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Uptake of Gold Nanoparticles in an Algae - Daphnid Food Chain

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Abstract

Nanoparticle entry into the environment can result in deleterious effects to exposed organisms, disruption of ecological processes, and accumulation within the wood web. Gold nanoparticles (AuNPs), which are classified as zero-valent metals, are of significant interest due to their use in a variety of applications including electrical, biomedical, catalytic, magnetic, and optical technology. The wide range of uses for AuNPs can be attributed to a combination of the unique physical properties of the element gold (i.e. density, conductivity, stability, etc.) and the diversity of sizes, shapes, and surface compositions that can be achieved through manipulation of AuNP synthesis. While previous studies have suggested that AuNPs can be bioconcentrated and bioaccumulated, these studies do not indicate the effects of AuNPs characteristics on trophic transfer.

The objectives of this study were to 1) quantify the uptake and depuration of 4nm and 18nm gold spheres by *D. magna*; 2) quantify the uptake of 4nm and 18nm AuNPs by the algae, *Selenastrum capricornutum*; 3) quantify the bioaccumulation of 4nm and 18nm AuNPs previously incorporated in algae; and 4) determine the bioconcentration factors (BCF) for 4nm and 18nm AuNps in *Selenastrum capricornutum* and *D. magna* and the bioaccumulation factors (BAF) for 4nm and 18nm AuNPs in *D. magna.* Bioconcentration factors for *D. magna* exposed to 4nm and 18nm AuNPs for 96hr were 6641 and 10207, respectively. Depuration followed first order kinetics for the *D. magna* exposed to 18 nm AuNPs with a rate of -0.67 μg Au/hr, however the slope for 4nm

depuration was not found to be significantly different from 0. Bioconcentration factors for *S. capricornutum* exposed to 4nm and 18nm AuNPs for 96hr were 79.8 and 146.3, respectively. Bioaccumulation factors for *D. magna* exposed to 4nm and 18nm AuNPs for 96hr were 3.9 and 7.5, respectively. In conclusion these data indicate that uptake and depuration of AuNPs by *D. magna* is dependent on particle size with larger AuNPs exhibiting increased depuration and that AuNP depuration is incomplete over the duration of these experiments.

Acknowledgements

I would like to acknowledge Dr. Steve Klaine, my advisor, and Dr.'s William Baldwin and Cindy Lee for the input and feedback I received while conducting this research. Thanks to my family and friends for providing encouragement and moral support during the tough times. Thanks to my labmates past and present for making the time in the lab interesting and for the help when I really needed it.

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1. Introduction

Generally defined as materials with at least one dimension smaller than 100 nm, nanomaterials are of growing interest to ecotoxicologists due to increasing risk of environmental contamination. This increased risk stems from innovation in nanomaterial design, increased production, and expanded use of nanomaterials [1]. Nanomaterials are generally categorized into five basic groups; metal oxides, carbonaceous nanomaterials, nanopolymers, semiconducting materials, and zero-valent metals. Metal oxide nanoparticles are commonly produced by the grinding of bulk materials. TiO_2 and ZnO are widely used nanoparticles in this class and are used in such applications as skin care products and bottle coatings due to their ultraviolet light blocking properties [2][3]. Carbonaceous nanomaterials include a variety of products including fullerenes, single-, and multi-walled nanotubes [1]. Single walled nanotubes are of particular interest to some industries due to strength to weight ratios 460 times greater than steel [4]. Nanopolymers describe a variety of different materials in which size topology, flexibility and molecular weight can be controlled. These materials include macrocapsules, nanolatex, colored glasses, chemical sensors, modified electrodes, DNA transfecting agents, therapeutic agents for prion diseases, hydrogels, and DNA chips [1]. Quantum dots are a class of nanomaterials made up of semiconducting nanocrystals. These materials are largely used in medical imaging and often consist of a metallic core such as cadmium surrounded by a shell that protects against oxidation, such as silica or ZnS [5]. Zero valent metal nanoparticles are used for a variety of purposes. Zero valent iron is

used in the remediation of ground water and soils contaminated with nitrates, polychlorinated biphenyls, and some pesticides [6]. Silver nanoparticles are widely used in consumer products including textiles, air filters, baby products, vacuum cleaners, and washing machines [7]. Gold nanoparticles (hereafter referred to as AuNps) are used in a wide variety of applications including medical imaging, cancer therapy, electronics manufacture, catalysts, and biosensor technology [8-12]. The wide range of uses for AuNps can be attributed to a combination of the unique physical properties of the element gold (i.e. density, conductivity, stability, etc.) and the diversity of sizes, shapes, and surface compositions that can be achieved through manipulation of the synthesis of AuNps [13].

2. History and Synthesis of AuNps

Gold nanomaterials have been used throughout much of history as dyes for glass and fabric and for presumed curative properties when ingested. One of the most famous historical uses is evidenced in the Lycurgus Cup, a cup dating from the 4^{th} to 5^{th} century B.C. that is red in transmitted light and green with reflected light due to use of colloidal gold in its manufacture [13]. Gold colloid solutions were described as curative for a variety of diseases and ailments as far back as the 17^{th} century and where characterized in a variety of solution compositions ranging from red, to purple, to gold in color [13]. The modern preparation of gold nanoparticles, however, was initiated by Faraday in 1857 when he described the formation of deep red gold solutions produced by a reduction of chloroaurate ($AuCl_4^-$) with phosphorous in CS_2 [14]. The method developed by

Turkevitch in 1951 [15] is still one of the most popular initial preparation methods and uses citrate to reduce $HAuCl_4$ in solution. Initially these particles were limited in size to approximately 20 nm but more recently careful adjustment of the reducing/stabilizing agents have allowed for the controlled synthesis of a variety of particle sizes [16]. The Brust-Schiffrin method [17] has enabled a larger variety of surface functional groups to be attached to gold nanoparticles using a thiol stabilizer. Particles made using this method can be isolated and re-dissolved in common organic solvents without losing solution stability [17]. Stabilization of gold nanoparticles can also be achieved using a variety of stabilizers such as xanthates, disulfides, di- tri- and tetra- thiols, phosphines, amines, and carboxylates [13].

In addition to different surface chemistry compositions gold nanoparticles can be synthesized in a variety of shapes including spheres, cubes, rods, and nanowires [13]. Gold nanorods are produced in a process that uses gold nanospheres as a seed for the surfactant directed growth of monodispersed nanorods [18]. Gold nanocubes are produced using electrochemical reduction in the presence of surfactants [19]. Gold nanowires can be produced using a technique that involves deposition of gold on pore walls of microporous membrane filters, this technique can be modified based on deposition time to produce either gold nanowires or gold nanotubes[13, 20]. Gold nanoparticles may also be produced in a range of different sizes from approximately 2nm to 100nm depending on the method and reaction conditions chosen during synthesis [13].

3. Uses of AuNps

A wide variety of uses have been developed for AuNps in recent years due to the differing physicochemical properties derived from surface chemistry, size, and shape compositions. Gold nanoparticles have a large range of applications including electrical, biomedical, catalytic, magnetic, and optical technology [1, 13]. While this is not intended to be a comprehensive review of the current state of gold nanoparticle technology, what follows are selected examples of gold nanoparticle technology to provide a brief overview of the variety of uses developed for gold nanoparticles in recent years.

3.1 Electrical Applications

Gold nanoparticles have recently been incorporated into carbon nanotube based transistors used in DNA detection. The use of gold nanoparticles in these devices has resulted in a significant increase in sensitivity allowing detection of DNA in the femtomolar range [21]. Gold nanoparticles are also being used to reshape the production of electronic circuitry. AuNps have been used to develop a production method that utilizes the conductive properties of the materials to print thin, flexible circuits in a manner similar to printing newspaper [10]. This process can dramatically reduce the cost of the production of low resistance circuits by removing the need for lithography and vacuum sealed production facilities [10]. This technology is also being adapted for use in radically inexpensive solar cells in which the AuN(laden ink is printed on the back of photovoltaic ‘paper’.

