Supporting Information

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Effects of Soluble Cadmium Salts Versus CdSe Quantum Dots on the Growth of Planktonic *Pseudomonas aeruginosa*

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Table of Contents

Supporting Tables

Supporting Figures

1. Materials and Methods

Quantum dot synthesis and washing. CdSe QDs were prepared according to Rogach *et al*. (1) which yields fully-dispersed red (peak emission of 600 – 650 nm), citrate-stabilized "bare" QDs (mean dia. 5 nm) in water. QD suspensions were stored in the dark at room temperature, and were dialyzed 24 h before each use in Spectra/Por 1, flat-width 10-mm membranes (MW cutoff 6,000 to 8,000; Fisher Scientific, Hampton, NH) against 4 mM sodium citrate to remove excess Cd(II) ions.

Culture conditions. Bacterial culturing was in Luria Bertani (LB) broth (200 μL) under aerobic conditions in the dark (200 rpm; 30 ^{o}C) within 96-well plates using a BioTek Synergy 2 microplate reader (UV/vis/fluorescence; Winooski, VT) that automatically measured optical density (OD, 600 nm) over time. Prior to each experiment, archived bacteria (maintained at -80 ^{o}C in 70% LB plus 30% glycerol v/v) were freshly streaked for inocula onto LB agar and incubated at 30 ^{o}C for 18 h.

Biochemical analysis. Culture subsamples were centrifuged (12,000 × *g* for 30 min) to separate cells from extracellular polymeric substances (EPS) as before (2). The EPS-containing supernatants were stored (-20 ^{o}C) until analysis. Pelletized cells were either untreated for microscopy, dried under an N_2 stream for XRD analysis, immediately transported for XANES analysis, or resuspended in 0.5 ml of 1.0 N NaOH then lysed by heating (80 ^{o}C, 1 h), neutralized with 0.5 ml 1.0 N HCl and stored (-20 ^{o}C) until analysis for DNA, total protein, or dissolved Cd(II). Samples for ICP-AES were prepared by dissolving in hot 10% (v/v) aqua regia. DNA was quantified by the PicoGreen method (Invitrogen, Carlsbad, CA) using calf thymus DNA as the standard. Total protein was quantified using the Bradford method (3) using Bio-Rad (Hercules, CA) reagents. Cellular carbohydrates were quantified by the phenol-sulfuric acid method using glucose as the standard (4).

DCFH-DA assay. DCFH-DA (Sigma Chemical; dissolved in ethanol at 1mM) was first freshly deacetylated to dichlorofluorescein (DCFH) by reacting 2 mL with 8 mL 0.01 N NaOH for 30 min. in the dark (5). The reaction was halted by adding 40 mL of 25 mM sodium phosphate buffer (pH 7.2), and the solution immediately placed on ice in the dark until use. Mid-exponential phase (15 hours for Cd acetate concentrations from 0 – 125 mg/L and QD concentrations from 0 – 75 mg/L; 27 hours for 125 mg/L QDs) ***P. aeruginosa*** cells were harvested from cultures amended with either CdSe QDs or cadmium acetate. Cell pellets were resuspended in 7.5 mL sterile 0.9% (w/v) NaCl, and triplicate 88 μL aliquots were deposited into a 96 well plate, then 167 μL of the DCFH + phosphate buffer solution was added immediately. Cultures were also subsampled (1 mL) to quantify intracellular DNA as a proxy for biomass. Abiotic controls (sterile LB broth with QD and cadmium amendments as above) were similarly sampled and assayed as were H_2O_2 and dichlorofluorescein (DCF) standards (10 concentrations each, prepared in water, up to 750 and 0.5 mg/L, respectively). After incubating in the dark (30 min.), well fluorescence was measured using the BioTek Synergy 2 plate reader (excitation/emission = 485/528 ± 20 nm).

Abiotic QD dissolution. CdSe QDs were evaluated for abiotic dissolution in sterile nanopure H_2O under ambient light at room temperature, and also in the dark in either sterile nanopure H_2O, sterile LB, or filter-sterilized (0.22 μm) supernatant from 24 hr LB-grown cultures, all in triplicate. For the former, 25 mL of a 75 mg/L (total cadmium) CdSe QD suspension in nanopure water were dialyzed and immediately transferred to a sterile test tube surrounding a Spectra/Por DispoDialyzer® tube (MW cutoff 6,000 to 8,000; Fisher Scientific, Hampton, NH) containing 2.5 mL sterile 4 mM sodium citrate. The dialysis tube contents were sampled (2.5 μL) at regular times and measured for dissolved Cd(II), as above. For the latter, individual 75 mg/L (total cadmium) QD suspensions were prepared from freshly-dialyzed QDs using each aqueous condition above. Each suspension was dialyzed in the dark against 4 mM sodium citrate for 24 h. Total cadmium remaining in each dialysis tube was measured and compared to the starting concentration to determine loss by dissolution.

