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AQUEOUS TOXICITY AND FOOD CHAIN TRANSFER OF QUANTUM DOTS™ IN FRESHWATER ALGAE AND *CERIODAPHNIA DUBIA*

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Abstract

Innovative research and diagnostic techniques for biological testing have advanced during recent years because of the development of semiconductor nanocrystals. Although these commercially available, fluorescent nanocrystals have a protective organic coating, the inner core contains cadmium and selenium. Because these metals have the potential for detrimental environmental effects, concerns have been raised over our lack of understanding about the environmental fate of these products. U.S. Environmental Protection Agency test protocol and fluorescence microscopy were used to determine the fate and effect of quantum dots (QDs; Qdot® 545 ITK™ Carboxyl Quantum Dots [Fisher Scientific, Fisher part Q21391MP; Invitrogen Molecular Probes, Eugene, OR, USA]) using standard aquatic test organisms. No lethality was measured following 48-h exposure of *Ceriodaphnia dubia* to QD suspensions as high as 110 ppb, but the 96-h median lethal concentration to *Pseudokirchneriella subcapitata* was measured at 37.1 ppb. Transfer of QDs from dosed algae to *C. dubia* was verified with fluorescence microscopy. These results indicate that coatings present on nanocrystals provide protection from metal toxicity during laboratory exposures but that the transfer of core metals from intact nanocrystals may occur at levels well above toxic threshold values, indicating the potential exposure of higher trophic levels. Studies regarding the fate and effects of nanoparticles can be incorporated into models for predictive toxicology of these emerging contaminants.

Keywords

Quantum dots; Aqueous toxicity; Food chain transfer; *Pseudokirchneriella subcapitata*; *Ceriodaphnia dubia*

INTRODUCTION

Engineered nanomaterials differ in size, shape, surface area, and chemical composition and currently are used in research, industrial, and consumer applications. These nanoscaled

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materials may be divided into two large subcategories, carbon-and metal-based particles. Carbon nanostructures have received some toxicological characterization [1–3]; however, few toxicological profiles have been performed for metal-based particles. Semiconductor nanocrystals are commercially available, metal-based nanoparticles and can be engineered to fluoresce at visible wavelengths. Semiconductor nanocrystals contain a Cd/Se or Cd/Te crystalline core (diameter, ~4 nm) coated with a shell of ZnS. An outer organic polymer coating is present and can be treated so that active residues are available for linking to other molecules (e.g., antibodies, lectins, and nucleic acids). The overall diameter of the resulting quantum dot (QD; Qdot® 545 ITK™ Carboxyl Quantum Dots (Fisher Scientific, Fisher part Q21391MP; Invitrogen Molecular Probes, Eugene, OR, USA) nanocrystal is approximately 10 to 25 nm—similar in size to small proteins. Stable fluorescence and surface treatments render these particles excellent replacements for labile fluorescent dyes using during in vivo and in vitro applications.

One example of these applications is the recognition of QD phagocytosis by cultured cells [4], leading to methodologies using this trait to determine the metastatic potential of cancer cell lines [5]. In vitro analysis with tumorigenic cell lines provides a valuable diagnostic tool in determining the invasive potential of in vivo human cancer cells. Some investigators, however, have reported that the surface coatings of QDs are subject to photolysis or oxidation [6–8], which may result in dissolution of the core so that the toxic metals are available as hydrated ions. The results of one in vitro study suggest that this significantly increases the toxicity associated with QD exposure in cultures of primary rat hepatocytes [6]. Intact QDs offer advantages as diagnostic tools for in vivo and in vitro testing because of their unique physical characteristics. The ease of suspension increases mobility as an aqueous contaminant, however, and little is known about the uptake of these water-miscible particles by aquatic organisms.