3.2 Biomedical Applications

Biomedical applications of gold nanoparticles are numerous and varied. The unique size and functional group accepting properties of gold nanoparticles make them ideal candidates for development for a variety of therapeutic purposes. Gold nanoparticles have been developed to incorporate functional groups that provide controlled release of nitric oxide. These nanoparticles are being further developed for use as in vivo sensors or as a way to increase blood flow to specific areas [22]. Gold nanoparticles are also being used in the development of potential therapies for serious human diseases such as HIV [23]. A study in published in 2008 describes 2nm mercaptobenzoic acid modified gold particles used as a platform to build a multivalent therapeutic. The resultant therapeutic effectively prevented binding of HIV-1 virus to human T-cells. Gold nanoparticle-peptide complexes are being developed to facilitate drug delivery to cell nuclei and for other potential uses [24]. Gold nanoparticles have also been developed as a potential detection and treatment method for certain types of cancer [9, 10]. Due to the unique resonance band in gold nanoparticles, they can be heated rapidly using an instrument such as an argon laser. Gold nanoparticles that are specifically functionalized to bind to cancer cells can be subsequently heated via laser to destroy the targeted cells. In one experiment this process was applied to oral carcinoma cells. The AuNP bound cells were killed by the application of low intensity laser light, while non-cancerous cells that did not bind the particles were unaffected at laser intensities up to four times higher than that which killed the cells containing AuNPs [9].

3.3 Catalytic and Magnetic Properties

The properties of gold nanoparticles smaller than 10nm have different catalytic properties than those observed in the bulk solid. Investigations into the catalytic properties of AuNps have included catalysis of CO oxidation, CH3OH, O2, hydrogenation reactions, and ethanol [12, 13]. Gold nanoparticles have been utilized by some researchers to increase the sensitivity of magnetic resonance imaging (MRI). In a study investigating the use of gold nanoparticles as a carrier of gadolinium chelates, commonly used as a contrast agent in MRI imaging, the incorporation of gold nanoparticles in this technology resulted in a higher proton relaxivity and subsequently an enhanced contrast in the imaging process [24].

3.4 Optical Properties

While the ability of gold nanoparticles to attach proteins and enzymes is certainly important for biomedical applications, there are other applications that arise from enzyme-gold .nanoparticle technology. For example, a colorimetric lead biosensor has been developed that can accurately detect lead at levels between 200µM and 4µM, a range that includes the toxicity threshold for humans [11]. This method is relatively inexpensive, field ready, and can even be tuned to specific detection ranges based on the type and quantity of DNAzymes used in AuNP preparation [11]. A similar strategy has been used to detect mercury in a one step method at room temperature [25]. This may eventually provide a simple, rapid test for mercury contamination in remote sites. Gold nanoparticles have also been coupled to thermosensitive polymers. This coupling leads

to reversible temperature-dependant light transmission which changes from 0% to 75% light transmittance between 25□C and 30□C [26]. This technology may be adapted to 'smart' window technology that effectively reduces incoming solar energy at higher ambient temperature. Gold nanoparticles have also been investigated for the formation of micro-scale mirrors for use in high end optical displays [27].

4. Growth of Nanotechnology

It is important to note that nanomaterial production is expected to grow rapidly in the coming years. Some estimates predict that the market for nanotechnology related products will grow by as much as 20% annually until at least 2011 with some sectors (e.g. nano-enabled drug delivery) growing by as much as 50% per year. Annual growth rates between 10-15% in worldwide nanotechnology have been reported. It is estimated that the market for nanotechnology may reach one trillion US dollars by 2015 and that the nanotechnology industry may surpass the biotechnology industry [28]. It is likely that gold nanomaterials will play a large role in much of this growth due to the number of applications being developed for AuNPs. With this increase in production, (and likely increase in the variety of materials produced) comes an inherent risk of environmental contamination. It is important, therefore, to understand the responses of potentially sensitive organisms to exposures of AuNPs.

5. Biological Responses from Exposure to AuNps

A study by Chithrani et al. in 2006 describes exposures of different size and shape gold nanoparticles in human cervical cancer (Hela) cells [29]. In this study Hela cells were exposed to gold nanospheres ranging in size from 14nm to 100nm and gold nanorods with aspect ratios of 1:3 and 1:5. Both types of AuNPs (spheres and rods) were surface functionalized with citric acid ligands. This study showed that uptake of gold nanoparticles into a mammalian cell line was influenced by both particle size and shape. Gold nanosphere uptake was maximal with 50nm gold spheres and decreased dramatically at particle sizes both greater and less than 50nm. Particle shape also affected uptake in this cell line. Gold nanorods had much lower uptake and slower uptake rates than similarly sized nanospheres. Specifically 14x17nm gold nanorods showed 350 and 500% less uptake than either 14nm or 74nm gold spheres, respectively. While the reason for this shape discrimination is unclear the authors did speculate that uptake of AuNPs in these cells is dependent on serum protein binding. A variety of serum proteins can bind to the citrate coated AuNPs and consequently facilitate uptake. The authors proposed that, since the gold nanorods can have more surface contact than the nanospheres, nanorods may effectively interfere with the protein receptors on the cell surface that facilitate the protein-assisted uptake.

Similarly, a study by Zhu et al. (2008) investigated the influence of surface chemistry on the uptake of AuNPs into mammalian cells [30]. The study exposed monkey kidney cells (COS-1) to 2nm AuNPs with 5 different surface chemistries. Cells were then lysed using an irradiation technique and the lysates were analyzed using laser

desorption/ionization mass spectrometry (LDI-MS). The LDI-MS method measures a 'mass barcode' based on the specific surface functionalities of the AuNPs. The authors concluded that the uptake of these AuNPs into the COS-1 cells was dependant on the surface functional groups of the AuNPs, and that further differential uptake may be found in future experiments involving subcellular fractionation of the lysate.

The bioconcentration and trophic transfer properties of small (10nm) amine coated AuNPs was investigated by Renault et al. (2008) [31]. In the study the green algae, *Scenedesmus subspicatus* were exposed to aqueous suspensions of the amine coated gold nanoparticles. The bivalve *Corbicula fluminea* were exposed trophically to algae containing the AuNPs. The results of the algae exposure were 20-50% algal mortality between the highest and lowest concentrations. Interestingly, no nanoparticles were found inside any of the algal cells but were found around the cell walls of contaminated cells. The authors concluded that the cells were killed by AuNPs smothering and weakening the cell walls. Bivalves were screened for biomolecular changes, specifically metallothionein concentrations and gene expression were quantified. In *C. fluminea* the AuNP were detected in the stomach epithelia inside the cells and nucleus. In gill cells, however, no AuNPs were found in the nuclei or cytoplasm but were confined to lysosomal vesicles. In low concentration exposures metallothionein and superoxide dismutase expression were induced. At the higher exposures repression of superoxide dismutase, glutathione S transferase, and cytochrome C oxidase were seen while catalase expression was increased. This study indicates that

amine coated AuNPs can cause adverse effects to exposed organisms and can be passed on through trophic transfer.

Another study involving the uptake of gold nanoparticles by aquatic organisms was conducted with *Daphnia magna* [32]. In this study *D. magna* were exposed to gold nanoparticles and imaged using transmission electron microscopy. Gold nanoparticles were observed in the gut tract of the organisms but, interestingly, no nanoparticles were seen to cross cellular boundaries. Gold was seen to move through the gut tract with time and some depuration was achieved when organisms were transferred to water not contaminated with AuNPs. Depuration of AuNPs was incomplete, however, with significant amounts of gold nanoparticles remaining in the gut tract after 24h.