XRD sample preparation. Cell pellets were washed 1× in sterile DI water to remove excess growth media, then deposited onto Al foil-covered glass XRD slides and dried overnight (O/N). Slides were placed in a Phillips Xpert Diffractometer and XRD profiles were generated using Cu Kα radiation (1.5405Å) at 40 kV and 50 mA. Diffraction peaks between 2θ values of 2° and 70° were recorded and compared to those from a standard slide prepared from dialyzed stock CdSe QDs.

XANES sample preparation. Cell samples were filtered into a concentrated spot of a 0.2 μm polycarbonate filter. Standards used for Se oxidation state measurements were grey metallic selenium (Se(0)), Na_2SeO_3 (Se(IV)), bulk CdSe (Se(-II)) and CdSe QDs (Se(-II)). X-ray energy was selected using a Si(220) double-crystal monochromator, detuned 20% for harmonic rejection. Energy was calibrated by defining the first derivative peak of a Se foil to be 12,658 eV. Samples were collected in fluorescence mode using a 30-element Ge array detector. The total incoming count rates from fluorescent photons were less than 30,000 s^{-1} per element, which is well within the linear response range of the detector. Spectra were collected over the range from 12,640 to 12,670 eV using a step size of 0.5 eV through the edge. Individual scans typically took 5 minutes for completion to minimize radiation exposure to the sample. All spectra were averaged, background subtracted, and normalized using SIXPACK (6).

STEM and EDXA. Specimen preparation involved resuspending cell pellets in 2% (v/v) glutaraldehyde, storing (4 ^oC), washing fixed pellets 2× in DI, and dispersing washed cell pellets in Noble agar. Staining was with 2% (v/v) osmium tetroxide and 2% (v/v) uranyl acetate (1 h each), except for samples containing cadmium which were stained only with 2% (v/v) osmium tetroxide because of EDXA interferences between the Cd and U peaks (data not shown). All samples were progressively dehydrated by ethanol (25%, 50%, 75%, 100% (v/v, 2×); 15 min. each), followed by washes with 50:50 ethanol:acetone and 100% acetone (v/v, 1×; 15 min. each), then incubated (O/N) in 50:50 acetone:Epon resin. Embedding was in 100% (v/v) Epon resin (cured 24 hr at 60 ^oC), followed by sectioning (60 nm) using an MT-X ultramicrotome with a 55^o Diatome diamond knife (7). The sections were placed on 400-mesh, carbon-coated copper grids (Ted Pella Inc., Redding, CA) followed by vacuum drying O/N (60 ^oC). Images were acquired using either an FEI

Tecnai G2 F30 FEG microscope (FEI Company, Hillsboro, OR) operating at 300 kV or an FEI Nano600 FEG microscope operating at 30 kV accelerating voltage, each with a STEM detector at a working distance of 6.7 mm. EDXA line scans were performed with a sub-nanometer (0.5 nm or less) electron beam to verify the locally arranged QDs and spectra were analyzed using Genesis software (EDAX, Inc., Mahwah, NJ).

Estimation of nanoparticle numbers per cell. The numbers and diameters for 25 nanoparticles (total) were scaled from three images per QD treatment, using 1 or more cells per image. These data were used to estimate the amount of nanoparticulate, perhaps QD-associated, cadmium that appeared in cells—as an additional estimate (besides calculations using the differences between total and dissolved cadmium) of QDs in cells. The total nanoparticle-associated cadmium mass with cells was calculated under two end-member scenarios, assuming: 1) QDs were only on cell surfaces, and 2) QDs were only in the cell interior. To determine nanoparticle surface density, 3 square regions from each cell were designated in Photoshop, and the total numbers of nanoparticles in each region were counted. To estimate the number of cadmium atoms in one nanoparticle (assuming CdSe composition), data from Kasuya *et al*. (8) was extrapolated to an 8 nm diameter CdSe particle yielding an estimated 109 cadmium atoms per nanoparticle, which was then used to calculate the total nanoparticulate cadmium mass per cell. The two end-member scenarios were compared to the measured amounts of cellular cadmium (by ICP-AES) to estimate the percentage of total cadmium in the form of intact QDs.

2. Supplementary Results

2.1 Effects on growth

The three growth parameters calculated (i.e. lag time, yield and growth rate calculated from growth curves; Figures S1 and S2) were related to cadmium concentration and appeared similar for the $Cd(CH_3COO))_2$ and QD –treated cultures (Tables S1 and S2; shown graphically in Figures 1 and S7). Consistently, total 24 h cell counts (by epifluorescence microscopy) at 75 mg/L total cadmium were statistically similar (P=0.19) and averaged $2.08 \pm 0.20 \times 10^{10}$ per mL, yet were significantly less than total counts in the LB control ($5.18 \pm 0.75 \times 10^{10,}$ per mL; P=0.02). Culture tube versus microplate formats yielded similar growth parameters (data not shown). Extensive damage to QD – treated cells was visible (Figure S9).

