to the high contrast and characteristic shape of the NPs. Elemental analysis confirmed that these particles were Au. Inside the gut cells of exposed *D. magna*, high contrast material

was found mainly in mitochondria and lipid droplets. High contrast precipitates in mitochondria gave mainly osmium signals (Figure 6C), as did particles found in lipid bodies (Figure 6D) and an aggregate of high contrast particles below the basal lamina contained iron and aluminum (Figure 6E). Of 64 particles found inside gut epithelial cells in 5 replicate animals and separately analyzed, none were confirmed to be Au. Furthermore, the 64 particles which were analyzed were only a fraction of the total number of particles observed inside cells. However, the majority of these were clearly not Au NPs when imaged in HAADF STEM and hence were not subjected to analysis. An additional source of artifacts in performing TEM of NP uptake can be imaging and analysis of particles inside dying cells. In this case, analysis is not a guarantee against false positives. The gut of *D. magna* undergo rapid turnover and holocrine cells in which the cell content is packed into vesicles and released by disruption of the cell wall into the gut lumen together with digestive enzymes [27] were seen throughout the sections. Within these cells, we could observe NPs which were confirmed to be Au (Figure 6F). This is not surprising, considering that the cell membrane was no longer intact, but in some images, the cells were early in the process and it was difficult to distinguish them from intact cells. This might lead to incorrect conclusions regarding uptake of NPs even when performing elemental analysis. No confirmed Au NPs (by STEM EDX) were identified inside the C1 and C2 control organisms or blank controls (Supplemental Data, Figure S4).

Internalization of NPs can take multiple routes depending on nanoparticle size, shape, surface charge and surface coating. Small particles are able to passively pass through cell membranes as shown in red blood cells which do not have an endocytic machinery [28, 29]. Both *in vivo* and *in vitro* studies have proposed NP uptake through endocytosis [7, 30, 31]. Larger NPs (approximately 100 nm) have been suggested to be taken up actively by endocytosis whereas smaller NPs (approximately 10 nm) have been suggested to accumulate on the cell membrane until the aggregates reach a certain size, resulting in uptake [32]. In case of long-time exposure to low numbers of NPs where threshold

densities of NPs on the cell membrane is insufficient, passive uptake might be a significant route of internalization [32]. Assuming that either aggregation of small particles on the surfaces of cells or long exposure time is necessary to facilitate uptake, it is possible that the small NPs in our study are simply not interacting with enough receptors on the cell surface to mediate an immediate response; especially since only single particles were seen to cross the PTM, across which they did not form large aggregates. Larger aggregates were isolated from the gut epithelial cells by the PTM.

Lovern et al. [6] found few ~20 nm Au NPs close to microvilli in *D. magna* in an intake experiment the animals were exposed up to 24 hours. This is in contrast to the results from Khan et al. that *D. magna* did neither internalize ~20 nm Au NPs in its gut cells nor were the NPs associated with the microvilli of the gut epithelium [9]. The animals were exposed for 5 hours and allowed to depurate before analysis. In contrast to these findings, we found a considerable amount of Au NPs very close to or associated directly with the microvilli. For a conservative estimate, if we assume that between 10 and 50 particles in each section are found to have crossed the peritrophic membrane and be either associated with or close to microvilli, assuming that each section is an exact 70 nm thick cross section of the gut and we estimate the length of the midgut to be 500 µm, then it would very roughly estimated amount to between 70000 and 350000 particles per animal. The difference between our findings and the study by Lovern et al. [6] may result from different NP sizes in the experiments, as the Au NPs in our study were smaller (10 nm). Heinlaan et al. [8] monitored *D. magna* at 6 time points up to 48h exposure to 30 nm CuO NPs and only at 48h found NPs close to microvilli. These differences indicate that the peritrophic membrane might act as an initial size-dependent barrier. Internalization *via* the gut epithelium in *D. magna* has been shown for ZnO NPs with the most efficient uptake of 10-30 nm particles [10]. The NPs were internalized and found inside microvilli and gut cells at various locations, as well as in the gut muscles indicating that they were able to cross the basal lamina as well. The animals in that study were also exposed for 48 hours. It is thus very likely that time and size are both

important factors for uptake of NPs when considering that particles have to cross the peritrophic membrane first. In contrast to these time-scales, Rosenkranz et al. [33] performed a study of uptake of negatively charged fluorescent polystyrene beads (20 nm and 1 µm) in the gut of *D. magna* where both particle sizes were shown to translocate from the gut to lipid storage bodies distant from the gut by confocal laser scanning microscopy within 30-60 minutes. This suggests that the translocation through the epithelial layer could also be relatively fast. Indeed fast translocation has recently been shown by the use of a layer of epithelial cells *in vitro* [34]. Together these findings suggest that future studies should include both short and long exposure times and include several particle sizes.