Recent aqueous toxicological studies of metal-based nano-particles have focused primarily on aqueous-phase exposures to test organisms [9,10], but most studies have failed to include toxic effects because of dietary intake of food-associated nano-particles. Realization that both water and dietary metals may invoke toxicity to aqueous test organisms continues to drive investigations [11,12]; however, metal bioavailability and up-take routes remain unclear. Sofyan et al. [12] reported that both water and dietary cadmium were important sources for exposure of *Ceriodaphnia dubia* but that dietary uptake of cadmium from green algae (*Pseudokirchneriella subcapitata*) resulted in a lower body burden than that with aqueous intake alone. This may be explained by uptake within the anterior midgut of the daphnids and subsequent distribution into internal soft tissues as compared to distribution of aqueous cadmium on outer body surfaces [13]. Studies of the biokinetics and development of metal tolerance in *Daphnia magna* include factors such as aqueous and dietary uptake, elimination, and growth [14] as well as the induction of a metallothionein-like protein [15,16]. Nonessential metals, such as cadmium, when transferred within nanocrystals, however, may exhibit properties different from those of the elemental cadmium used in the above-referenced toxicological studies. The coatings on QDs may provide protection to test organisms during aqueous toxicity tests, but QDs exposed to environmental factors, such as photolysis or oxidation, may lose their protective organic coatings [6–8] and expose aqueous organisms to bioavailable metals in the core of the nanocrystal. Additionally, the fate of intact QDs following organism uptake, via either food or the aqueous route, has yet to be established.

Product information provided by the manufacturer ([http:// www.invitrogen.com](http://www.invitrogen.com)) states that 1 ppb of QDs contains approximately 260 ppb of cadmium and a 4:1 ratio of cadmium to selenium [17], providing 65 ppb of selenium per 1 ppb of QDs. Because of the high concentration of cadmium within the core and the largely unknown environmental fate of

intact semiconductor nanocrystals, the potential exists for metal exposure to aquatic organisms following disposal or release of these nanoparticles into the environment. With increasing demand and commercialization of these and other nanomaterials [18], the environmental fate and effects to nontarget organisms need to be determined. Although specific uptake routes remain unclear, metals are accumulated within organisms from both dietary and aqueous uptake; hence, metal-based QDs also may transfer toxic metals via both matrices. The present study has been performed to determine transfer and uptake of QDs through aqueous and dietary exposures and to establish if a food chain transfer of intact particles exists. The freshwater alga *P. subcapitata* (formerly *Raphidocelis subcapitata* and *Selenastrum capricornutum*) and the cladoceran *Ceriodaphnia dubia* were selected because they are established model species in standard toxicological studies and ecological risk assessments [19,20]. These organisms provide a simple model for food chain transfer as well as published endpoints regarding nonessential metals for evaluation and comparison.

METHODS AND MATERIALS

Exposures used Qdot® 545 ITK™ Carboxyl Quantum Dots (Fisher Scientific, Fisher part Q21391MP; Invitrogen Molecular Probes, Eugene, OR, USA). Peak emission of these particles is between 541 and 549 nm, and the organic polymer coating of these nanoparticles was treated so that carboxyl groups were available on their surface. The QD concentration in the purchased suspensions, as stated by the manufacturer, was of 7.7 µM; this concentration was used in all nominal calculations. All test organisms were cultured in the Ecotoxicology Research Facility at Arkansas State University (Jonesboro, AR, USA) according to U.S. Environmental Protection Agency (EPA) [20] protocol. The Arkansas State University Ecotoxicology Research Facility enforces good laboratory practices and quality-assurance guidelines as set forth by the facility.

Aqueous toxicity exposures

The 48-h acute toxicological endpoints were assessed with *C. dubia* using standardized methods [19], and 96-h chronic endpoints were determined with *P. subcapitata* according to the U.S. EPA [20]. Serial dilutions for *C. dubia* used U.S. EPA [20] moderately hard water (hardness, 90 mg/L as CaCO₃), and *P. subcapitata* dilutions were made in Bold's media (Table 1). For this, media salts were dissolved in Milli-Q® pure water (Millipore, Bedford, MA, USA), and 2 ml of trace metal solution were added for each 1 L of media. Solution was autoclaved at 103,421 pascals for 15 min, and 2 ml of vitamin stock were added after cooling to room temperature. *Ceriodaphnia dubia* exposures were conducted in a controlled environment at 25 ± 1°C and a 16:8-h light dark photoperiod. Algal tests were performed in a Conviron growth chamber (model 8507; Controlled Environments Limited, Winnipeg, MB, Canada) on an orbital shaker. Constant rotation was maintained at 100 cpm, continuous illumination at 400 ± 40 foot-candles, and temperature at 25 ± 0.1°C. Microscopic cell counts on a hemocytometer were used to obtain 96-h algal endpoints.

