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Uptake of Nanoparticles of Cerium Oxide and Yttrium Oxide by *Acanthamoeba castellanii* (Protozoa) and *Daphnia magna* (Crustacea)

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ABSTRACT

Currently, nanoparticles are synthesized and used at an unprecedented rate for industrial, medical, and research applications. The use of cerium oxide nanoparticles (CeONP) and yttrium oxide nanoparticles (YtONP) results in their spread as contaminants into the environment. Once in the environment, CeONP and YtONP can be taken up by organisms in the food chain where they may pose a public health risk. In this study we determine whether *Acanthamoeba castellanii* and *Daphnia magna* uptake CeONP or YtONP from their environment and thereby play a role in the transmission of the nanoparticles. Using electron microscopy, organisms exposed to the nanoparticles were examined. Our results indicate that the nanoparticles are associated with cell and organelle membranes. These findings have implications for the health risks associated with environmental contamination by CeONP and YtONP.

INTRODUCTION

In this study we determine whether protists and crustaceans play a role in the transfer of cerium oxide nanoparticles (CeONP) and yttrium oxide nanoparticles (YtONP) from the environment to other organisms within the aquatic food chain. *Acanthamoeba castellanii*, a common protist, and *Daphnia magna*, a planktonic crustacean, are important components in many aquatic ecosystems. Because acanthamoebae, such as *A. castellanni*, are aggressive feeders they consume inorganic and organic compounds from their environment, thereby serving as a link by transferring normally unavailable inorganic components to the food chain (Weekers et al. 1993). *D. magna* feeds on acanthamoebae and other protists found in lower trophic levels. Because of the high reproductive potential of *D. magna*, these planktonic crustaceans can substantially alter the structure and functioning of microbial food webs in freshwater ecosystems , such as acidic swamps, freshwater lakes, ponds, rivers and streams (Guisande 1993). *D. magna* and other aquatic crustaceans have the ability to filter particles of a variety of sizes including nanoparticles (Rosenkranz et al. 2009, Kim et al. 2010, Zhu et al. 2010). The authors hypothesized that CeONP or YtONP in

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the natural environment could be taken up by these organisms. Particles engineered at dimensions between 1-100 nm, are referred to as nanoparticles. Currently, nanoparticles are synthesized and used for industrial, medical, and research applications. CeONP are used as diesel fuel additives, in automotive catalytic converters, and are a by-product of many industrial processes, including the polishing of glass and semi-precious stones. CeONP also have potential uses for medical applications acting as an antioxidant (Elswaifi et al. 2009). Yttrium oxide and cerium oxide belong to the rare earth elements. Yttrium oxide nanoparticles (YtONP) are used in the manufacturing of cathode ray tubes for computer monitors and televisions and have potential medical applications due to their ability to act as antioxidants (Schubert et al. 2006, Cotton 2006, Okuyama et al. 2007, and Gilmore et al. 2008). The use of CeONP and YtONP results in their release into the environment (Biswas and Wu 2005, Chow et al. 2005) where they may exist in concentrations and forms that are toxic. This release may result from the process of their synthesis, as a by-product of their use, or from their indiscriminate disposal after use. Presently, CeONP and YtONP are released into the environment from diesel engine emissions, from improper disposal of automotive catalytic converters, and from improper disposal of old TV and computer monitors. These nanoparticles may then make their way into air, soil, or ground water (Biswas and Wu 2005, Chow et al. 2005). As with many of the engineered nanoparticles, CeNOP and YtONP may also have toxic effects on humans and animals (Gatti and Montanari 2008). The toxicity of CeONP and YtONP has been recently investigated *in vivo* and *in vitro* (Gojova et al. 2007, Gatti and Montanari 2008, Andelman et al. 2009, Hardas et al. 2010). Toxic effects include reduced cell viability, increased cellular oxidative damage, and apoptosis. Effects also include vascular inflammation that may lead to pulmonary thromboemblism resulting in stroke or myocardial infarction. Chronic inflammation may also lead to rare earth pneumoconiosis or lung cancer (Gojova et al. 2007, Gatti and Montanari 2008, Andelman et al. 2009, Hardas et al. 2010, Lin et al. 2006). However, the routes of exposure of humans to these nanoparticles are poorly understood. Examples of routes of exposure of CeONP and YtONP may be through the contamination of organisms in the food-chain or through contamination of drinking water (Holbrook et al. 2008). In a typical food chain, there is usually a maximum of four or five trophic levels, although food chains in aquatic ecosystems frequently contain more levels than those in terrestrial ecosystems (Pimm and Lawton 1977). As organisms in lower trophic levels are consumed by those in a higher level, nanoparticles may become concentrated in top level consumers, level consumers, namely fish and animals that consume fish, including humans. Many protists, including acanthamoebae, are voracious feeders of organic and sometimes inorganic materials as they occupy the bottom of aquatic and some terrestrial ecosystems (Khan 2009).