CONCLUSIONS

10 nm Au NPs can be taken in and accumulate in the gut lumen of *D. magna*. These particles are able to pass the PTM mainly as single particles, thus gaining access to the microvilli of the gut epithelial cells and attaching to these as observed after 24 hours of exposure. It is becoming increasingly recognized that TEM images of various nanomaterials in biological samples can be misinterpreted [11, 12, 35]. Our findings show that some form of elemental analysis is necessary for the identification of internalized NPs using TEM. As presence of Au NPs was confirmed in cells with disrupted membranes, there are two possibilities of false positive observations of NP internalization, either resulting from preparation artefacts or from mistaking holocrine cells for intact cells. By comparison to other similar studies our study also highlights the need for limiting the number of variable conditions in this type of experiments as both exposure time and NP size may influence the uptake.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data Availability—Data available on request to the authors (louise.jensen@epfl.ch)

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Figure 1. Cryo-TEM image of Au NP stock solution. The particles are non-aggregated, scale bar = 300 nm, * = lacey carbon support.

Figure 2. *D. magna* after (A) 0 h, (B) 2 h, (C) 4 h and (D) 24h exposure to Au NPs (0.4 mg Au/L) using a 5x magnification water immersed objective. The red and white color in the image corresponds to FITC tag (505-545 nm) and auto fluorescence of the daphnia (575-615 nm) respectively. Scale bars 0.5 mm.

Figure 3. FIB-SEM BSE images of *D. magna* gut epithelium after 24h exposure to Au NPs (0.4 mg Au/L). Electron dense structures appear white. Examples of Au NP-like objects are marked with arrows. (A) Scale bar = 1 µm, (B) scale bar = 0.5 µm. GC = gut cells, LU = Lumen, MV = Microvilli, PTM = Peritrophic membrane.

Figure 4. *D. magna* gut epithelium after 24h exposure to Au NPs (0.4 mg Au/L). Overview of gut lumen and microvilli, scale bar = 0.5 µm. *= Au NP- like objects. GC = gut cells, MV = Microvilli, PTM = Peritrophic membrane. Arrows point to electron dense particles and aggregates.

Figure 5. TEM images of *D. magna* gut epithelium exposed to Au NPs (0.4 mg Au/L) for 24h. (A) Electron dense particles attached to microvilli (arrows). Scale bar = 200 nm and (B) intracellular structures and objects which resemble NPs (inserts). Scale bar = 200 nm and inserts = 5 nm.

Figure 6. HAADF STEM images of *D. magna* gut epithelium exposed to Au NPs (0.4 mg Au/L) for 24h showing intracellular structures and possible NPs. Corresponding spectra are superimposed. For clarity the C-peaks are capped and only up to 2.5 keV is depicted. (A) Au NP at microvilli, scale bar = 100 nm, (B) Au NP at base of microvilli, scale bar = 100 nm, (C) Os-rich particles in mitochondrion, scale bar = 300 nm, (D) Os-rich particles in lipid droplet, scale bar = 50 nm, (E) Aggregate of Fe-rich particles below the basal lamina, scale bar = 0.5 µm, (F) Au NP in a holocrine cell, scale bar = 200 nm.