2.2 QD dissolution

The analytical approach for distinguishing nanoparticulate QD-cadmium from dissolved Cd(II) involved measuring total cadmium by ICP-AES and dissolved cadmium by the Measure iT assay, then calculating nanoparticulate QD-cadmium as the difference. By this approach, CdSe QD dissolution in water was rapid and followed the Noyes – Whitney relation for sparingly soluble solids in aqueous solutions (9) (Figure S4). The Measure iT assay was performed similarly in LB broth, spent (24 h) culture supernatant and water (Figure S5), and the QD dissolution extent (by dialysis) was also similar across these solutions (data not shown). As expected, higher initial Cd masses (as QDs) resulted in higher dissolved Cd(II) concentrations (Figure S6). The amount of dissolved Cd(II) from the QDs was also measured intra and extra –cellularly in *P. aeruginosa* cultures (Table S3).

2.3 Intra and extra –cellular QD concentrations

Assuming a cell width of 1 μm (Fig. 4), and the aspect ratio for QD-treated cells (above) of 3.3, the cellular concentration of these "intracellular QDs" is then approximately 7.2×10^{19} QDs/liter. Cellular (i.e. a combination of membrane-associated and internally-distributed), QD-associated cadmium was then estimated, assuming 109 cadmium atoms per nanoparticle by extrapolating from Kasuya *et al*. (8), to be 0.0083 pg of cadmium per cell. The presence of intracellular QDs is supported by the observed co – localization of Cd and Se inside cells (Figure S8). Using this same density of cadmium atoms per nanoparticle (109) and the dissolution data (Fig. S5) for QDs in LB broth, then the extracellular concentration of QDs is approximately 2.5×10^{18} QDs /liter, which is approximately 30 fold less than the intracellular concentration.

2.4 Biomacromolecule contents

For the 75 mg/L (total cadmium) concentration, intracellular DNA was similar (P=0.31) for the QD-treated ($2.52 \pm 0.11 \times 10^{-6}$ ng/cell) and $Cd(CH_3COO))_2$-treated ($2.03 \pm 0.88 \times 10^{-6}$ ng/cell) cultures but significantly (P=0.00) greater than the no-metal controls (1.12 ±

0.18×10^{-6} ng/cell). Intracellular protein contents for the QD and Cd(II) treatments were similar and averaged 92.6 ± 9.8 pg/cell which was significantly (P = 0.01) greater than the controls (51.6 ± 5.1 pg/cell). Similarly, intracellular carbohydrate contents for the QD and Cd(II) treatments were similar and averaged 4.6 ± 0.3 pg/cell which was significantly (P = 0.01) greater than the controls (1.7 ± 0.1 pg/cell).

2.5 Abiotic ROS generation

After correcting for background fluorescence (as above) and again converting fluorescence to H_2O_2 equivalents, sterile LB broth plus DCFH yielded an ROS concentration of 534 ± 183 mg/L H_2O_2. When added to either LB or to water, CdSe QDs also generated ROS abiotically, albeit to a much lesser extent in water as compared to LB broth (Fig. S12) and to a much greater extent than LB alone (above). Cadmium salts added to either sterile LB or water did not result in ROS formation.

Supporting Tables

Table S1. Growth parameters for *P. aeruginosa* cultures exposed to varying cadmium acetate concentrations. Like letters in each column indicate no significant difference (T-test, $p > 0.05$).

Cd(II) (mg/L)	Lag Time (h)	Specific Growth Rate (h^{-1})	Max. OD_{600}
0.0	3.69 ± 0.13^{a}	0.92 ± 0.02^{a}	$1.60 \pm 0.02^{a,b}$
5.0	5.43 ± 0.14^{b}	0.67 ± 0.04^{b}	$1.70 \pm 0.02^{c,d}$
10.0	5.56 ± 0.11^{b}	0.63 ± 0.02^{b}	1.60 ± 0.02^{a}
20.0	5.24 ± 0.37^{b}	0.51 ± 0.01^{c}	$1.58 \pm 0.07^{a,b,d}$
37.5	7.15 ± 0.07^{c}	0.46 ± 0.02^{d}	$1.47 \pm 0.05^{b,e}$
75.0	9.93 ± 0.84^{d}	0.44 ± 0.00^{d}	$1.45 \pm 0.07^{a,e}$
115.0	10.55 ± 0.14^{d}	0.29 ± 0.02^{e}	1.21 ± 0.03^{f}
150.0	13.24 ± 0.07^{e}	0.24 ± 0.02^{f}	1.24 ± 0.06^{f}

Table S2. Growth parameters for *P. aeruginosa* cultures exposed to varying CdSe quantum dot concentrations. Shown are both the total Cd(II) concentration, as well as the Cd(II) ion concentration at 6 hours. Like letters in each column indicate no significant difference (T-test, $p > 0.05$).