Considering the numerous uses of AuNPs in such a diverse range of fields there is curiously little information on the biological and ecological effects of gold nanoparticle exposure in non-target organisms and systems. With the accelerating growth in the field of gold nanoparticle technology it is important to evaluate the potential impacts of exposure to these materials may have on aquatic organisms and the subsequent risk that organism effects may have on the ecology of the system as a whole.

References

1. Klaine S, Alvarez P, Batley G, Fernandes T, Handy R, Lyon D, Mahendra S, MeLaughlin M, Lead J. 2008. Nanomaterials in the Environment: Behavior, Fate, Bioavailability, and Effects. *Environ Toxicol Chem* 27:1825-1851.

2. Sass J. 2007. Nanotechnology's Invisible Threat: Small Science Big Consequences. *NRDC Issue paper* .

3. Pitkethly M. 2004. Nanomaterials-The Driving Force. *Nano-today* 7:20-29.

4. ASTM. American Society for Testing and Materials. 2006. Standard Terminology Relating to Nanotechnology. E 2456-06.

5. Dabbousi B, Rodriguez-Viejo J, Mikulec F, Heine J, Mattoussi H, Ober R, Jensen K, Bawendi M. 1997. (CdSe)ZnS core-shell quantum dots: Synthesis and characterization of a size series of highly luminescent nanocrystals. *J Phys Chem B* 101:9463-9475.

6. Zhang W. 2003. Nanoscale iron particles for environmental remediations: An overview. *Journal of Nanoparticle Research* 5:323-332.

7. Klopffer W, Curran M, Frankl P, Heijungs R, Kohler A, Olsen S. 2007. Nanotechnology and life cycle assessment. Woodrow Wilson Center Project on Emerging Nanotechnologies.

8. Liu X, Dai Q, Austin L, Coutts J, Knowles G, Zou J, Chen H, Huo Q. 2008. A One-Step Homogeneous Immunoassay for Cancer Biomarker Detection Using Gold

Nanoparticle Probes Coupled with Dynamic Light Scattering. *J Am Chem Soc* 130:2780-2782.

9. El-Sayed I, Huang X, El-Sayed M. 2006. Selective laser photo-thermal therapy of epithelial carcinoma using anti-EGFR antibody conjugated gold nanoparticles. *Cancer Letters* 239:129-135.

10. Huang D, Liao F, Molesa S, Redinger D, Subramanian V. 2003. Plastic-Compatible Low Resistance Printable Gold Nanoparticle Conductors for Flexible Electronics. *Journal of the Electrochemical Society* 150:G412-G417.

11. Liu J, Lu Y. 2003. A Colorimetric Lead Biosensor Using DNAzyme-Directed Assembly of Gold Nanoparticles. *J Am Chem Soc* 125:6642-6643.

12. Zheng N, Stucky G. 2006. A General Synthetic Strategy for Oxide-Supported Metal Nanoparticle Catalysts. *J Am Chem Soc* 128:14278-14280.

13. Daniel M, Astruc D. 2004. Gold Nanoparticles: Assembly, Supramolecular Chemistry, Quantum-Size-Related Properties, and Applications toward Biology, Catalysis, and Nanotechnology. *Chem Rev* 104:293-346.

14. Faraday M. 1857. Experimental Relations of Gold (and other Metals) to Light. *Philos Trans* 147:145-181.

15. Turkevitch J, Stevenson P, Hillier J. 1951. Nucleation and Growth Process in the Synthesis of Colloidal Gold. *Discuss Faraday Soc* 11:55-75.

16. Frens G. 1973. Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Suspensions. *Nature: Phys Sci* 241:20-22.

17. Brust M, Walker M, Bethell D, Schiffrin D, Whyman R. 1994. Synthesis of Thiol-Derivatized Gold Nanoparticles in a Two-phase Liquid-Liquid System. *J Chem Soc , Chem Commun* :801-802.

18. Busbee B, Obare S, Murphy C. 2003. An Improved Synthesis of High Aspect Ratio Gold Nanorods. *Adv Mater* 15:414-416.

19. Huang C, Wang Y, Chiu P, Shih M, Meen T. 2006. Electrochemical Synthesis of Gold Nanocubes. *Mat Lett* 60:1896-1900.

20. Lee W, Scholz R, Nielsch K, Gosele U. 2005. A Template-Based Electrochemical Method for the Synthesis of Multisegmented Metallic Nanotubes. *Angew Chem Int Ed* 44:6050-6054.

21. Dong X, Lau C, Lohani A, Mhaisalkar G, Kasim J, Shen Z, Ho X, Rogers J, Li L. 2008. Electrical Detection of Femtomolar DNA via Gold-Nanoparticle Enhancement in Carbon-Nanotube-Network Field-Effect Transistors. *Adv Mater* 20:2389-2393.

22. Rothrock A, Donkers R, Schoenfisch M. 2005. Synthesis of Nitric Oxide-Releasing Gold Nanoparticles. *J Am Chem Soc* 127:9362-9363.

23. Bowman M, Ballard T, Ackerson C, Feldheim D, Margolis D, Melander C. 2008. Inhibition of HIV Fusion with Multivalent Gold Nanoparticles. *J Am Chem Soc* 130:6896-6897.

24. Debouttiere P, Roux S, Vocanson F, Billotey C, Beuf O, Favre-Reguillon A, Lin Y, Pellet-Rostaing S, Lamartine R, Perriat P, Tillement O. 2006. Design of Gold Nanoparticles for Magnetic Resonance Imaging. *Adv Funct Mater* 16:2330-2339.

25. Xue X, Wang F, Liu X. 2008. One-Step, Room Temperature, Colorimetric Detectionof Mercury (Hg^{2+}) Using DNA/Nanoparticle Conjugates. *J Am Chem Soc* 130:3244-3245.

26. Zhu M, Wang L, Exarhos G, Li A. 2004. Thermosensitive Gold Nanoparticles. *J Am Chem Soc* 126:2656-2657.

27. Kim J, Osterloh F. 2006. Planar Gold Nanoparticle Clusters as Microscale Mirrors. *J Am Chem Soc* 128:3868-3869.

28. Hullman H. 2006. The economic development of nanotechnology-An indicators based analysis. 28. European Commission, DG Research,Unit “Nano S&T - Convergent Science and Technologies”

29. Chithrani B, Ghazani A, Chan W. 2006. Determining the Size and Shape Dependence of Gold Nanoparticle Uptake into Mammalian Cells. *Nano Letters* 6:662-668.

30. Zhu Z, Ghosh P, Miranda O, Vachet R, Rotello V. 2008. Multiplexed Screening of Cellular Uptake of Gold Nanoparticles Using Laser Desorption/Ionization Mass Spectrometry. *J Am Chem Soc* 130:14139-14143.

31. Renault S, Baudrimont M, Mesmer-Dudons N, Gonzalez PM, S., Brisson A. 2008. Impacts of gold nanoparticle exposure on two freshwater species: a phytoplanktonic alga (*Scenedesmus subspicatus*) and a benthic bivalve (*Corbicula fluminea*). *Gold Bulletin* 41:116-126.

32. Lovern S, Owen H, Klaper R. 2008. Electron microscopy of gold nanoparticles in the gut of *Daphnia magna*. *Nanotox* 2:43-48.