Acanthamoeba spp. are free-living amoebae that are ubiquitous in aquatic and terrestrial ecosystems. Acanthamoebae exist as trophozoites, the feeding stage, and as cysts. Presently, 23 species of *Acanthamoeba* are reported and their biology and pathogenicity reviewed by Marciano-Cabral and Cabral (2003) and Khan (2009). At least three species of *Acanthamoeba* have been reported as parasites of animals, and humans (Marciano-Cabral and Cabral 2003 and Khan 2009). It is unknown whether CeONP or YtONP particles are taken up and concentrated by protists or by crustaceans in contaminated aquatic ecosystems. In this report we investigate whether CeONP and

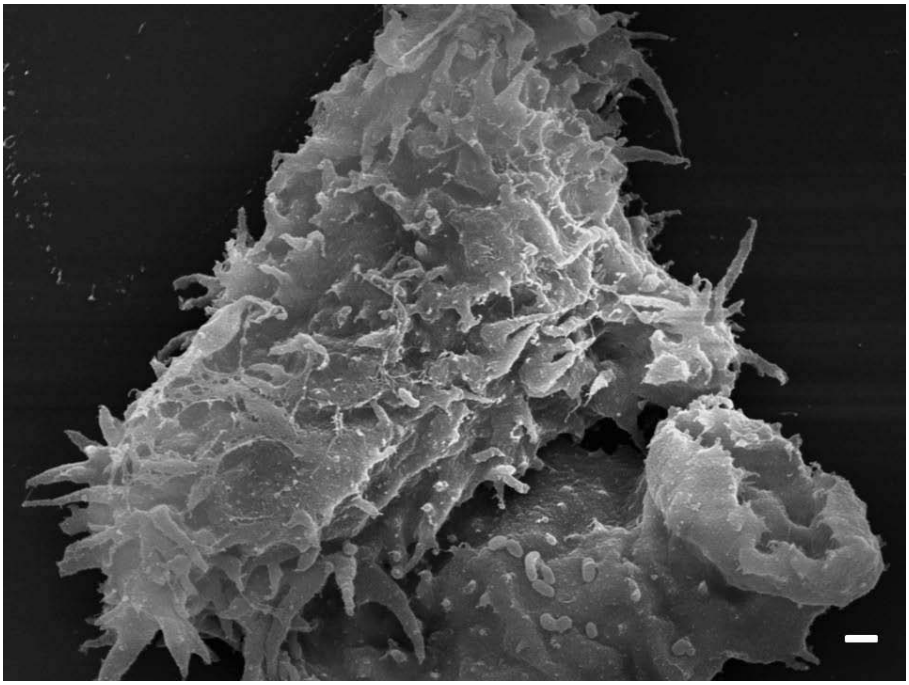

FIGURE 1. Scanning electron micrograph showing two trophozoites feeding stages of *Acanthamoeba castellanii.*

YtONP may be incorporated into the protest *Acanthamoeba castellanii* (Fig. 1) and into the arthropod *Daphnia magna*, two integral components representing organisms at two different trophic levels of the aquatic food chain.