Total Cd (mg/L)	Cd(II) at 6 h (mg/L)	Lag Time (h)	Specific Growth Rate (h^{-1})	Max. OD_{600}
0.0	0.0	3.69 ± 0.13^{a}	0.92 ± 0.02^{a}	1.60 ± 0.02^{a}
10.0	4.9	4.89 ± 0.15^{b}	0.63 ± 0.02^{b}	1.54 ± 0.01^{a}
20.0	8.6	5.67 ± 0.08^{c}	0.69 ± 0.03^{b}	1.84 ± 0.07^{b}
37.5	14.6	6.97 ± 0.08^{d}	0.55 ± 0.04^{c}	1.76 ± 0.04^{b}
50.0	18.2	7.41 ± 0.06^{e}	$0.49 \pm 0.04^{c,d}$	$1.68 \pm 0.05^{a,b}$
75.0	23.6	7.96 ± 0.11^{f}	0.43 ± 0.02^{d}	1.41 ± 0.05^{c}
100.0	29.1	8.41 ± 0.06^{g}	0.31 ± 0.03^{e}	1.04 ± 0.02^{d}
125.0	33.1	27.66 ± 3.87^{h}	0.27 ± 0.05^{e}	0.51 ± 0.01^{e}

Table S3. Distribution of total cadmium (by ICP-AES) and dissolved Cd^{2+} (by Measure iT assay) across intracellular (Cells) and extracellular (Supernatant) fractions for QD- and $Cd(CH_3COO))_2$ –treated *P. aeruginosa* PG201 grown in LB broth. Controls were "Sterile" LB amended with either $Cd(C_2H_3O_2)_2$ or CdSe QDs at a total Cd(II) concentration of 75 mg/L. Assays were performed immediately after inoculation (t = 0 h) and at the end of exponential phase (t = 24 h). Experiment volume was 10 mL with all treatments performed in triplicate.

	Initial (t = 0 h)		**Final (t = 24 h)**	
	Total Cd (mg)	**Cd^{2+} (mg)**	**Total Cd (mg)**	**Cd^{2+} (mg)**
Cd(II) Sterile	0.79 ± 0.04	0.75 ± 0.07	0.70 ± 0.03	0.62 ± 0.05
Cd(II) Cells	~	~	0.03 ± 0.00	0.03 ± 0.00
Cd(II) Supernatent*	0.73 ± 0.03	0.66 ± 0.04	0.55 ± 0.04	0.57 ± 0.03
QD Sterile	0.77 ± 0.01	0.18 ± 0.04	0.73 ± 0.01	0.50 ± 0.01
QD Cells	~	~	0.02 ± 0.01	0.03 ± 0.00
QD Supernatent*	0.76 ± 0.01	0.24 ± 0.07	0.70 ± 0.01	0.40 ± 0.01

*At 0 h of growth all Cd was in the media/supernatant.

Supporting Figures

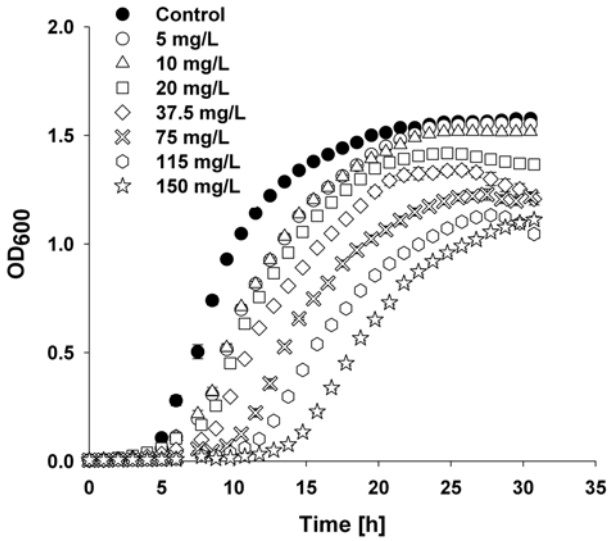

Figure S1. Growth of *P. aeruginosa* versus time with varying concentrations of total cadmium delivered as cadmium acetate.

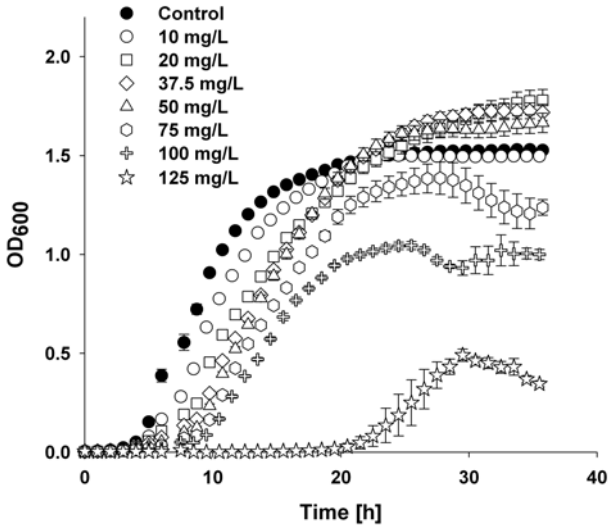

Figure S2. Growth of *P. aeruginosa* versus time with varying concentrations of total cadmium delivered as bare CdSe QDs