Uptake of Gold Nanoparticles in an Algae – Daphnid Food Chain

Introduction

Nanotechnology promises exciting possibilities for almost every sector of society. New products incorporating nanotechnology are being released at a rate of 3-4 per week according to the Woodrow Wilson Center Project on Emerging Nanotechnologies. This rapid growth of products is accompanied by an equally rapid increase in the risk of environmental contamination either through product production or product use[1][2].

Nanomaterials are generally categorized into five basic groups; metal oxides, carbonaceous nanomaterials, nanopolymers, semiconducting materials, and zero-valent metals. Gold nanoparticles (AuNPs), which are classified as zero-valent metals, are of significant interest due to their use in a variety of applications including electrical, biomedical, catalytic, magnetic, and optical technology [2, 3]. The wide range of uses for AuNPs can be attributed to a combination of the unique physical properties of the element gold (i.e. density, conductivity, stability, etc.) and the diversity of sizes, shapes, and surface compositions that can be achieved through manipulation of AuNP synthesis. Initially these particles were limited in size to approximately 20 nm but more recently refinement of the reducing/stabilizing agents have resulted in synthesis of a variety of sizes, surface charges, and shapes [4-6].

Nanoparticle entry into the environment can result in deleterious effects to exposed organisms, disruption of ecological processes, and accumulation within the wood web. Renault et al [7} investigated the uptake of gold nanoparticles by an alga and a bivalve and reported significant toxicity. The AuNPs used in this study had an amine

surface chemistry that probably contributed to the toxicity. Petersen et al [8] quantified the movement of carbon nanotubes through the gut tract of *Daphnia magna* while Roberts et al [9] demonstrated that *D. magna* could strip the lipid coating from lysophospholipid surface-modied multi-walled carbon nanotubes.

In vitro studies have demonstrated that AuNPs could be taken up in cell cultures and that uptake was a function of particle size and shape [10, 11]. Another study investigating uptake of gold nanoparticles concluded that uptake of AuNPs by *D. magna* was possible and depuration was only partially achieved over the duration of the test [12]. However, while previous studies have suggested that AuNPs can be bioconcentrated and bioaccumulated, these studies do not indicate the effects of AuNPs characteristics on trophic transfer.

The objectives of this study were to 1) quantify the uptake and depuration of 4nm and 18nm gold spheres by *D. magna*; 2) quantify the uptake of 4nm and 18nm AuNPs by the algae, *Selenastrum capricornutum*; 3) quantify the bioaccumulation of 4nm and 18nm AuNPs previously incorporated in algae; and 4) determine the bioconcentration factors (BCF) for 4nm and 18nm AuNps in *Selenastrum capricornutum* and *D. magna* and the bioaccumulation factors (BAF) for 4nm and 18nm AuNps in *D. magna.*

Materials and Methods

Experimental Design

This study is comprised of three sets of experiments: waterborne exposures of AuNPs to *D. magna*, waterborne exposures of AuNPs to *S. capricornutum*, and, food borne exposures in which *S. capricornutum* previously exposed to AuNPs was fed to *D. magna*.

Gold Nanoparticles

Spherical AuNPs of approximately 18nm and 4nm in diameter were obtained from Professor Catherine Murphy, University of South Carolina. Nanoparticles were produced using a modified form of the Turkevitch citrate reduction method [4]. Organisms were exposed to 7 μg/L Au and 8.4 μg/L Au for 4 nm and 18 nm AuNP, respectively. Preliminary bioassays revealed no deleterious effects at concentrations up to 2000 μg/L Au.

Daphnia magna Uptake and Depuration Exposures

D. magna were obtained from cultures maintained at the Clemson University Institute of Environmental Toxicology (CU-ENTOX) and exposed to AuNPs suspended in synthetic moderately hard water (~80mg/l as $CaCO_3$) 14]. Organisms were <7d old (pre-gravid) at the time of exposure initiation and exposed in 100 ml polypropylene beakers. Uptake exposures consisted of 12 replicate exposure beakers and three control beakers, each containing 40 organisms. Three exposure beakers were sampled at 12h, 24h, 48h, and 96h; *D. magna* were removed and washed in clean moderately hard water for approximately one minute then dried at 60 C for >24h. Depuration exposures

consisted of 15 replicate exposure beakers containing 40 organisms per beaker. After 24h of initial exposure three replicate beakers were sampled and *D. magna* were removed and washed in clean moderately hard water for approximately one minute then dried at 60 C for >24h. *D. magna* from remaining replicate beakers were then transferred to 100 ml polypropylene beakers containing uncontaminated, moderately hard water. Three beakers were then sampled at 1h, 6h, 12h, and 24h after transfer to clean water, *D. magna* were then removed and washed in clean moderately hard water for approximately one minute then dried at 60 C for >24h. Sampled and processed organisms were digested and analyzed for gold as described below.

Waterborne Algal Exposures

Algal cultures of *S. capricornutum* were cultured at CU-ENTOX. Algae were isolated via centrifuge and diluted to a concentration of 3×10^4 cells per ml in two replicate four liter ehrlenmeyr flasks filled with three liters of moderately hard water that contained either 18nm or 4nm AuNPs. Flasks were placed under a continuous light source with magnetic stir plates and forced aeration for agitation. Three replicate 30 ml samples from each flask were collected at 12h, 24h, 48h, and 96h. Samples were vacuum filtered with 0.45 micron nitrocellulose filters and dried at 60□ C for >24h. Samples were then immersed in 10ml of 10% solution of aqua regia (3:1) in Milli-Q, capped overnight, then placed in a boiling water bath for approximately one hour for digestion. Digestate was then diluted to a final acid concentration of 5% and analyzed using a Thermo Scientific X Series 2 ICP-MS.

Food borne *D. magna* Exposures

Algae exposed to 4nm and 18nm AuNPs for 96 h were concentrated via centrifugation to a final concentration of 3×10^6 cells per ml. *D. magna* were fed these algae in 12 replicate polypropylene beakers per AuNP size, containing 40 organisms in 100 ml of moderately hard water per replicate. *D. magna* were >7d old (pre-gravid) at test initiation and were fed 2 ml of the concentrated algae per beaker per day. Organisms were collected from 3 replicate beakers at 12h, 24h, 48h, and 96h and dried at 60□ C for >24h. Dried *D. magna* were weighed and placed in centrifuge tubes with 500 µl of aqua regia, centrifuge tubes were then capped and digested in a boiling water bath for one hour. Digestate was then diluted with Milli-Q water to achieve a final concentration of 5% acid and analyzed using a Thermo Scientific X Series 2 ICP-MS.

Gold Analysis

Water samples collected at each time point were preserved with aqua regia for gold analysis. Dried algal samples were immersed in 10ml of 10% solution of aqua regia (3:1) in Milli-Q, capped overnight, then placed in a boiling water bath for approximately one hour for digestion. Digested samples were diluted to a final acid concentration of 5% and analyzed using a Thermo Scientific X Series 2 ICP-MS. Dried *D. magna* were weighed and placed in centrifuge tubes with 500 µl of aqua regia, centrifuge tubes were then capped and digested in a boiling water bath for one hour. Digestate was then diluted with Milli-Q water to achieve a final concentration of 5% acid and analyzed on the ICP-MS.

Calculations

The slopes of uptake and depuration curves for 18nm and 4nm AuNPs were statistically compared using a Students t-test where the test statistic was calculated as follows:

$$t = \frac{b_1 - b_2}{s_{b_1 - b_2}}$$

Where b_1 and b_2 are the slopes being compared and $S_{b1\text{-}b2}$ is the standard error of the difference between the two slopes.