MATERIALS AND METHODS

Exposure of Organisms to Nanoparticles: CeONP and YtONP stock solutions were prepared by suspension of nanoparticles in distilled water. Samples of the nanoparticles suspended in distilled water were placed in a vortex apparatus for 5 minutes before and after preparation of the solution to minimize formation of nanoparticle aggregates. *D. magna* cultures were exposed to nanoparticles by the addition of the respective solution to make a final concentration of 10 μM of CeONP or YtONP. *D. magna* viability was determined by observing motility and gill movement. Distilled water without nanoparticles was used in control samples. CeONP or YtONP were added to cultures of *A. castellanii* containing >95% trophozoites, making a final nanoparticle concentration of 100 nM. Equal amounts of distilled water were used for exposure of control samples. Cultures were incubated for 24 hours at 25C, washed two times in Page's saline solution (Petry et al. 2006), then processed for electron microscopy using standard techniques. Control groups consisted of organisms treated with only distilled water. To define the appearance CeONP and YtONP alone, suspensions were used to prepare samples for observation by transmission and scanning electron microscopy.

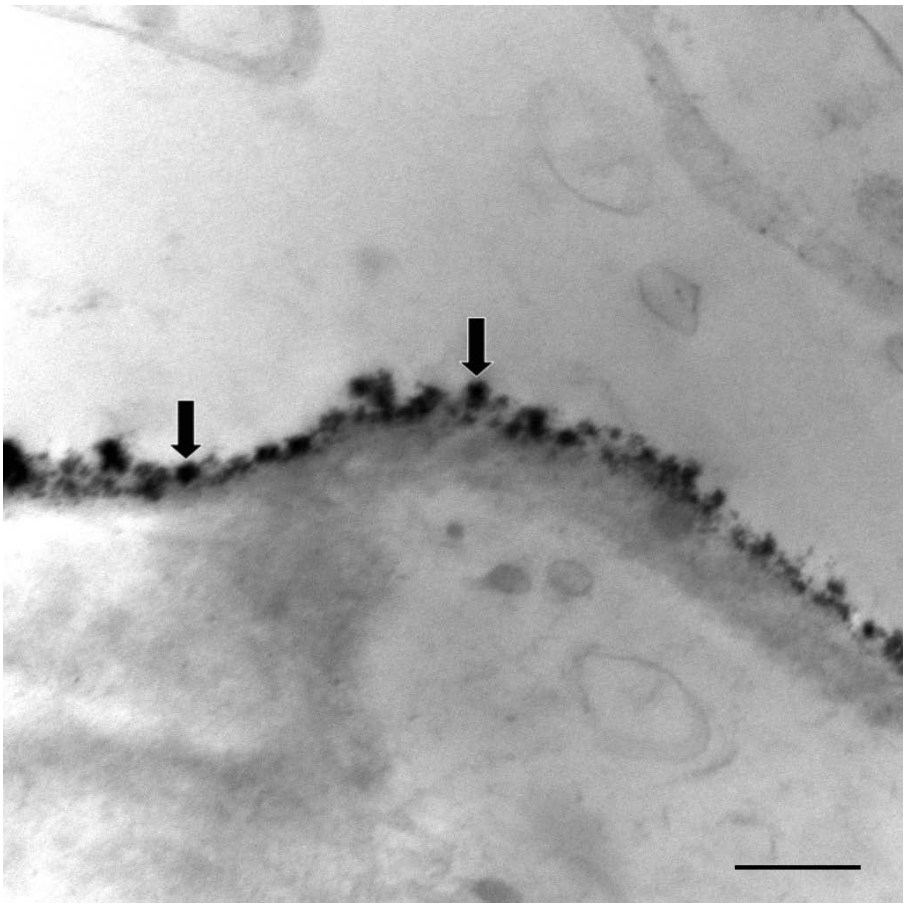

FIGURE 2. Outer membrane of *Acanthamoeba castellanii* demonstrating accumulations of yttrium oxide nanoparticles attached to outer surface. Arrows indicating yttrium oxide nanoparticles (Bar = 500nm).

The appearance of CeONP and YtONP in cells was compared to those reported in other studies. Each experiment was performed twice including control groups. There were sixteen experimental group cultures; eight cultures of *A. castellanii* and eight cultures of *D. magna*. There were four control groups run for each species; two cultures each, exposed to CeONP for *D. magna* and A. *castellanii* and 2 cultures each, exposed to YtONP for *D. magna* and A. *castellanii* .