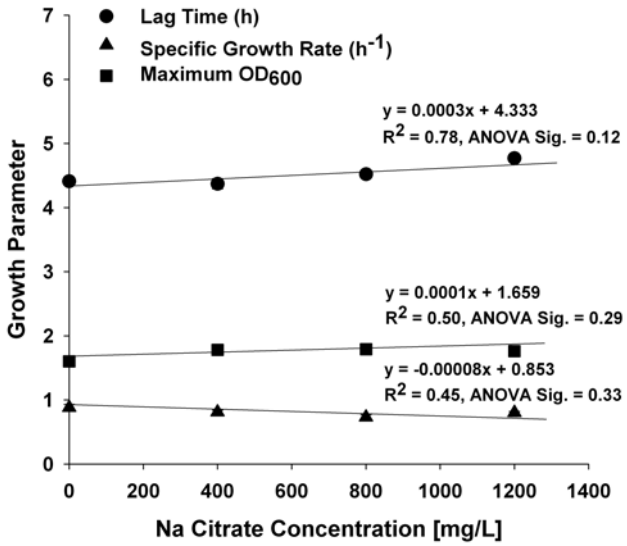

Figure S3. Growth parameters for planktonic *P. aeruginosa* cultivated with sodium citrate amended at varying concentrations, showing that citrate is unlikely to affect growth when cultures are amended with citrate-stabilized bare QDs (i.e. Figure S2).

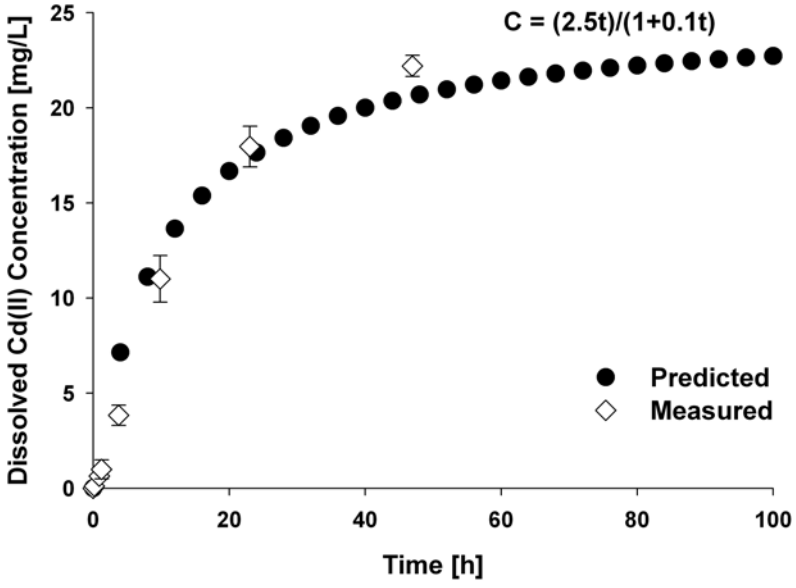

Figure S4. Time course of dissolution for 75 mg/L (total cadmium basis) citrate-stabilized CdSe QDs in de-ionized water. Predicted values are modeled using the Noyes – Whitney relation for sparingly soluble solids in aqueous solutions [57], as described in the Results.

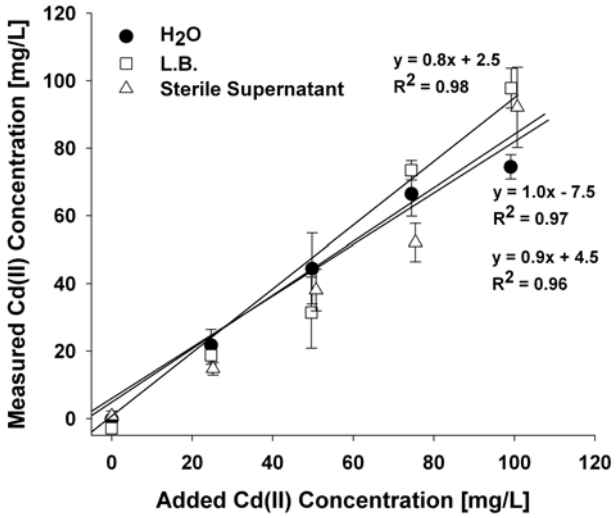

Figure S5. Relationships between measured vs. added Cd(II) concentrations where cadmium acetate was dissolved into three sterile aqueous solutions relevant to this study, showing that dissolved Cd(II) is measured equivalently across these three conditions at all relevant concentrations.

