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Relative Susceptibility and Transcriptional Response of Nitrogen Cycling Bacteria to Quantum Dots

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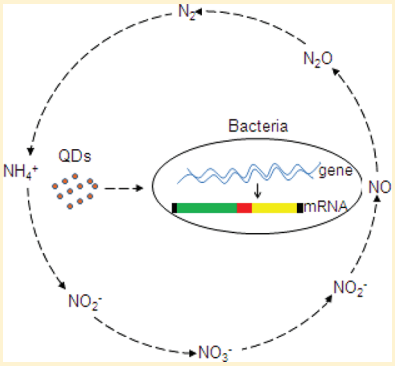
Supporting Information

ABSTRACT: Little is known about the potential impacts of accidental or incidental releases of manufactured nanomaterials to microbial ecosystem services (e.g., nutrient cycling). Here, quantum dots (QDs) coated with cationic polyethylenimine (PEI) were more toxic to pure cultures of nitrogen-cycling bacteria than QDs coated with anionic polymaleic anhydride-alt-1-octadecene (PMAO). Nitrifying bacteria (i.e., *Nitrosomonas europaea*) were much more susceptible than nitrogen fixing (i.e., *Azotobacter vinelandii*, *Rhizobium etli*, and *Azospirillum lipoferum*) and denitrifying bacteria (i.e., *Pseudomonas stutzeri*). Antibacterial activity was mainly exerted by the QDs rather than by their organic coating or their released QD components (e.g., Cd and Zn), which under the near-neutral pH tested (to minimize QD weathering) were released into the bacterial growth media at lower levels than their minimum inhibitory concentrations. Sublethal exposure to QDs stimulated the expression of genes associated with nitrogen cycling. QD-PEI (10 nM) induced three types of nitrogenase genes (*nif*, *anf*, and *vnf*) in *A. vinelandii*, and one ammonia monooxygenase gene (*amoA*) in *N. europaea* was up-regulated upon exposure to 1 nM QD-PEI. We previously reported up-regulation of denitrification genes in *P. stutzeri* exposed to low concentrations of QD-PEI.[1] Whether this surprising stimulation of nitrogen cycling activities reflects the need to generate more energy to overcome toxicity (in the case of nitrification or denitrification) or to synthesize organic nitrogen to repair or replace damaged proteins (in the case of nitrogen fixation) remains to be determined.



INTRODUCTION

Quantum dots (QDs) are semiconductor nanoparticles (2−100 nm) consisting of a metalloid crystalline core, such as CdSe or CdTe, and an encapsulating ZnS or CdS shell that is generally coated by organic molecules to enhance particle stability and biocompatibility.[2,3] QDs exhibit unique physicochemical and optical properties such as narrow emission spectra and photostability, which are useful for biomedical imaging, therapeutic applications, and solar cells.[4,5] These beneficial applications are challenged by the concern that QDs and other manufactured nanomaterials (MNMs) could become hazardous pollutants, and their incorporation into a wide variety of products is outpacing the development of knowledge and regulations to mitigate their potential impacts to public and ecosystem health.[6−11] QDs can be toxic to bacteria,[12,13] invertebrates,[14] and to animal[15] and human cells,[16] and can damage DNA[17] and enzymes.[18] However, little is known about how accidental or incidental releases of QDs would impact microbial ecosystem services, such as nitrogen cycling.

The nitrogen cycle is of great relevance to ecosystem health, water quality, agricultural productivity, and climate change. Most steps in the nitrogen cycle are predominantly mediated by bacteria that supply different forms of nitrogen compounds to higher organisms. Model bacteria that are commonly used to study the nitrogen cycle include *Azotobacter vinelandii*, which can fix nitrogen aerobically as a diazotroph;[19] *Nitrosomonas europaea*, which is an autotrophic nitrifying bacterium that oxidizes ammonia to nitrite;[20] and *Pseudomonas stutzeri*, which respires nitrate to N_2 via NO_2^-, NO, and N_2O during denitrification[21] (Figure 1). These bacteria are relatively well understood at the physiological and genetic levels, making them convenient models to study the potential effect of QDs on microbial nitrogen cycling.