Bioconcentration factors were calculated based on the following equation:

$$\text{BCF} = \frac{\text{(mean organism whole body Au concentration ug/g dry weight)}}{\text{(aqueous AuNp exposure total Au concentration)}}$$

Bioaccumulation factors were calculated based on the following equation:

$$\text{BAF} = \frac{\text{(mean } \textit{D. magna} \text{ Au concentration (µg/g dry weight))}}{\text{(mean } \textit{S. capricornutum} \text{ Au concentration (µg/g dry weight))}}$$

Results

AuNP Characterization

Exposure concentrations 7 μg/L and 8.4 μg/L as Au for 4nm and 18nm AuNps, respectively, were measured via ICP-MS. Size of 18nm and 4nm AuNPs was verified using transmission electron microscopy and were in the appropriate size range (Figures 1 and 2). Zeta potential of the AuNP suspensions was measured to be -27.0 mV and -25.7 mV for 18nm and 4nm AuNPs respectively.

AuNP Uptake by *D. magna*

Exposures of both 18nm and 4nm AuNps resulted in progressive increases in whole body Au concentration in *D. magna*, however *D. magna* exposed to 4nm AuNPs accumulated more AuNPs than *D. magna* exposed to 18nm AuNps (Figure 3). Whole body Au concentrations in control organisms were below the instrument detection limit. In general, *D. magna* accumulated larger quantities of 4 nm AuNPs than 18 nm AuNPs (Table 1). Uptake of AuNPs followed first order kinetics (r^2 for semi logarithmic plots of Au as a function of time were greater than 0.96) with rates of 0.018 and 0.017 AuNP/hr for 4nm and 18 nm AuNPs, respectively. Statistical comparisons of the uptake curves showed that while total AuNP uptake by *D. magna* was greater in 4nm AuNP exposures, the uptake rates for 18nm and 4nm AuNPs by *D. magna* was not different. Interestingly, when whole organism Au uptake rates are compared, rather than particle uptake rate, the whole body Au uptake rate is greater in 18nm AuNP exposures than in 4nm exposures.

Bioconcentration factors for *D. magna* exposed to 4nm and 18nm AuNPs for 96hr were 6641 and 10207, respectively.

D. magna AuNP Depuration

D. magna exposed to 18nm AuNPs showed a time dependent decrease in AuNP concentrations after being placed into clean water for 24h (Table 1). The trend was not statistically significant for *D. magna* exposed to 4nm AuNP (Figure 4). The slopes of the depuration curves for 18nm and 4nm AuNPs were statistically different ($p < 0.05$) and the slope for 4nm depuration was not found to be significantly different from 0. Depuration followed first order kinetics for the *D. magna* exposed to 18 nm AuNPs with a rate of -0.67 μg Au/hr.

S. capricornutum AuNP Uptake

Exposure to AuNPs resulted in measurable uptake of both 4nm and 18nm AuNP in *S. capricornutum* (Figures 5 and 6). Bioconcentration factors for *S. capricornutum* exposed to 4nm and 18nm AuNPs for 96hr were 79.8 and 146.3, respectively. Whole body Au concentrations in control organisms were below the instrument detection limit and were rounded to 0 for data analysis. Slopes for both 18nm and 4nm AuNP uptake in *S. capricornutum* were not statistically different than 0 and could not be compared, however intercepts for the slopes were compared and found to be statistically different ($p<0.05$).

D. magna Food Borne AuNP uptake

Uptake of AuNPs followed first order kinetics (r^2 for semi logarithmic plots of AuNPs as a function of time were greater than 0.85) with rates of 0.0125 and 0.0069 AuNP/hr for 4nm and 18 nm AuNPs, respectively(Figure 7). Bioaccumulation factors for *D. magna* exposed to 4nm and 18nm AuNPs for 96hr were 3.9 and 7.5, respectively. While whole body AuNP concentrations in *D. magna* exposed to 18nm AuNPs were consistently lower than whole body Au concentrations in *D. magna* exposed to 4nm AuNPs, the slope of the uptake curves were not significantly different ($p < 0.05$).

Discussion and Conclusions

D. magna exposed to aqueous suspensions of AuNPs accumulated significantly more 4nm AuNPs than 18nm AuNPs. However, whole body gold measurements show the opposite; *D. magna* exposed to 18nm AuNPs accumulated more total Au than *D. magna* exposed to 4nm AuNPs. Uptake slopes for 18nm and 4nm AuNP were significantly different indicating that, while the uptake rate was greater in exposures of 18nm AuNPs, the extent of AuNP uptake was greater in 4nm AuNP exposures. Chithrani et al [12] reported that uptake of gold nanospheres by Hela cells was highest at approximately 50nm diameters while both larger and smaller nanoparticles were not taken up as readily [12]. While this study tested only 4nm and 18nm AuNPs, and not AuNPs in the larger range as in Chithrani et al., our results indicate that larger nanospheres may have preferential uptake rates in *D. magna* compared to smaller

diameter nanoparticles. In addition, since we were not able to differentiate between AuNPs in the gut tract and those in the body, these results might be explained by longer residence times in the gut tract by 18 nm AuNPs. Gold crystallizes in a cubic unit cell with an edge length of 4.08 angstroms per side, with four gold atoms per unit cell. Based on this configuration it was calculated that for a μg of Au there are 1.701×10^{10} 18nm AuNPs or 1.55×10^{12} 4nm AuNPs. So, while 18nm AuNP exposures resulted in higher rates of total Au uptake in *D. magna* and *S. capricornutum*, the uptake of total individual nanoparticles was much higher for 4nm AuNP exposures.

Results of depuration experiments indicated that while 18nm AuNps were depurated by *D. magna*, little depuration was observed with 4nm nanoparticles. Though depuration of 18nm AuNP by *D. magna* was significant, depuration appeared to slow significantly between 12 and 24 hr (Figure 4). This was consistent with findings by Lovern et al. in which 20nm gold nanoparticles were observed to depurate from D. magna during 24 hours in control water but that depuration was not complete [13]. This effect may be a function of body burden as depuration of 18 nm AuNPs slowed drastically body burden approached that of the D. magna exposed to 4 nm AuNPs. This would also explain the lack of significant depuration of 4 nm AuNPs. These results may signify that multiple binding sites or mechanisms are involved and that once a certain low level of contamination is reached depuration no longer occurs.

Algae exposed to 18nm AuNPs generally had greater whole body gold concentrations than algae exposed to 4nm AuNPs; however, there was significant variability between replicates particularly in algae exposed to 4nm AuNPs. Algal Au

uptake curves were not significantly different from 0 for either 18nm AuNPs or 4nm AuNPs but total organism gold was statistically different than controls, indicating that uptake of AuNPs by *S. capricornutum* occurred within 12h of test initiation. This makes sense based on findings by Renault et al. [7] that indicate AuNPs bind to the outer cell wall in freshwater algae. If AuNPs are bound only to the outside of the algae it is possible that uptake will be limited after the binding areas are saturated.

Daphnids fed algae previously exposed to 4nm AuNP exhibited greater AuNP concentrations at each time point than daphnids that were fed 18nm AuNP exposed algae. This may have been a function of the algal body burden of Au being higher in the 4 nm treatment than in the 18 nm treatment. Bioaccumulation factors for *D. magna* exposed to both sizes of AuNPs were notably smaller than bioconcentration factors calculated from water only exposures of the same AuNPs in previous experiments. This may indicate that the presence of food in the gut tract decreases the rate or extent that AuNPs are bound or absorbed by the *D. magna*, though it is not clear why that might be. It is possible that the food particulates are physically inhibiting uptake in the gut and facilitating movement of AuNPs out of the gut tract. Ferry et al. (2009) concluded that food borne uptake was somewhat mitigated compared to water column borne exposure and that while biofilms in the experiment had some of the highest gold uptake factors, organisms that primarily fed on biofilms had some of the lowest uptake factors [14].