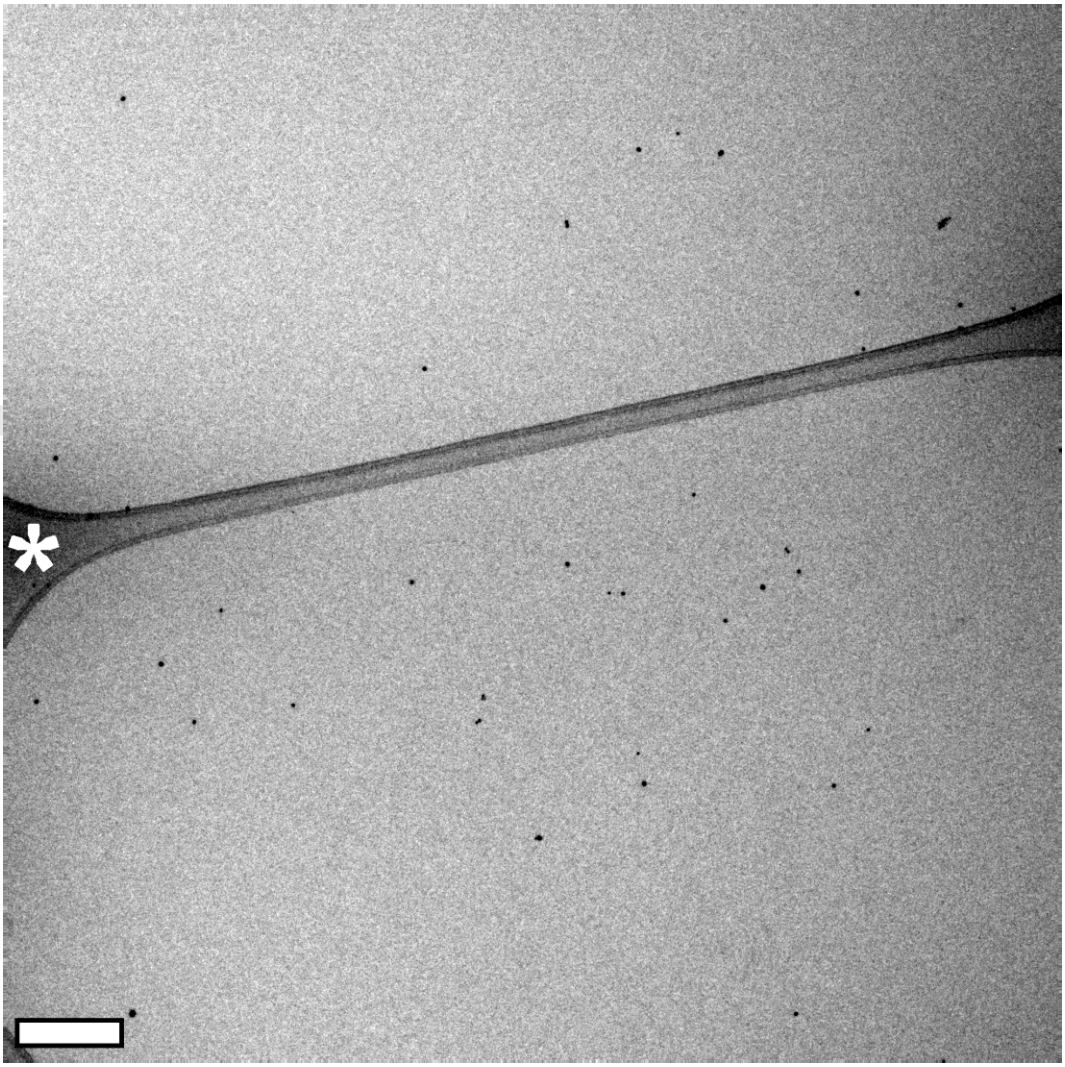

Figure 1

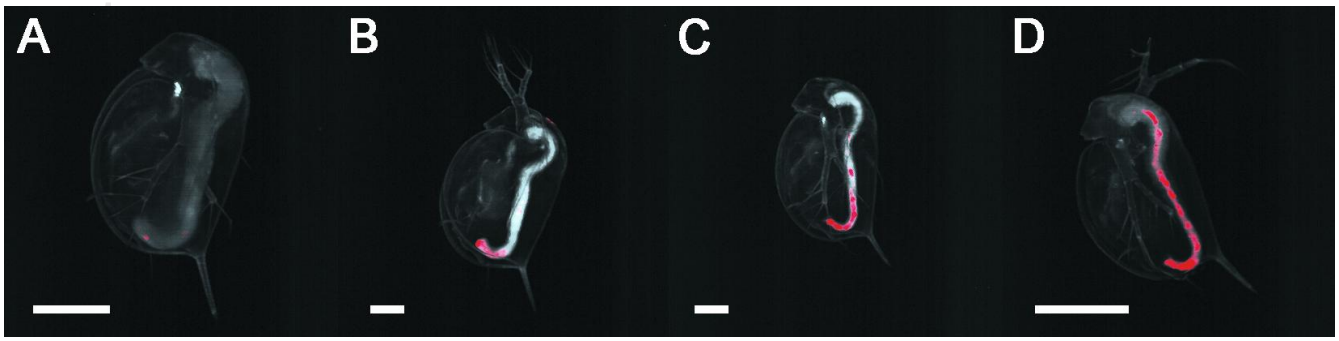