This is the first paper to address how a class of MNMs (i.e., QDs) may affect bacteria involved in the nitrogen cycle, and to quantify their cellular and transcriptional responses. The coating of QDs plays a key role in their biocompatibility, stability, weathering, and mobility.[12] Thus, we investigated the potential impacts of two types of QD coatings: anionic polymaleic anhydride-alt-1-octadecene (PMAO, M_n = 30 000−50 000), and polycationic polyethylenimine (PEI, M_n = 10 000) QDs. The effect of toxic heavy metals (Cd and Zn) released from QDs was also considered. Minimum inhibitory concentrations (MIC) and dose−response patterns were determined to assess the relative susceptibility of different N-cycling phenotypes. Reactive oxygen species (ROS) production and the expression of superoxide dismutase (*sod*) genes were

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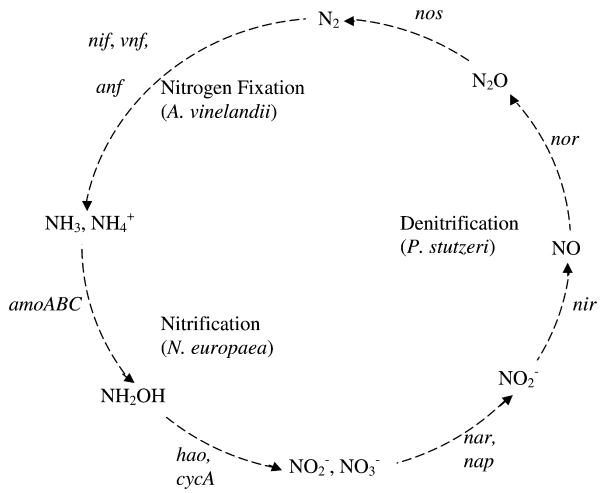

Figure 1. The nitrogen cycle and model bacteria and functional genes used in this study.

characterized to assess the importance of oxidative stress as a toxicity mechanism. We also quantified the expression of functional genes involved in nitrogen fixation, nitrification, and denitrification (and the associated enzymatic activities) during sublethal exposure to QDs to investigate microbial adaptation mechanisms and its implications on N cycling.

MATERIALS AND METHODS

QD Preparation and Characterization. QD-PMAO and QD-PEI were synthesized as described previously.[1,22] QD size distribution and zeta-potential were measured by a Zen 3600 Zetasizer Nano (Malvern Instruments, U.K.). QD sizes in 50 mM borate buffer (pH = 10) were 32.6 ± 2.7 nm for QD-PMAO and 41.6 ± 3.4 nm for QD-PEI. Spectral profiles and size distributions of the QDs in different microbial growth media are shown in Supporting Information (SI) Figures S1 and S2, respectively. The zeta-potential (ζ) of QD-PEI in borate buffer was positive (82.5 ± 7.2 mV), while that of QD-PMAO was negative, about −29.4 ± 7.3 mV.[12] The metal content of QD-PEI was 94.0 ± 1.0 μg/L/nM-QD for Cd and 36.7 ± 1.2 μg/L/nM-QD for Zn, whereas QD-PMAO contained 64.5 ± 0.8 μg/L/nM-QD for Cd and 28.7 ± 0.5 μg/L/nM-QD for Zn.

Microorganisms and Chemicals. All the bacteria were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Model bacterial used for dose−response and transcriptomic analyses of N cycling processes were *P. stutzeri* (ATCC 17588) for denitrification, *A. vinelandii* (ATCC 13705) for nitrogen fixation, and *N. europaea* (ATCC 19718) for nitrification. Additional bacteria whose genetic pathways are not as well understood were considered to assess the relative toxicity of QDs to different groups involved in N cycling. These include the nitrogen fixing bacteria *Rhizobium etli* (ATCC 51251) and *Azospirillum lipoferum* (ATCC 29707). The growth media compositions and growth conditions are described in the SI, as well as the reagents and chemicals used as ingredients.