This study indicated that both 4nm and 18nm citrate coated AuNps can be taken up from the water and transferred from *S. capricornutum* to *D. magna*. This is similar to findings by Renault et al. that showed that 10nm amine-coated could be trophically

transferred from freshwater algae to freshwater bivalves [7]. It is not appropriate to compare levels of gold contamination between these studies, however, since Renault et al. obtained gold concentrations from specific portions (gill tissue and visceral mass) of the bivalve while this study considered pooled mass whole body Au concentrations in *D. magna*. Interestingly while *D. magna* uptake rates of Au in waterborne exposures of 18nm and 4nm AuNPs were significantly different, uptake rates for food borne exposures of 18nm and 4nm AuNPs were not different. This indicates that food in the gut tract influences both the rate and extent AuNP uptake in *D. magna*, and seems to overwhelm or in some way mitigate the particle size effect observed in waterborne exposures. These results are similar to findings by Petersen et al. (2008) in which carbon nanotubes were found to bioconcentrate in *D. magna*. Peterson et al. found that carbon nanotubes did not readily depurate when organisms were placed in uncontaminated water which is similar to the lack of depuration observed in 4nm AuNP exposures in this study. Petersen et al. also found that when food was administered, depuration of carbon nanotubes was observed but was incomplete [8]. This is interesting in that we found a similar trend of incomplete depuration in 18nm AuNP exposures, except in the absence of food. Furthermore, the enhanced depuration seen with the application of food by Petersen et al. suggests that food in the gut tract of *D. magna* has a significant influence on nanoparticle behavior similar to our observation in this study that food borne exposures of AuNPs resulted in much smaller uptake than in waterborne exposures. Nanoparticles are mainly incorporated in the gut [9, 10] and there is evidence that *in vivo* biomodification can occur [10], there is also evidence that indicates that surface chemistry is related to uptake

of nanoparticles [11]. It is possible that the change in uptake rate seen in this study in the food borne exposures was a result of surface chemistry modification either in the gut of *D. magna* or related to prior exposure in *S. capricornutum*. Though it is not possible to determine if that is the case in this study, there may be an opportunity to explore this question in future research.

In conclusion these data indicate that uptake and depuration of AuNPs by *D. magna* is dependent on particle size with larger AuNPs exhibiting increased depuration. Nanoparticle depuration is incomplete over the duration of these experiments for 18nm AuNPs and was insignificant for 4nm AuNPs. Furthermore, this study shows that 4nm and 18nm AuNps can bioconcentrate in *S. capricornutum* and can be transferred from the algae to *D. magna* via ingestion. Incomplete depuration in 18nm AuNP exposures and the lack of any observable depuration by *D. magna* in 4nm AuNP exposures raise questions about the internal fate of AuNPs in *D. magna* and the potential effect particle size has on this fate. Further research may involve exposures of a greater variety of AuNP sizes and different shapes or surface chemistries to *D. magna* and attempts to elucidate binding and uptake mechanisms.

Tables and Figures

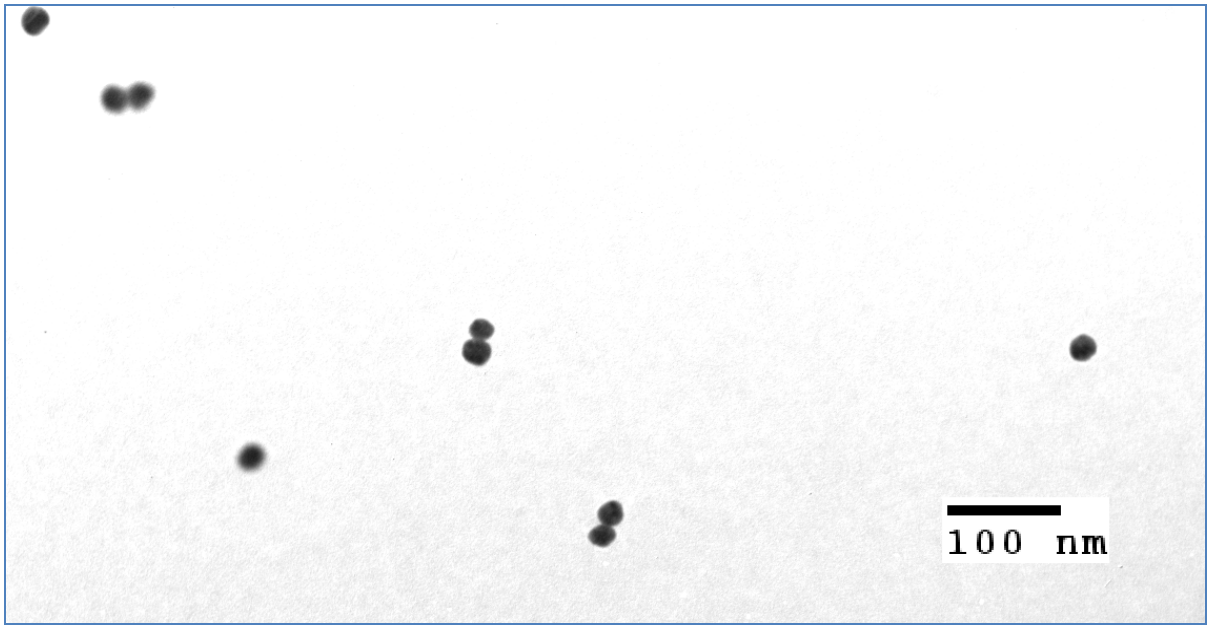

Figure 1. TEM microscopy image of AuNPs of approximately 18nm diameter.

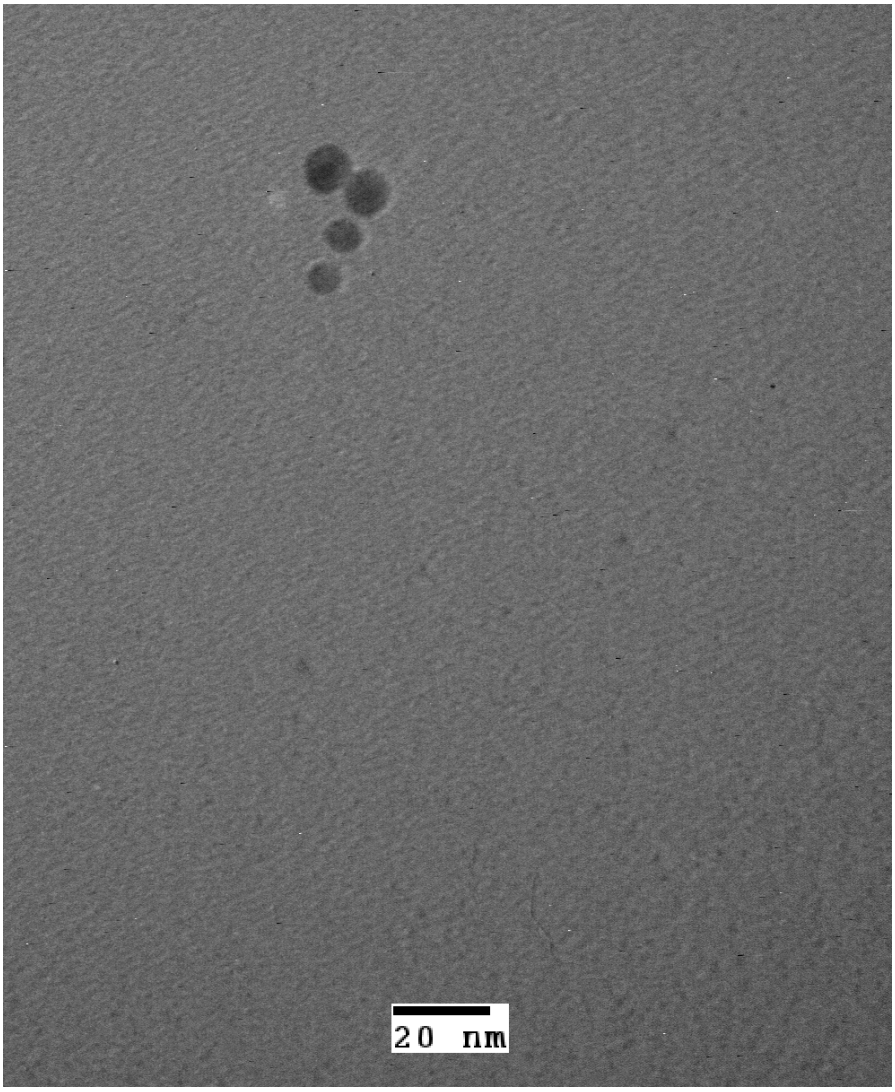

Figure 2. TEM microscopy image of AuNPs of approximately 4nm diameter.