Figure 2

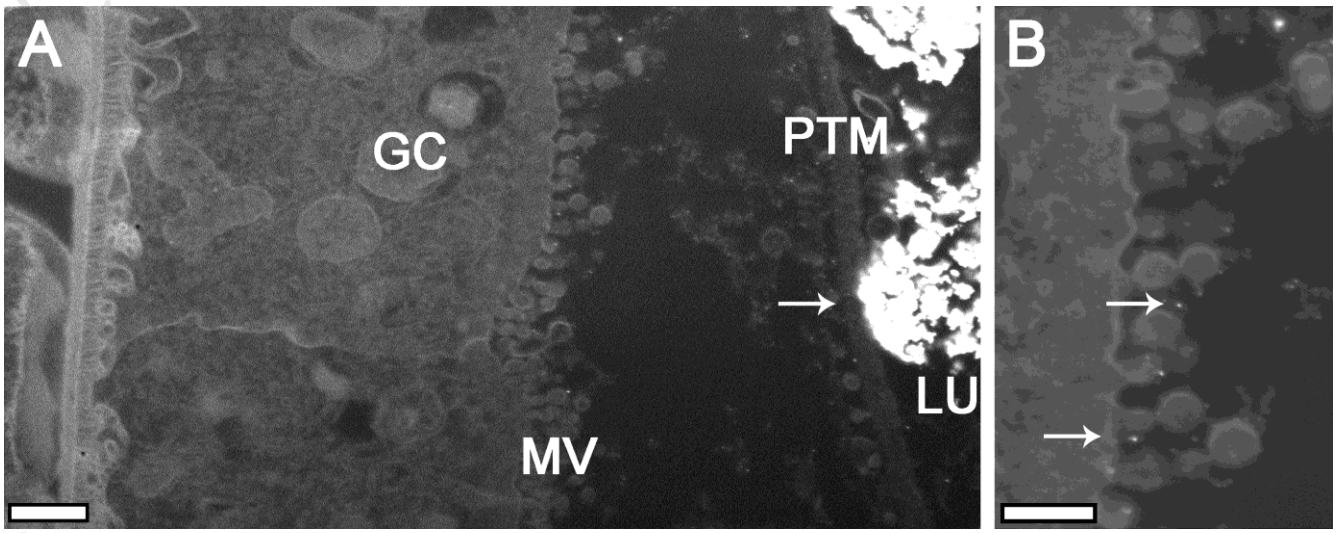

Figure 3