MIC of QDs to Bacteria. The minimum inhibitory concentration (MIC) is the lowest concentration of an antibacterial agent that can inhibit the visible growth of a bacterium.[23] The MIC of QD-PEI and QD-PMAO was measured for different bacteria as described earlier.[24,25] Briefly, sterile test tubes contain 1 mL of strain-specific growth medium and serial dilutions of QD-PEI or QD-PMAO were prepared in triplicate, inoculated under aerobic conditions with the test bacteria, and incubated overnight at 37 °C for *P. stutzeri*, and at 26 °C for *A. vinelandii*, *R. etli*, *A. lipoferum*, and *N. europaea*. The tubes were then visually inspected for turbidity development. Controls (bacteria only) and blanks (broth only) sets were also prepared in triplicate.

Dose−Response Assays. *A. vinelandii* was exposed to QD-PEI (10−200 nM) or QD-PMAO (100−800 nM) for 48 h at 26 °C. A 7-day exposure period (also at 26 °C) was used for slower-growing *N. europaea* (as recommended by ATCC), with concentrations ranging from 1 to 100 nM for QD-PEI or 10 to 500 nM for QD-PMAO. For *P. stutzeri*, exposure was for 24 h at 37 °C, and concentrations ranged from 1 to 80 nM for QD-PEI or from 50 to 800 nM for QD-PMAO. To avoid background (optical density) noise contributed by QDs, cell densities were measured by flow cytometry with a BD LSRFortessa cell analyzer and bacteria counting kit from Invitrogen. The experiments were conducted at least in triplicate and repeated. Half maximal inhibitory concentration (IC_{50}) was calculated using the following equation:

$$y = y_0 + \frac{(y_{max} - y_0)}{1 + (x/IC_{50})^{slope}}$$

where y is the observed growth inhibition, y_0 is the baseline in unamended controls, y_{max} is the maximum extent of inhibition observed (e.g., 100% for a lethal dose), slope (also known as the Hill efficiency) refers to the linear part of the QD concentration versus inhibition curve, and x is the QD concentration.[26] SigmaPlot software (San Jose, CA) was used to fit this equation to the observed data.

For dose−response tests with metal salts, cells were exposed to similar or even higher concentrations of dissolved Cd or Zn (added as $ZnCl_2$ and $Cd(NO_3)_2$) as the highest metal concentrations released during exposure to QDs. Similar to the QD dose−response tests, exposure times were 1 day for *P. stutzeri*, 2 days for *A. vinelandii* and 7 days for *N. europaea*. Microbial growth was monitored by measuring optical density (600 nm) with a SpectraMax plus spectrometer (Molecular Device, Sunnyvale, CA).

Release of Metals from QDs and Speciation Modeling. To quantify the concentration of heavy metals released during microbial exposure to QDs, the supernatant of the exposure broth was separated by ultracentrifugation (35 000 rpm for 3.5 h)[1,27] after 1 day exposure for *P. stutzeri*, 2 days for *A. vinelandii*, and 7 days for *N. europaea*. The total dissolved Cd and Zn concentrations were measured by inductively coupled plasma-mass spectrometry (ICP-MS) using an Elan 9000 instrument (Perkin-Elmer, Waltham, MA). Speciation modeling was performed using Visual MINTEQ version 3.0 to determine the likelihood of metal precipitation in different growth media and identify the chemical species that would prevail in solution at equilibrium. For microbial media with poorly defined organic substrates (i.e., denitrifiers and N_2 fixers media), Stockholm Humic Model was used to assess potential carbon source−metal interactions.[28]