To ensure test sensitivity to toxic metals, organisms also were exposed to dilutions of CdCl₂·2.5H₂O in moderately hard water to determine *C. dubia* 48-h survival endpoints and in Bold's media for *P. subcapitata* 96-h survival/growth endpoints. All CdCl₂·2.5H₂O dilutions were made from a stock solution of 1.0 g Cd/L Milli-Q pure water. Results from all toxicity assays were statistically analyzed using Toxcalc® (Ver 5.0.25; Tidepool Scientific Software, McKinneyville, CA, USA). Initial test dilutions in reference toxicant assays were calculated as nominal cadmium concentrations, with values measured by inductively coupled plasma-mass spectrometry used in final endpoint calculations. All median lethal concentrations (LC50s) and related confidence intervals were determined using probit analysis with Toxcalc. All data were tested using α = 0.05. Assumptions of normality were

tested using the Shapiro-Wilk test, and the Steel many-one rank test was used to determine significance in survival.

The QD 48-h toxicity to *C. dubia* was determined with concentrations up to 110 ppb following the protocol described above. The QD 96-h toxicity to *P. subcapitata* was determined with concentrations up to 55.0 ppb following the aforementioned protocol.

Food chain transfer

Algae exposed to concentrations of QDs during 96-h toxicity tests were washed three times in Bold's media as described in Table 1 following 96-h test shutdown to remove unbound QDs remaining in aqueous suspension. Centrifugation following resuspension was at 1,000 rpm for 5 min. Algal suspensions were then reconstituted to 3.5×10^7 cells/ml in Bold's media. Algae exposed to QDs at concentrations of 11.1 and 55.0 ppb were introduced to 12 *C. dubia* adults, which were allowed to feed for 24 h (targeted algal concentration, 2.0×10^6 cells/ml). Exposed *C. dubia* were from a 24-h age cohort to minimize size variation and had been unfed for 24 h. Subsamples of three to four live organisms were randomly chosen and examined under bright-field and fluorescence microscopy to determine pixel intensity as described below. Two controls accompanied food chain exposures: Unfed organisms, and *C. dubia* exposed to control algae during 96-h toxicity testing. Endpoints of food chain exposure were calculated as the mean pixel intensity of the organism for QD/algal concentration. Results from observations were analyzed using one-way analysis-of-variance techniques. All tests for significance were conducted using $\alpha = 0.05$. For all analyses, data were tested for normality using the Kolmogorov–Smirnov statistic and for homogeneity of variance using the Bartlett test (Minitab™, 2000; Minitab, Inc., State College, PA, USA).

Bright-field and fluorescence microscopy

All images were taken of living organisms using an epifluorescence microscope (excitation wavelength, 465–495 nm; emission wavelength, 515–555 nm; Eclipse E800; Nikon Instruments, Melville, NY, USA) equipped with mercury-vapor illumination and a Cascade Photometric® digital camera (Roper Scientific®, Tucson, AZ, USA). A bright-field image of each *C. dubia* or algal field was taken. Following a 24-h incubation with QD-dosed algae, organisms were immediately placed on a glass slide in a small drop of suspension media (moderately hard water for *C. dubia* and Bold's media for *P. subcapitata*). To ensure consistent focus for *C. dubia*, the postabdominal claw was placed in clear focus for the bright-field imaging. A fluorescence image was then taken without altering the focus (Fig. 1). Algal images were focused with the largest number of cells per microscopic field (Fig. 2). Images were digitized using ACT-1© software (Nikon Instruments) and stored for further analysis. All imaging was conducted within 60 s of removal from incubation suspensions.