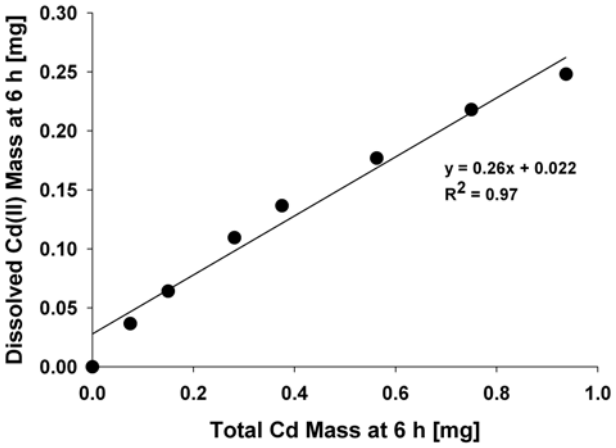

Figure S6. Relationship between (dissolved) cadmium ion mass and total (QD + dissolved) cadmium mass measured in *P. aeruginosa* cultures amended with the associated total mass of cadmium as CdSe QDs. The line of regression and fit parameter suggest a linear relationship, and the slope supports that most QDs are undissolved after 6 h (entry into exponential phase).

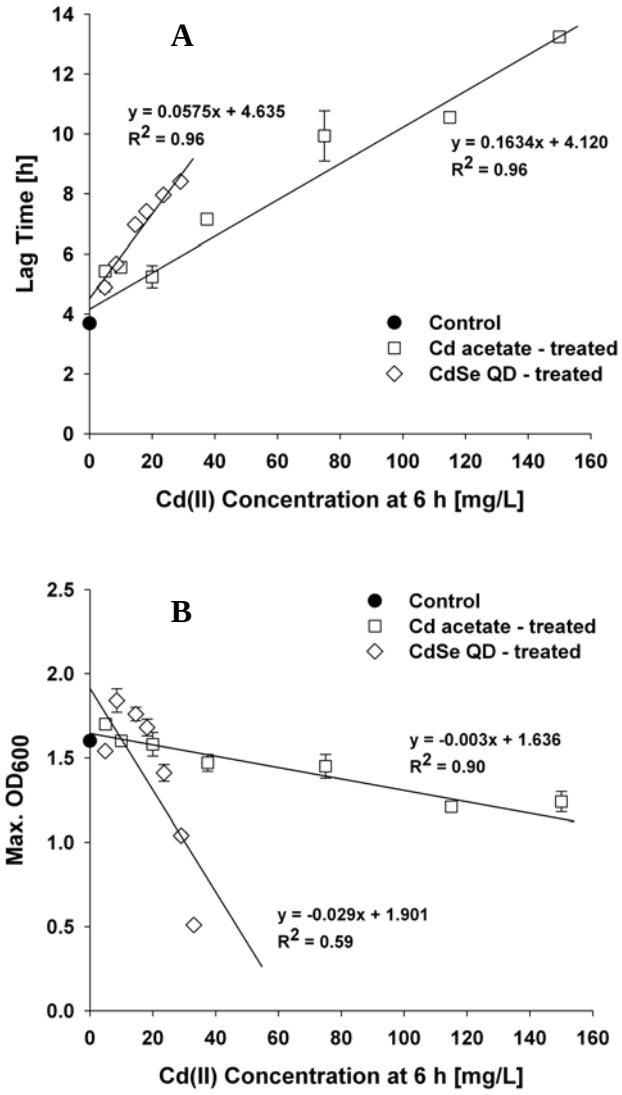

Figure S7. Lag time (A) and yield (by maximum OD, B) of planktonic *P. aeruginosa* grown either without (control, dark circle) or with cadmium in the form of either cadmium acetate (open square) or CdSe QDs (open diamond). QDs appear to cause increased lag times and, above 50 mg/L (total cadmium basis), lower yields relative to cadmium acetate when each form is expressed as dissolved Cd(II) concentration at the end of exponential phase (6 h).

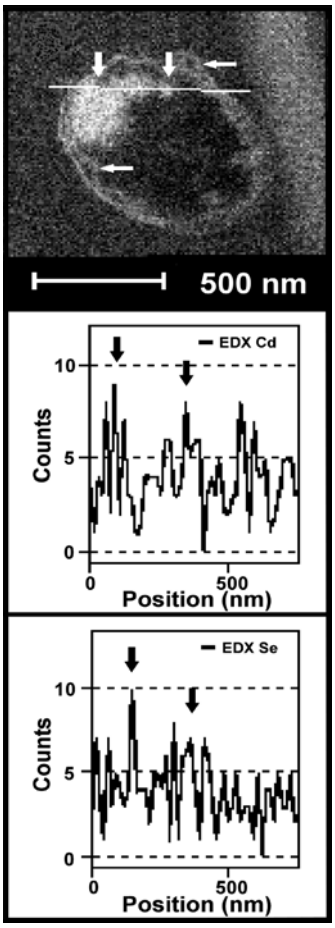

Figure S8. Representative STEM image of *P. aeruginosa* PG201 cell grown in the presence of 75 mg/L CdSe QDs (top). The EDS spectra, acquired for the line scan across the cell (white horizontal line), are for Cd (middle) and Se (lower). Vertical arrows indicate regions rich in Cd and Se; the horizontal arrows show cell envelope damage (blebbing).