Electron Microscopy: After exposure of *A. castellanii* and *D. magna* to CeONP or YtONP, specimens were prepared for electron microscopic examination. *A. castellanii* was washed two times using Page's saline solution, centrifuged and fixed in 0.5% buffered glutaraldehyde at 4C for 72 hours. *D. magna* was pre-fixed in FAA fixative solution for 5 minutes at 4C and post-fixed in 0.5% buffered glutaraldehyde at 4C for

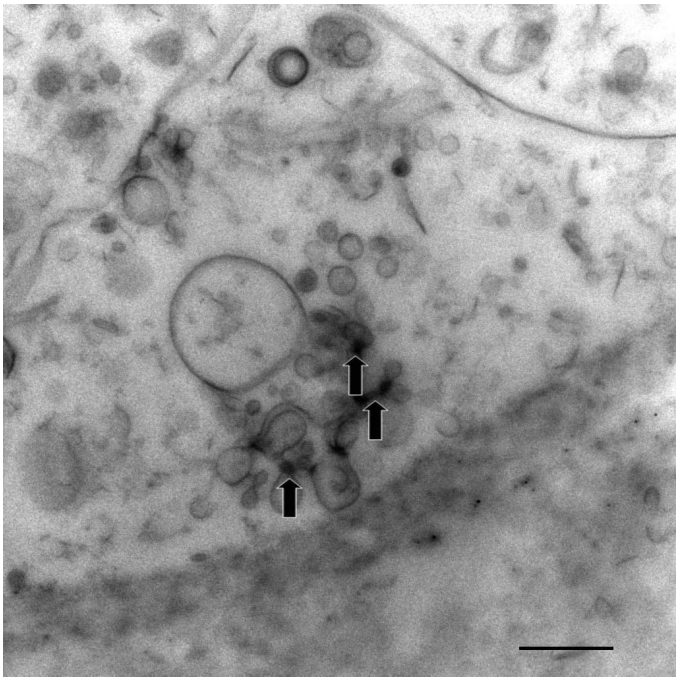

FIGURE 3. Golgi apparatus of *Acanthamoeba castellanii* demonstrating cerium oxide nanoparticles associated with Golgi vesicles. Arrows indicating cerium oxide nanoparticles (Bar = 500nm).

72 hours. *A. castellanii* and *D. magna* were then processed for ultra-thin sectioning and examination using transmission electron microscopy according to standard methods used by the Virginia-Maryland Regional College of Veterinary Medicine Morphology Services Laboratory. Electron micrographs of thin sections of *A. castellanii* and *D. magna* were examined for densely stained nanoparticles of CeONP and YtONP to determine if nanoparticle uptake had taken place. For scanning electron microscopy, samples of *A. castellanii* were isolated and processed according to standard methods used by the Virginia-Maryland Regional College of Veterinary Medicine Morphology Services Laboratory.

RESULTS

Our results demonstrate that CeONP and YtONP can be readily taken up by A. *castellanii* and *D. magna*. We have also demonstrated that after uptake, CeONP and YtONP were associated with cell and organelle membranes in these organisms. *Acanthamoeba castellanii*: CeONP and YtONP were observed in association with cell and organelle membranes of *A. castellanii*. These nanoparticles were observed on the outer surface of the cell membrane (Fig. 2) and on the membranes of intracellular organelles, including Golgi apparatus (Fig. 3) and various vesicles (Fig. 4). Vesicles