MetaMorph© 6.10 Meta Imaging Series Environment (Universal Imaging Corporation™, West Chester, PA, USA) was used to measure average pixel intensity in the fluorescence image. The outline of each *C. dubia* (not including the dorsal brood pouch) was traced on the corresponding bright-field image and transferred to the fluorescence image. Area was calculated for each organism, and treatment groups were compared statistically to detect possible differences in organism size. Individual *P. subcapitata* cells were traced within each microscopic bright-field image and transferred to the corresponding fluorescence image. Both the number and average intensity of the pixels within the outline on the fluorescence image were measured. Significant differences in outlined areas and pixel intensity for each organism was determined using one-way analysis-of-variance techniques as described above.

RESULTS

Aqueous toxicity exposures

The reference toxicant ($\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$) elicited a LC50 for *C. dubia* of 31.9 $\mu\text{g/L}$ following exposure to measured cadmium concentrations ranging from 23.8 to 82.1 $\mu\text{g/L}$. The determination of reference toxicant endpoints to *P. subcapitata* included exposure to measured cadmium concentrations ranging from 32.2 to 330 $\mu\text{g/L}$. The LC50 for *P. subcapitata* to measured cadmium was 129 $\mu\text{g/L}$.

Exposures to *C. dubia* to QDs were calculated from molar concentrations provided by the manufacturer and ranged from 11 to 110 ppb. No toxicological endpoints were measured for *C. dubia* exposed to these QD suspensions (LC50, >110 ppb of QDs). As calculated by the manufacturer's stated 260:1 Cd: QD molar ratio to QDs, *C. dubia* were exposed to cadmium levels of up to 28,600 $\mu\text{g/L}$ and selenium levels of up to 7,150 $\mu\text{g/L}$ within QDs, which did not elicit a toxic response to test organisms. Exposures of *P. subcapitata* to QDs included nominal QD concentrations from 11.1 to 55.0 ppb, with a measured LC50 of 37.1 ppb of QDs. Intact QDs elicited a LC50 (calculated according to the manufacturer's ratio of cadmium to QD) of 9,638 $\mu\text{g/L}$ of cadmium and 2,410 $\mu\text{g/L}$ of selenium to *P. subcapitata* in 96-h exposures.

Algal QD uptake

Pixel intensity measured in algal cells exposed to QDs during 96-h toxicity testing revealed significant differences in fluorescence among treatments and control (Fig. 3). Pixel intensity in algae ranged from 0.47 ± 0.13 for controls to 11.14 ± 17.48 for cells exposed to 55.0 ppb; significant differences were found among each treatment and controls. Significant differences were measured among polygon areas measured for pixel intensity for control algae ($2,808 \pm 801$), algae exposed to 11.1 ppb ($3,332 \pm 767$), and algae exposed to 55.0 ppb ($43,800 \pm 1,522$).

Food chain transfer

No *C. dubia* mortality was noted following the 24-h feeding regime with QD-exposed algae, and the mean area of organism used for calculations of pixel intensity did not differ significantly among treatment groups. Untreated *C. dubia* exhibited autofluorescence, as has been reported previously [21]; unfed organisms exhibited a pixel intensity (mean $\pm$ standard deviation) of 9.45 ± 1.69 (Fig. 4). Following food assimilation, *C. dubia* fed control algae had a pixel intensity of 9.97 ± 0.89 . *Ceriodaphnia dubia* fed algae exposed to 11.1 ppb of QDs had a measured 24-h pixel intensity of 9.71 ± 0.91 , and those fed algae exposed to 55.0 ppb of QDs had a pixel intensity of 13.27 ± 0.53 .