Measurement of ROS. Intracellular ROS production during QD exposure was investigated as a potential toxicity mechanism. Two hundred microliters of *A. vinelandii*, *N. europaea*, or *P. stutzeri* suspension (OD_{600} = 0.001−0.005) was transferred to each well on an opaque 96-well plate, after they were washed twice and suspended in PBS buffer, and blank

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Table 1. Minimum Inhibitory Concentrations (MIC) and Average Aggregate Sizes of QD-PEI and QD-PMAO (100 nM) in Various Microbial Growth Media[a]

		QD-PEI		QD-PMAO			
	species	MIC (nM)	average QD aggregate size (nm)	MIC (nM)	average QD aggregate size (nm)	ionic strength (μS/cm)	carbon sources and their concentrations (g/L)
denitrification	*P. stutzeri*	500	19.6 ± 2.0	>1000	41.3 ± 4.5	1192.5 ± 3.5	peptone (5) beef extract (3)
nitrogen fixation	*A .vinelandii*	600	2430.5 ± 607.4*	>1000	1729 ± 555.1*	1019.5 ± 0.7	sucrose (20) yeast extract (3)
	R. etli	500	812.1 ± 78.9*	400	295.1 ± 9.8*	420.0 ± 1.4	yeast extract (1) mannitol (10) soil extract (77 g soil in 200 mL H_2O)
	A. lipoferum	120	27.7 ± 5.7	>1000	4027 ± 1023*	2305.0 ± 7.1	yeast extract (0.05)
nitrification	*N. europaea*	40	35.9 ± 2.8	>1000	43.5 ± 2.2	5515.0 ± 21.2	N/A

[a]Asterisks (*) indicate significant increase compared to the original size in borate buffer ($p < 0.05$).