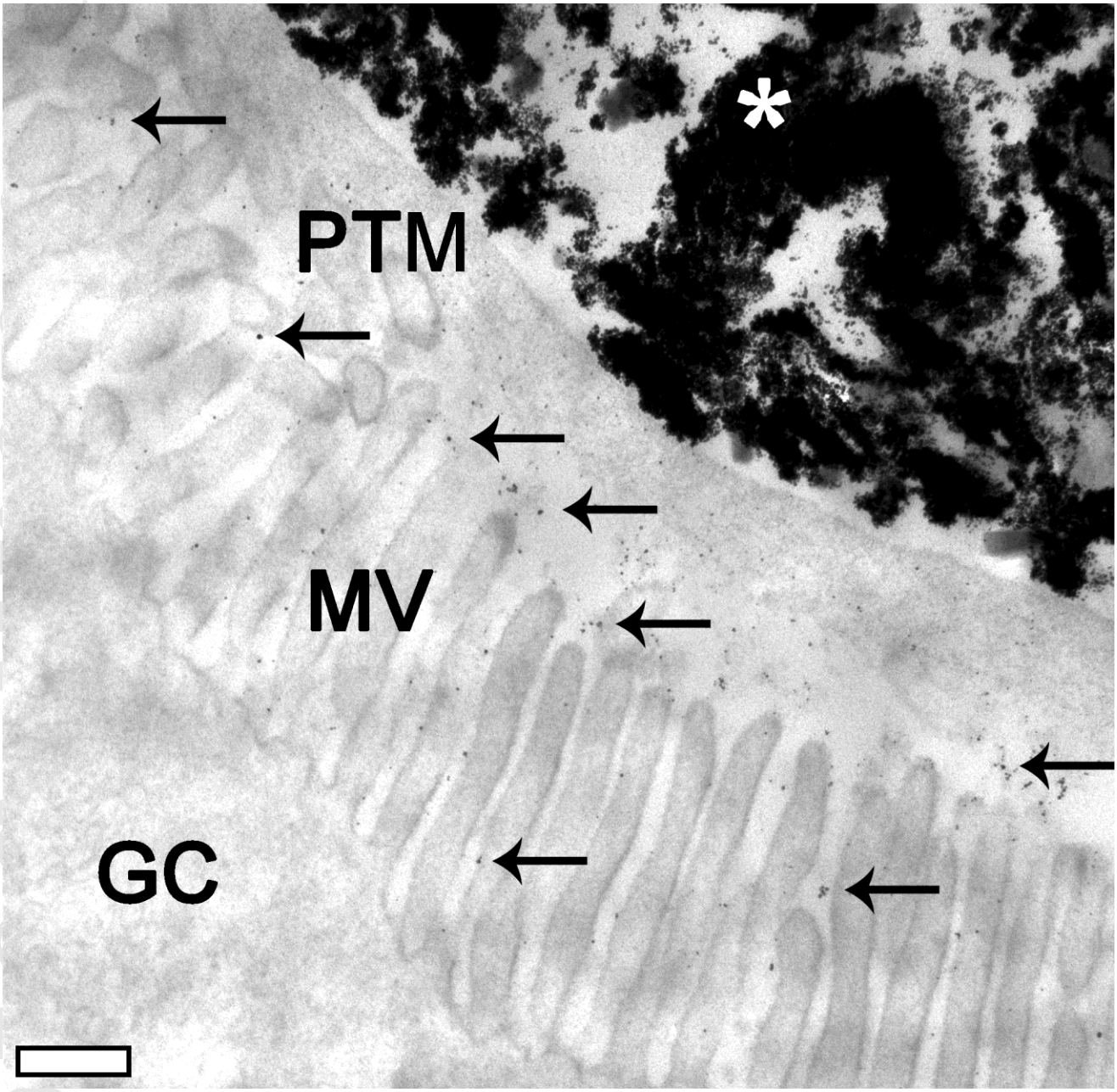

Figure 4

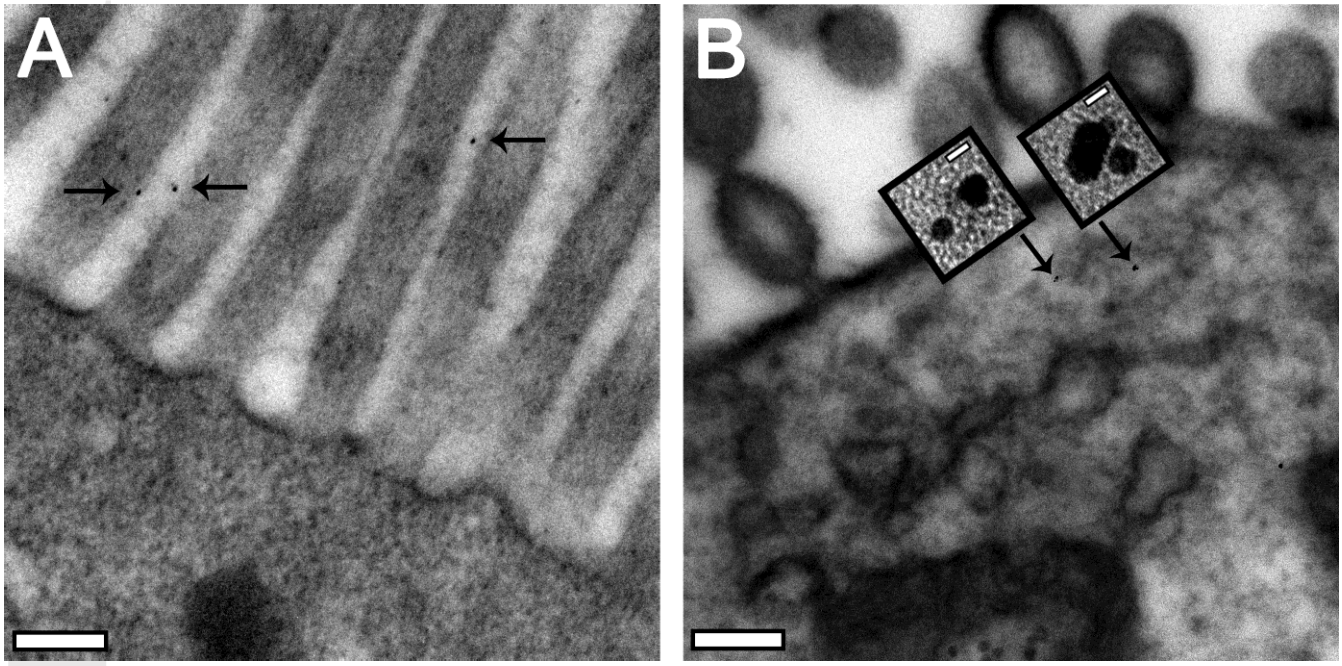