DISCUSSION

Intact QDs provided a measure of protection to aquatic test organisms against toxic core metals. *Ceriodaphnia dubia* survived in the presence of cadmium at concentrations of greater than 500-fold that of previously published LC50s of 54 to 59 $\mu\text{g/L}$ [22–24] and 1,000-fold that of the reference toxicity LC50 measured in the present study. Although a lethal endpoint of QDs to the freshwater alga *P. subcapitata* was determined, intact nanocrystals offered protection up to 190-fold that of the previously published cadmium LC50 of 50 $\mu\text{g/L}$ [25] and 75-fold that of the LC50 measured in the present study. The transfer of QDs from the algal food source to primary consumers in the present study, as measured with increased pixel intensity, provides evidence of dietary ingestion of nanocrystals. Whereas aqueous uptake of QDs provided evidence of bioconcentration by *C. dubia* against an environmental gradient [21], the present study measured uptake from a

food source. Fluorescence intensity at 24 h after being fed algae exposed to 55.0 ppb of QDs was comparable to measured fluorescence following 24-h aqueous exposure to 400 ppt of QDs (pixel intensity, 12 ± 6.4) [21]. Because whether the organic polymer coating of QD facilitates binding to outer exchange sites on organisms during aqueous exposures is yet to be determined, the possibility exists that the QD body burden may include external binding. In any case, the combined effect of exposure through both food and aqueous matrices will determine the bioaccumulation factor of QDs for these organisms.

Higher cadmium body burdens in daphnids have been measured from water sources compared with those measured from diet [12,26]; however, it also has been noted that total body burden is not a good predictor of sublethal toxicity (e.g., re-productive effects) [12]. Barata et al. [26] reported that cadmium accumulation and body burden from aqueous exposure was twice that from dietary exposure. In the present study, QDs from aqueous sources contributed to the same body burden (as measured by pixel intensity) following 400 ppt of aqueous QDs as that following use of algae exposed to 55.0 ppb as a food source. Toxic metals in the core obviously are not bioavailable to *C. dubia*, because no response was measured in the present study. Given that acidic conditions within the gut of primary consumers have the potential to release food-bound toxins [27], feeding spiked algae to *C. dubia* has the potential for transfer of toxic core metals to grazing organisms. If the digestive tract, changes within algal cells, or external environmental factors facilitate the release of core metals, then toxic exposures of aquatic organisms are inevitable, because these concentrations are considerably higher than the known toxic endpoints to most aquatic organisms. Work by Taylor et al. [11] supported the results of previous studies, and those authors described possible gut poisoning in *D. magna* exposed to sublethal cadmium concentrations [28,29], resulting in subsequent decreased daphnid feeding. Although diminished feeding rates of organisms exposed to environmental contaminants will reduce the potential for food chain uptake, unavoidable aqueous exposure persists.

Many algae/contaminant adsorption studies have been performed at high cell densities, but algal densities in the present food chain exposure were similar to those in *C. dubia* toxicity testing. Taylor et al. [11] reported that cadmium adsorption increased in algal cells at approximately the same densities as found in the present study, with metal uptake leveling off at concentrations above approximately 100 $\mu\text{g/L}$. In the present study, QD concentrations resulted in total cadmium adsorption in algal cells up to the LC50 of 9,638 $\mu\text{g/L}$ (37.1 ppb of QDs), providing evidence that the carboxyl protective coating allowed at least temporary protection to the cells during adsorption. Metal uptake onto algal cell surfaces is facilitated by a mixture of cationic and anionic exchange sites [30] and may have provided initial binding sites to QDs, followed by slower absorption into cells. This process also may have contributed to the morphological changes of algal cells noted in the present study.

Following 96-h QD exposure, we noted a change in structure from the typical sickle or crescent shape of many *P. subcapitata* cells to a more spherical shape, indicating a possible change in structural integrity. This observation was most apparent in the exposure with 55.0 ppb of QDs and was reflected in the significant increase in the polygon area as measured for calculations of pixel intensity. It has been shown that free metal binding occurs rapidly by physical adsorption to algal cells with slower uptake into the cell [31,32]. Although the metals in the QD core were not in the free ionic form, we suspect that QDs similarly entered *P. subcapitata* cells, resulting in a change of cell integrity and structure. Structural changes such as these may offer a sublethal endpoint of toxicity that can be measured by increased cell area.