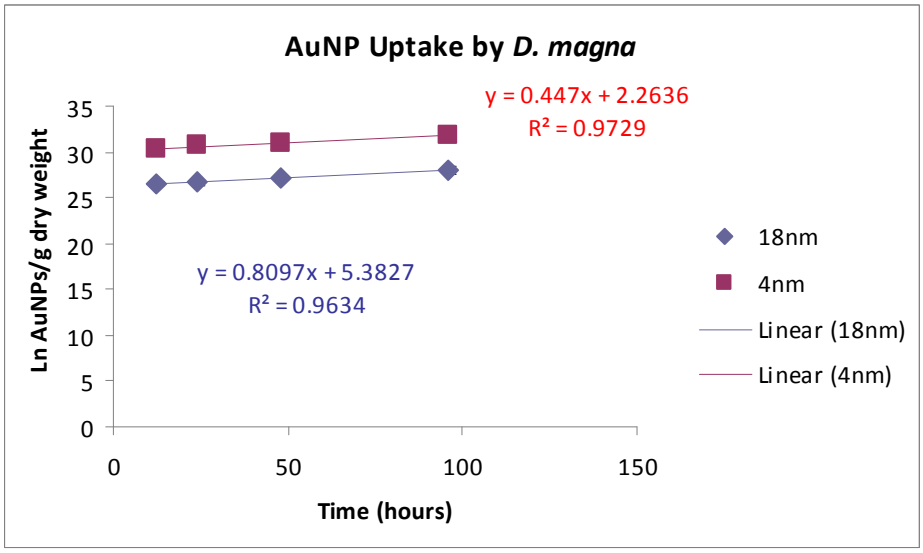

Figure 3. AuNP concentrations in *D. magna* exposed to 4nm and 18nm gold nanospheres.

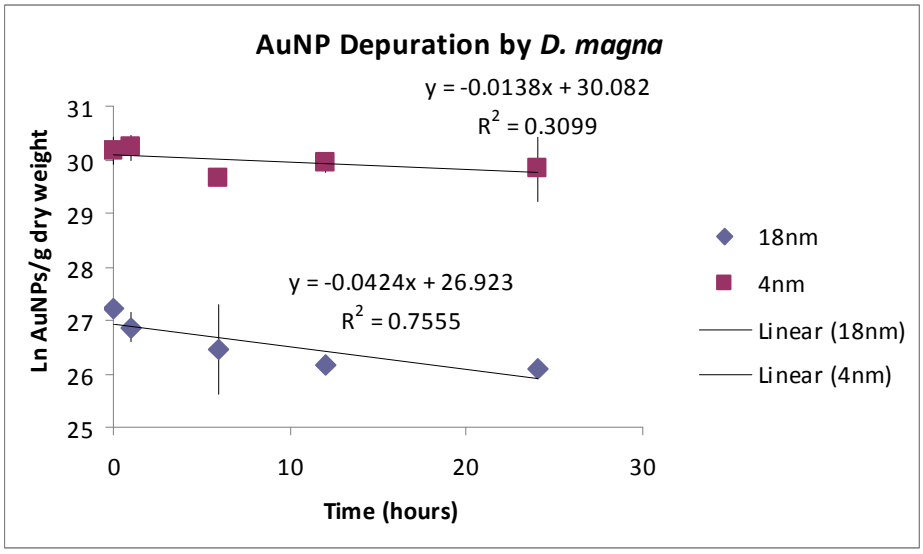

Figure 4. Whole body gold concentrations in D. magna after exposure to 4nm and 18nm AuNps and transfer to uncontaminated water. (error bars represent $^{+}$/- one standard deviation)

Table 1. AuNP and whole body Au concentrations (µg/g) in
D. magna and *S. capricornutum* exposed to 4nm and 18nm gold nanospheres.

Exposure		**18nm**		**4nm**
	Time	Au body burden µg/g	Time	Au body burden µg/g
	12	20.30	12	9.40
	24	24.05	24	13.64
	48	36.31	48	19.63
Water only	96	86.63	96	46.83
		AuNPs/g		AuNPs/g
	12	3.45376E+11	12	1.45749E+13
	24	4.09191E+11	24	2.11504E+13
	48	6.17798E+11	48	3.04321E+13
	96	1.4741E+12	96	7.25938E+13
		18nm		**4nm**
	time	Au body burden µg/g	time	Au body burden µg/g
	0	39.32	0	8.42
	1	27.86	1	8.85
	6	23.22	6	4.89
	12	13.74	12	6.56
Depuration	24	12.59	24	6.59
		AuNPs/g		AuNPs/g
	0	6.6911E+11	0	1.30478E+13
	1	4.74087E+11	1	1.37175E+13
	6	3.9512E+11	6	7.5753E+12
	12	2.33809E+11	12	1.01639E+13
	24	2.14161E+11	24	1.02194E+13
		18nm		**4nm**
	Time (hours)	Au body burden µg/g	Time	Au body burden µg/g
	12	0.56	12	0.71
	24	1.30	24	0.60
	48	2.79	48	0.72
Algae	96	1.24	96	0.56
		AuNPs/g		AuNPs/g
	12	9464646206	12	1.10777E+12
	24	22142283617	24	9.26411E+11
	48	47482029190	48	1.11168E+12
	96	21174692270	96	8.66054E+11

		18nm		4nm
	Time	Au body burden µg/g	Time	Au body burden µg/g
	12	2.45	12	1.52
	24	3.41	24	1.83
	48	3.62	48	2.89
Food borne	96	4.87	96	4.20
		AuNPs/g		AuNPs/g
	12	41653653230	12	2.36138E+12
	24	58035179386	24	2.84003E+12
	48	61636770004	48	4.48124E+12
	96	82948287211	96	6.514E+12

Table 1. (Continued)

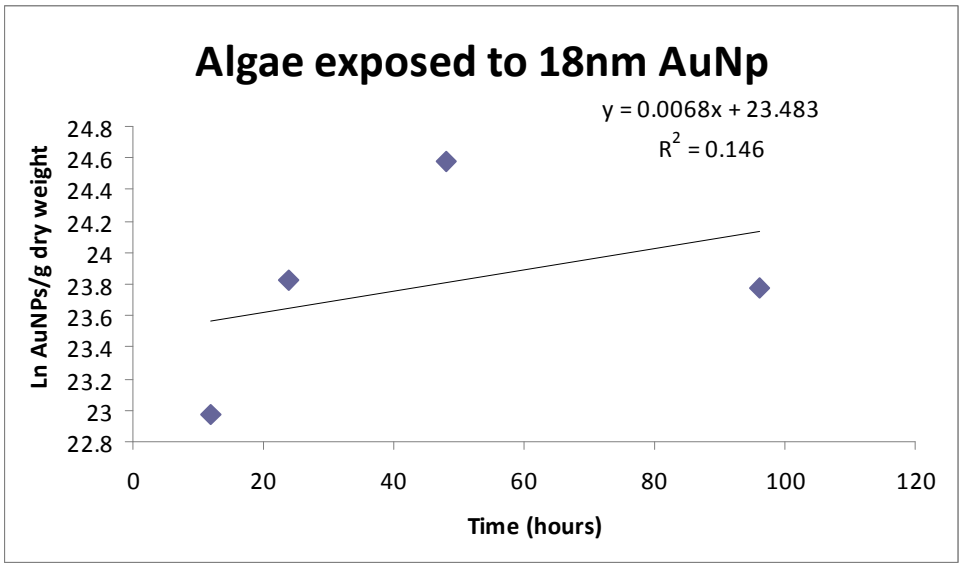

Figure 5. Whole organism Au concentrations for *S. capricornutum* exposed to 18nm AuNps (error bars represent +/- one standard deviation).