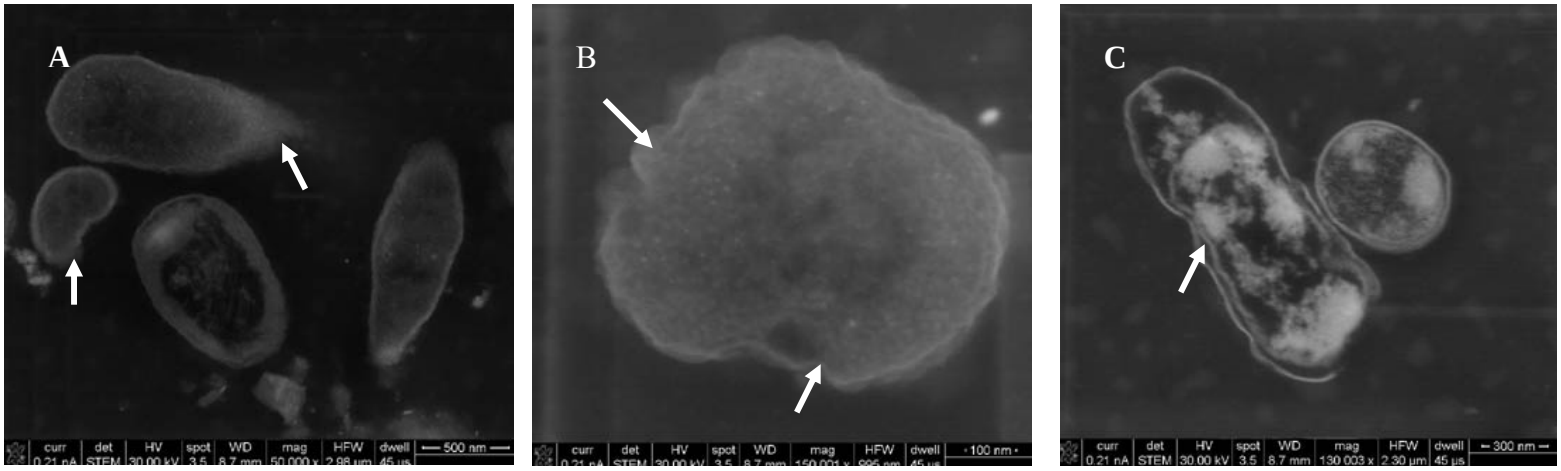

Figure S9. STEM micrographs of CdSe QD-cultured cells showing membrane holes (A), blebbing (B), and cell wall detached from the plasma membrane (C). White arrows indicate the region of damage.

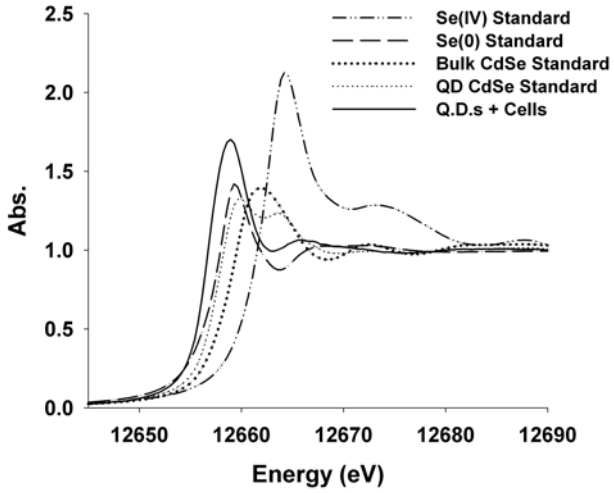

Figure S10. XANES spectra for cells amended with CdSe QDs (Q.D.s + cells) and all relevant standards, showing that the oxidation state of selenium associated with cells grown with CdSe QDs is more similar to elemental selenium, Se(0), as compared to the other relevant standards tested here.