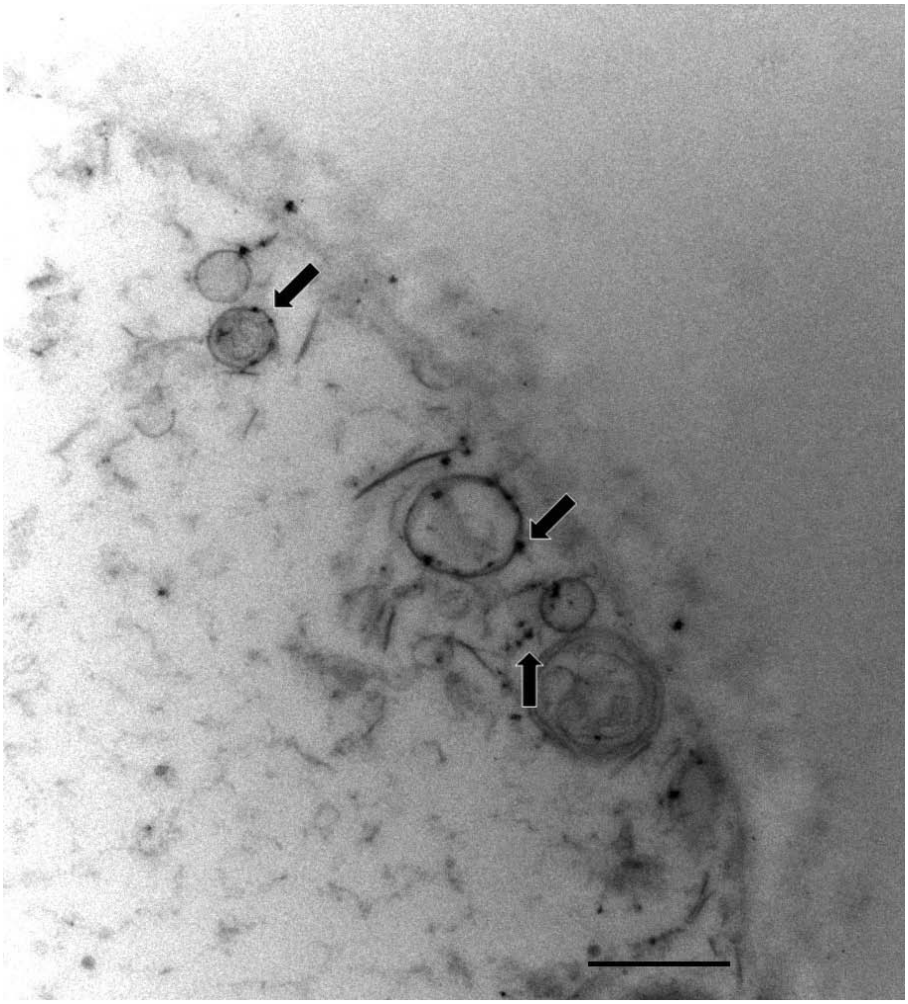

FIGURE 4. *Acanthamoeba castellanii* demonstrating yttrium oxide nanoparticles within organism outer membrane and organelle membranes. Arrows indicating yttrium oxide nanoparticles (Bar = 500nm).

containing nanoparticles were observed lining the inner surface of the cell membrane (Fig. 4). In addition, *A. castellanii* treated with YtONP revealed that the nanoparticles blanketed the outer cell surface and sometimes appeared as aggregates, some crystalline and some with ill-defined edges throughout the organism.

Daphnia magna: CeONP and YtONP were observed throughout the tissues of *D. magna*. These nanoparticles were observed in multiple organs including reproductive and digestive, and were also observed in gill filaments. Observation of both cross sections and longitudinal sections of gill filaments revealed a close association with