The development of toxicological models (e.g., quantitative structure–activity relationship, biotic ligand, and fugacity) has been an invaluable step toward predictive toxicology;

however, engineered nanoparticles not following elemental physico-chemical properties create new challenges in this field. Food and aqueous exposure to aquatic organisms increases the complexity of developing predictive nanoparticle models. Models continue to be investigated to determine aqueous routes of uptake for ionic metals [12–14]. Tsui and Wang [14] reported assimilation efficiency of *D. magna* as a rate of aqueous uptake and food assimilation, with cadmium assimilation resulting primarily from the aqueous phase and selenium assimilation mostly from food uptake. Bioconcentration in phytoplankton was exponentially higher for selenium than for cadmium, resulting in higher selenium transfer via the food chain [14]. Intact QDs transferred to zooplankton via food or aqueous uptake, however, will transfer core elements of cadmium and selenium at stoichiometric levels present within these nano-crystals. Such transfers in the presence of protective outer coating of nanocrystals will increase overall body burden of these toxic metals to primary consumers, increasing the risk of higher trophic levels to biomagnifications through food webs. In addition, if algal uptake ultimately results in QD breakdown and core metal release, then lethality to primary producers also may lead to food chain disruption. Such prediction of QD breakdown and exposure to toxic core metals may be determined with metallothionein-like protein assays and help to determine the timing or site of metal release. Additionally, future data accrual and simple models for assimilation efficiencies of nanocrystals within aqueous organisms will help to build predictive toxicological models for environmental risk assessments of metal-based particles.

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Fig. 1. Polygons drawn in a bright field and transferred to a fluorescence image to determine the pixel intensity of unexposed *Ceriodaphnia dubia* showing slight autofluorescence of the digestive tract and heart. Polygons were drawn to exclude the brood pouch.

Fig. 2. Polygons drawn in a bright field and transferred to fluorescence images to determine pixel intensity of *Pseudokirchneriella subcapitata* showing increased fluorescence of exposed cells.

Fig. 3. Measured pixel intensity (mean $\pm$ standard deviation) of algal cells following 96-h Qdot® 545 ITK™ Carboxyl Quantum Dots (Fisher Scientific, Fisher part Q21391MP; Invitrogen Molecular Probes, Eugene, OR, USA) exposure. Sample size included control (■; $n = 936$), 11.1 ppb (▨; $n = 257$), and 55.0 ppb (▤; $n = 176$). An asterisk denotes a significance difference from control ($p = 0.005$).

Fig. 4.

Measured pixel intensity (mean $\pm$ standard deviation) of *Ceriodaphnia dubia* following 24-h exposure to algae dosed with Qdot® 545 ITK™ Carboxyl Quantum Dots (Fisher Scientific, Fisher part Q21391MP; Invitrogen Molecular Probes, Eugene, OR, USA). An asterisk denotes a significant difference from control ($p = 0.005$). □ = unfed; ■ = unexposed; ▨ = 11.1 ppb; ▩ = 55.0 ppb.

Table 1

Bold's media for quantum dot algae toxicity tests^a

Salts	mg/L	Trace metals ^b	mg/L	Vitamin stock ^b	mg/L
NaNO ₃	250	ZnSO ₄ ·7H ₂ O	22	Thiamine	200
MgSO ₄ ·7H ₂ O	75	MnCl ₂ ·4H ₂ O	180	Biotin	10
KH ₂ PO ₄	175	CoCl ₂ ·6H ₂ O	10	B ₁₂	10
CaCl ₂ ·2H ₂ O	25	CuSO ₄ ·5H ₂ O	10		
K ₂ HPO ₄	75	NaMoO ₄ ·2H ₂ O	6.4		
NaCl	25	Dissolve metals in 1 L of deionized water in the sequence listed			

^aModified from Pollio et al. [33] and Stein [34].

^bAdd 2 ml for each 1 L of working solution.