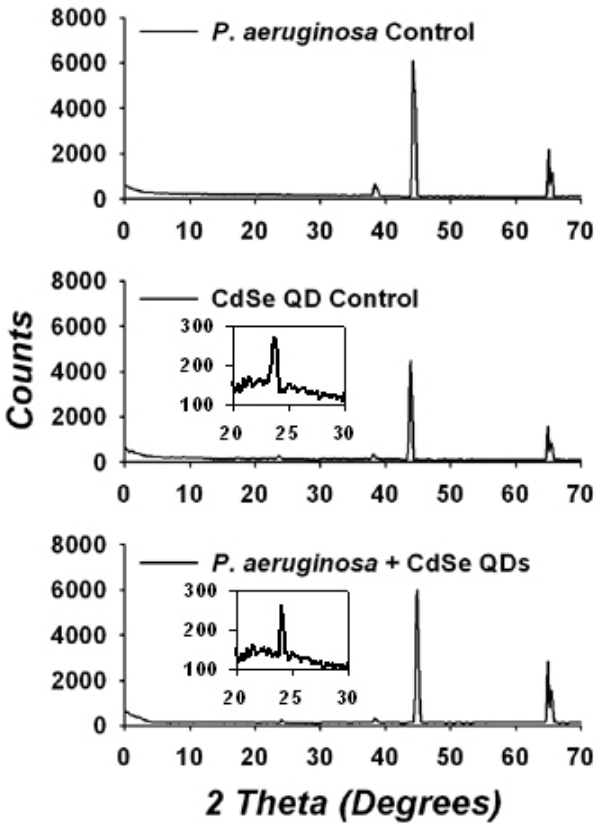

Figure S11. XRD spectra for *P. aeruginosa* control (top), CdSe QD control (middle), and *P. aeruginosa* + CdSe QD (bottom) samples. The weak peaks at $2\theta \approx 24^{o}$ in the QD control and *P. aeruginosa* + QD samples are shown as insets (middle and bottom).

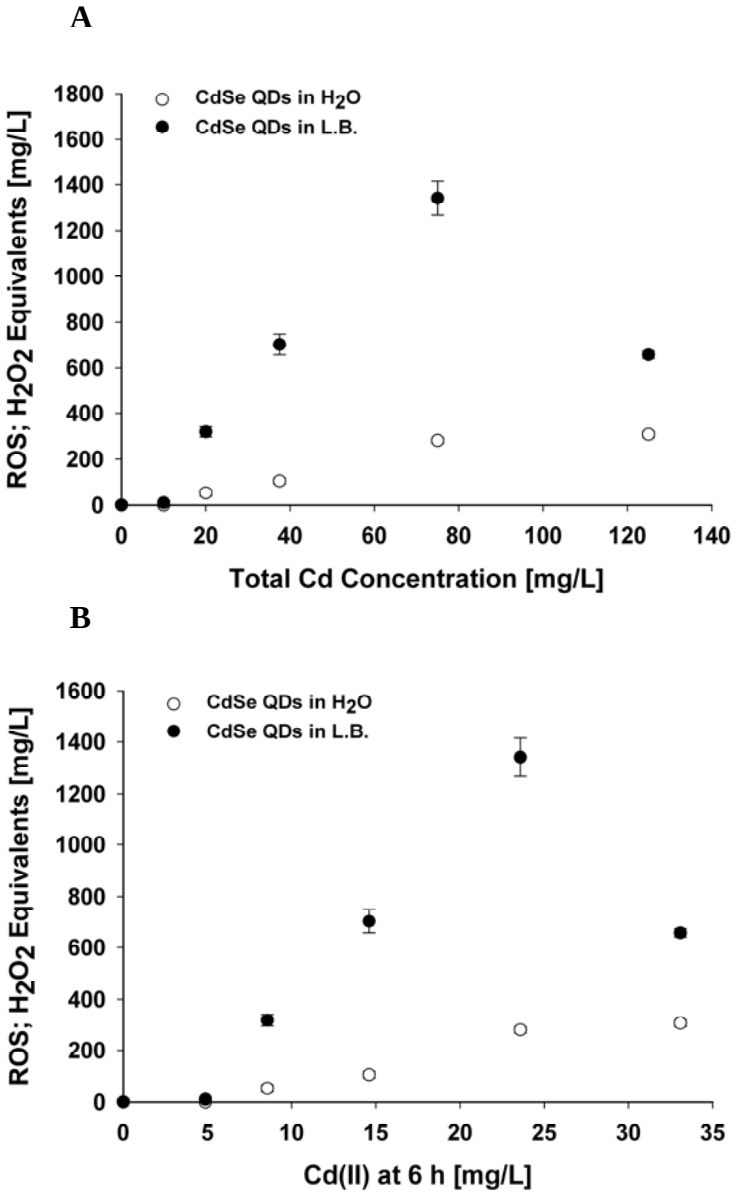

Figure S12. Reactive oxygen species (ROS, as H_2O_2 equivalents per liter) produced abiotically with various concentrations of CdSe QDs added to either sterile water or sterile LB broth. ROS is related to either A) total cadmium concentration in the form of QDs or B) dissolved cadmium after 6 hours, i.e. the time of entry into exponential phase when cells are present. Similar measurements were made for cadmium acetate in both aqueous conditions, but no ROS were produced across the same cadmium concentration range (not shown).

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