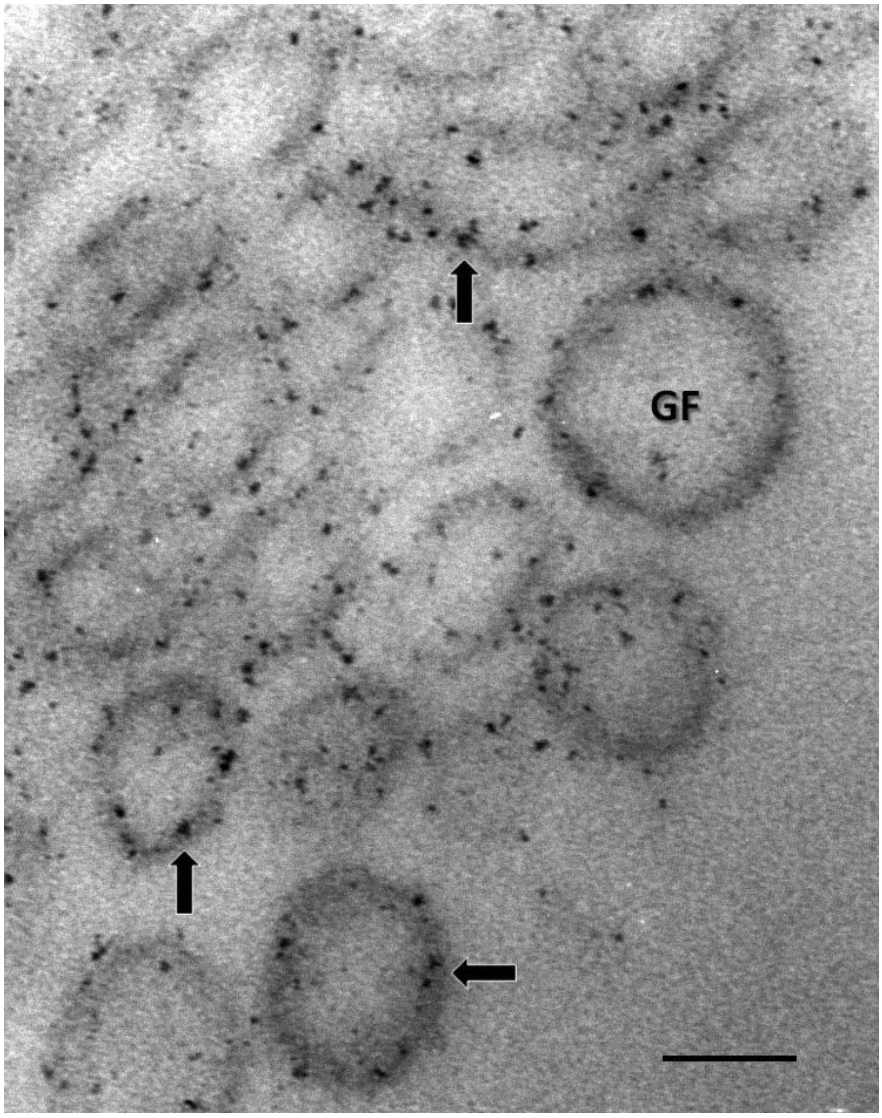

FIGURE 5. Cross sections of gill filaments (GF) of *Daphnia magna* showing yttrium oxide nanoparticles on gill filament borders. Arrows showing yttrium oxide nanoparticles on gill filament boarders (Bar = 100nm).

YtONP (Fig. 5 and Fig. 6). YtONP were also observed in the space underlying the gill filaments (Fig. 6). CeONP were observed at the site of muscular attachment to the cuticle. At the sub-cellular level, CeONP and YtONP were observed in mitochondria, nuclei, muscle fibers, vesicles, and within the multilamellar bodies (Fig. 7). CeONP