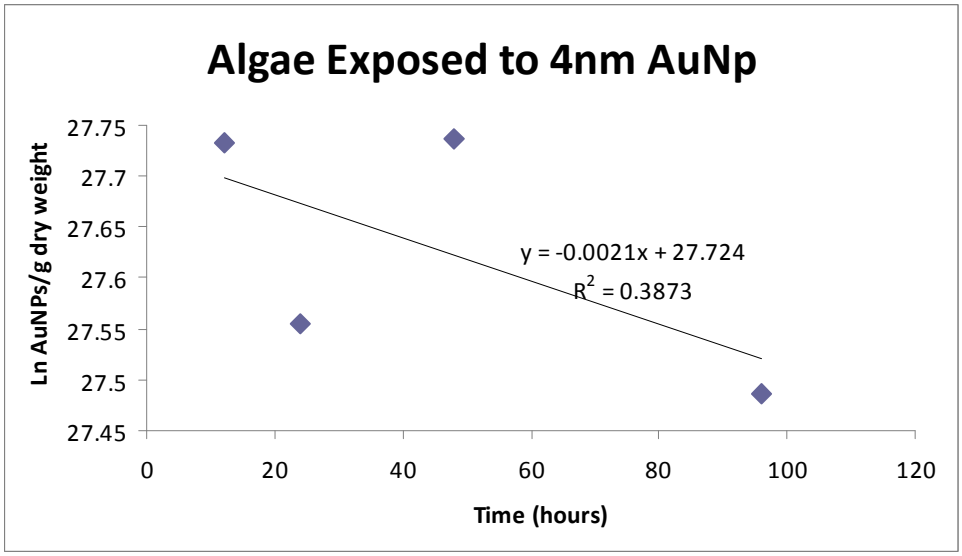

Figure 6. Whole organism Au concentrations for *S. capricornutum* exposed to 4nm AuNps (error bars represent +/- one standard deviation).

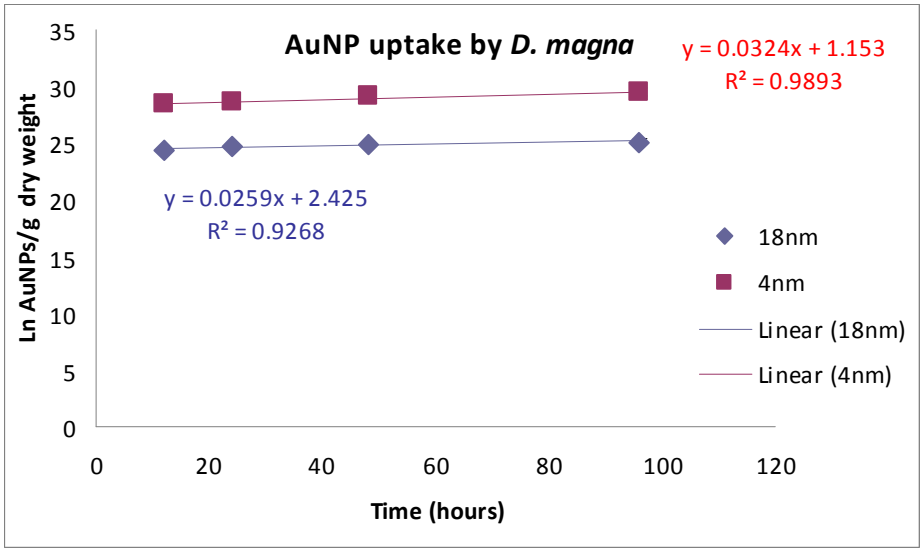

Figure 7. Whole body Au concentrations for *D. magna* fed algae contaminated with 4nm and 18nm AuNps (error bars represent +/- one standard deviation).

References

1. Handy RD, Owen R, Valsami-Jones E. 2008. The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges and future needs. *Ecotoxicology* 17:315-325.

2. Klaine S, Alvarez P, Batley G, Fernandes T, Handy R, Lyon D, Mahendra S, MeLaughlin M, Lead J. 2008. Nanomaterials in the Environment: Behavior, Fate, Bioavailability, and Effects. *Environ Toxicol Chem* 27:1825-1851.

3. Daniel M, Astruc D. 2004. Gold Nanoparticles: Assembly, Supramolecular Chemistry, Quantum-Size-Related Properties, and Applications toward Biology, Catalysis, and Nanotechnology. *Chem Rev* 104:293-346.

4. Turkevitch J, Stevenson P, Hillier J. 1951. Nucleation and Growth Process in the Synthesis of Colloidal Gold. *Discuss Faraday Soc* 11:55-75.

5. Frens G. 1973. Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Suspensions. *Nature: Phys Sci* 241:20-22.

6. Brust M, Walker M, Bethell D, Schiffrin D, Whyman R. 1994. Synthesis of Thiol-Derivatized Gold Nanoparticles in a Two-phase Liquid-Liquid System. *J Chem Soc , Chem Commun* :801-802.

7. Renault S, Baudrimont M, Mesmer-Dudons N, Gonzalez PM, S., Brisson A. 2008. Impacts of gold nanoparticle exposure on two freshwater species: a phytoplanktonic alga (*Scenedesmus subspicatus*) and a benthic bivalve (*Corbicula fluminea*). *Gold Bulletin* 41:116-126.

8. Petersen E, Akkanen J, Kukkonen J, Weber W. 2009. Biological Uptake and Depuration of Carbon Nanotubes by *Daphnia magna*. *Environ Sci Technol* 43:2969-2975.

9. Roberts A, Mount A, Seda B, Souther J, Qiao R, Lin S, Ke PC, Rao A, Klaine S. 2007. In vivo biomodification of lipidcoated carbon nanotubes by Daphnia magna. *Environ Sci Technol* 41:3025-3029.

10. Zhu Z, Ghosh P, Miranda O, Vachet R, Rotello V. 2008. Multiplexed Screening of Cellular Uptake of Gold Nanoparticles Using Laser Desorption/Ionization Mass Spectrometry. *J Am Chem Soc* 130:14139-14143.

11. Chithrani B, Ghazani A, Chan W. 2006. Determining the Size and Shape Dependence of Gold Nanoparticle Uptake into Mammalian Cells. *Nano Letters* 6:662-668.

12. Lovern S, Owen H, Klaper R. 2008. Electron microscopy of gold nanoparticles in the gut of *Daphnia magna*. *Nanotox* 2:43-48.

13. Ferry J, Preston C, Hexel C, Sisco P, Frey RP, P., Fulton M, Scott G, Decho A, Kashiwada S, Murphy C, Shaw T. 2009. Transfer of gold nanoparticles from the water column to the estuarine food web. *Nat Nano* 4:441-444.