Figure 5

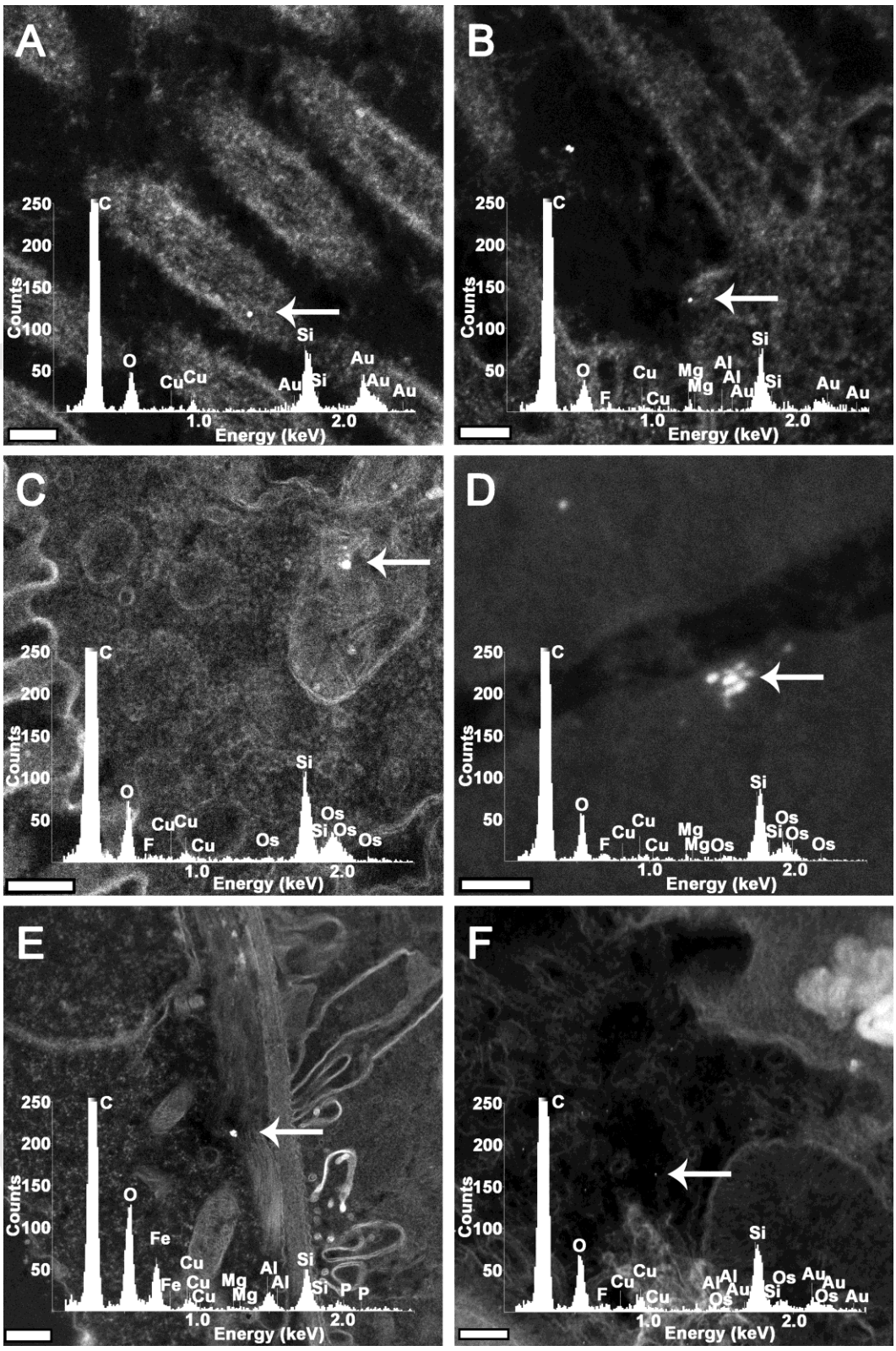

Figure 6