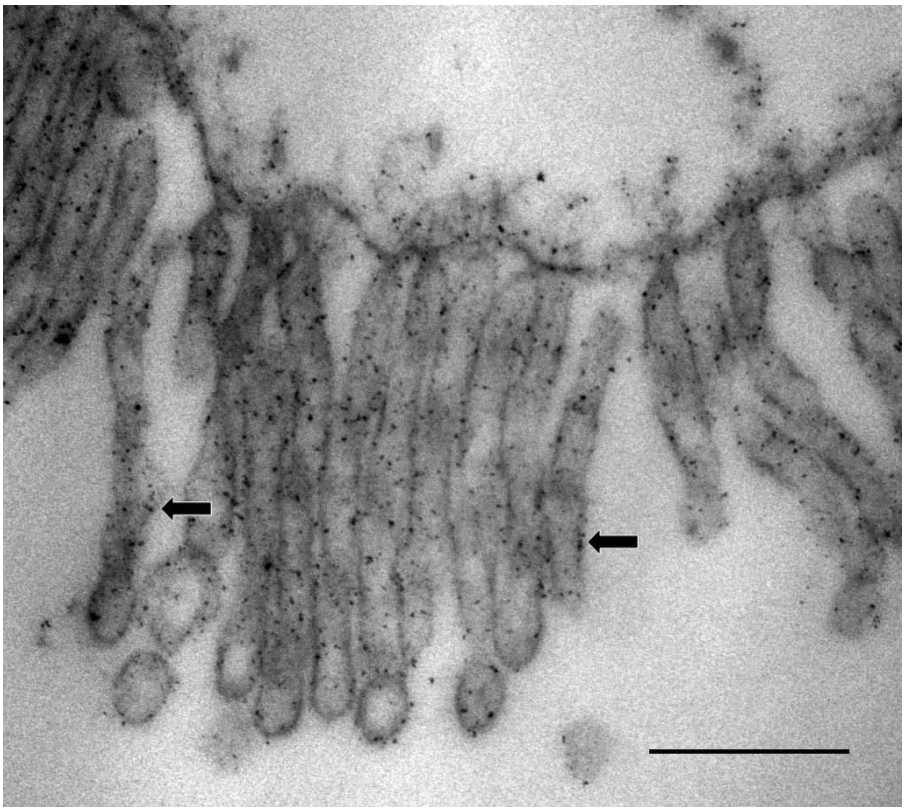

FIGURE 6. Longitudinal section of gill filaments of *Daphnia magna* demonstrating yttrium oxide nanoparticles along gill filament borders. Arrows showing yttrium oxide nanoparticles on gill filament boarders (Bar 500 nm).

and YtONP were observed on the surface of various organelle membranes, on mitochondrial cisternal membranes, and on the membrane surfaces and internal contents of vesicles.

DISCUSSION

The observations of nanoparticles in both *A. castellanii* and *D. magna* indicate that nanoparticles are associated with cells and their membranes. The association of CeONP and YtONP with cellular membranes of *A. castellanii* and *D. magna*, may indicate the first step in the diffusion of the nanoparticles into the cell. The association of the nanoparticles with vesicles found along the cell surface (Fig. 4) may indicate that the nanoparticles are actively transported into the cells by endocytosis or possibly delivered through membrane channels to vesicles for further breakdown. Alternatively, the nanoparticles may diffuse passively through the membranes. The presence of proteins

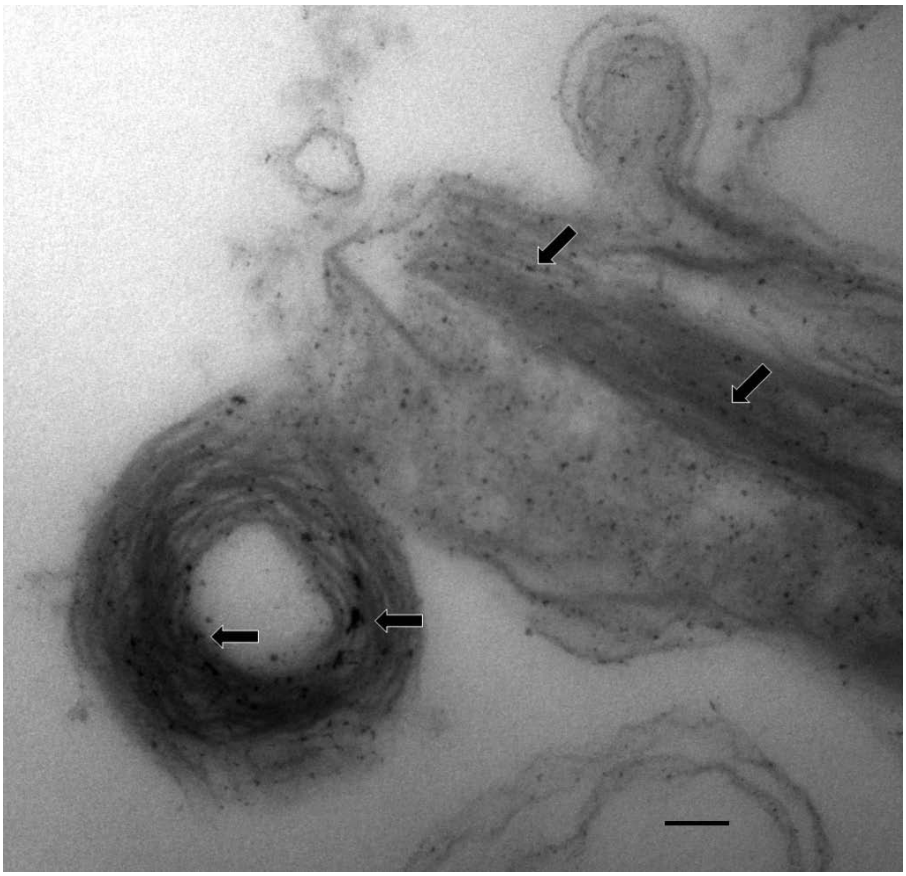

FIGURE 7. Multilamellar body and associated structures of *Daphnia magna* showing cerium oxide nanoparticles throughout. Arrows showing cerium oxide nanoparticles (Bar = 100nm).

on the cells surface of *A. castellanii* may play a role in enhancing the uptake of nanoparticles by coating the nanoparticles and enhancing phagocytosis (Andelman et al. 2009, Lynch et al. 2009). Both CeNOP and YtNOP were associated with cell and organelle membranes. However, the amount and distribution of YtONP in cells appeared to be greater than that for CeONP.

Acanthamoebae and planktonic crustaceans are important components in aquatic food chains (Jürgens 1994, Zöllner et al. 2003). Acanthamoebae interact directly with bacterial and fungal decomposers, and with autotrophic and heterotrophic microplankton. Our observation of YtONP in the gill filaments of *D. magna* (Fig. 5. and Fig. 6) supports the ability of these crustaceans to filter particles in the nanoscale range. The presence of *D. magna* may influence the food chain structure because they

consume microplankton from lower trophic levels. In turn they are consumed by fish, making *D. magna* a key factor in the propagation of trophic cascades from bacteria to fish (Jürgens 1994). It is possible in the natural environment that nanoparticles could be transferred to *Daphnia* following the ingestion of acanthamoebae containing nanoparticles; fish could then feed on these *Daphnia*. Fish containing nanoparticles may transfer them directly to humans who consume the fish, or indirectly through the feeding of forge-fish to livestock, which are then consumed by humans.

The ability of *A. castellanii* and *D. magna* to uptake CeONP and YtONP from their environment has implications on the organisms' ability to deliver nanoparticles to higher trophic levels, including human who consume fish species. The potential for transfer of CeONP and YtONP to humans through the food chain poses a significant public health risk (Biswas and Wu 2005, Chow et al. 2005, Gojova et al. 2007, Gatti and Montanari 2008, Gojova et al. 2009) that requires further investigation. The ability of *A. castellanii* to uptake CeONP and YtONP also has implications for transmission of microbial diseases. Acanthamoebae are ecologically important as natural grazers that feed on organic and inorganic matter in the soil and water, thereby contributing to the natural recycling of nutrients and minerals. Acanthamoebae also consume bacteria, fungi, and other protists. Therefore, acanthamoebae are important for ecological balance and help regulate microbial populations in nature (Sinclair et al. 1981, Foster and Dormaar 1991, Kreuzer et al. 2006, Khan 2009). The ability of CeONP and YtONP to act as cellular antioxidants, to protect cells, and to elongate their lifespan (Rzigalinski 2005, Schubert et al. 2006, Rzigalinski et al. 2006, Elswaifi et al. 2009) means that these effects may apply to bacteria engulfed by the acanthamoebae. Therefore, environmental contamination by these nanoparticles may lead to the development of bacteria that resist killing in the phagosome and become "superbugs" (Elswaifi et al. 2009) thereby posing a further risk to the environment and to human health. On the other hand, bacteria may be directly exposed to the nanoparticles in the environment, then, if consumed by humans, those bacteria may become more resistant to killing by the immune system or by antibiotics (Elswaifi et al. 2009).

CONCLUSIONS

In conclusion, our results demonstrate that CeONP and YtONP can be readily taken up by a protozoan (*A. castellanii*) and a planktonic crustacean (*D. magna*), two integral components in an aquatic food chain. We have also demonstrated that after uptake, CeONP and YtONP are associated with cell and organelle membranes. These finding have implications for the health risks associated with environmental contamination by CeONP and YtONP.

AUTHORS' CONTRIBUTIONS

JRP conceived of the study, helped design the study, participated in growing the organisms and exposing them to nanoparticles, participated in interpreting the results, and drafted the manuscript. SFE conceived of the study, helped design the study, participated in interpreting the results, and assisted in drafting the manuscript. CM prepared *Acanthamoeba* for electron microscopy and collected and photographed specimens. GG prepared *Daphnia* for electron microscopy and collected and photographed specimens. All authors read and approved the final manuscript.

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