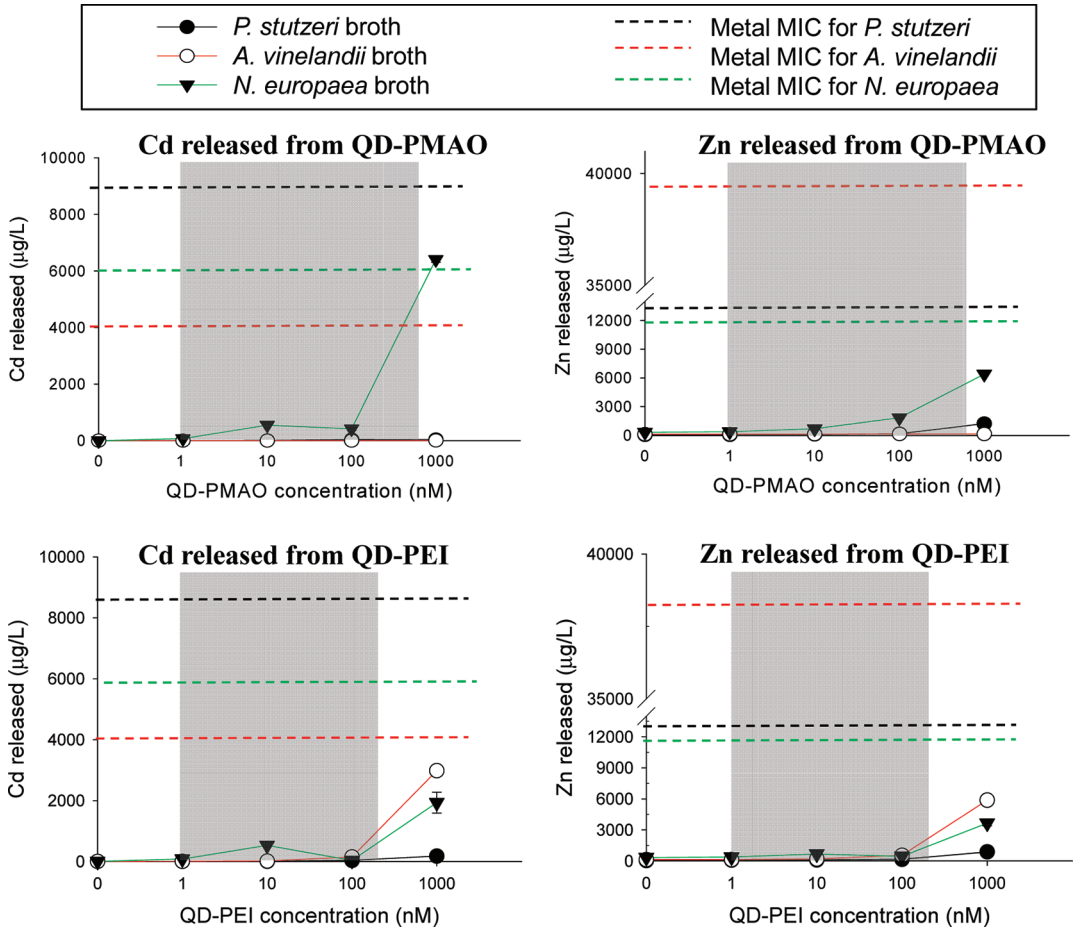

Figure 2. Released heavy metal concentrations (solid lines) from QD-PEI and QD-PMAO in *P. stutzeri*, *A. vinelandii*, and *N. europaea* media, and corresponding minimum inhibitory concentrations (MIC). Shadowed domains show the range of QD concentrations used for dose−response assay and transcriptomic studies. The released metals were below their MIC for the model bacteria within the relevant (shadowed) domain.

controls were amended with the same volume of PBS buffer.[29] The bacteria were then exposed to QD-PEI or QD-PMAO (10 nM and 100 nM) for 1 h and a positive control was treated with H_2O_2 (100 μM). One microliter dichlorodihydrofluorescein diacetate (H_2DCFDA, 4 mM in dimethyl sulfoxide) was subsequently added to each well, and after 30 min fluorescence was measured with an Infinite M1000 fluorometer (Tecan Systems Inc., San Jose, CA) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm. Two types of method controls were prepared. Negative controls with QDs and H_2DCFDA alone (no cells) were prepared to ensure that H_2DCFDA did not yield false positives.[30] Positive controls were prepared with cells exposed to QDs, H_2DCFDA, and H_2O_2 to obviate false negatives. Bacterial controls (bacteria without QDs) were also prepared to provide a baseline for signals from different treatment samples. Background QD

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fluorescence was subtracted from the signals. All the samples were replicated at least three times.

Effect of QDs on Gene Expression. For the transcriptomic analysis, *A. vinelandii* was exposed to 10 nM QD-PEI or 250 nM QD-PMAO for 1 day, while *N. europaea* was exposed to 1 nM QD-PEI or QD-PMAO for 3−4 days. All bacteria were collected at mid-log phase for RNA extraction. The specific housekeeping gene *gapA* (which codes for D-glyceraldehyde-3-phosphate dehydrogenase) was used as an internal standard for each bacterium. For *A. vinelandii*, the transcriptional levels of *cadR*, *sodA*, *nifD*, *nifH*, *vnfD*, *vnfH*, *anfD*, and *anfK* were determined. We also quantified the expression of *amoA1*, *amoA2*, *amoB2*, *amoC2*, *hao2*, and *cycA1*, as well as superoxide dismutase gene *sodB* in *N. europaea*.[31] Bacteria were collected by 10 min centrifuging at 5000 rpm and resuspended in RNAprotect bacteria reagent. Cells were treated with 3 mg/mL lysozyme in TE buffer for 10 min and RNA was extracted using RNeasy Mini Kit according to the manufacturer's protocol. RNA concentrations were determined by Nanodrop ND-1000 from Nanodrop products Inc. (Wilmington, NE). cDNA was synthesized overnight at 42 °C by reverse transcription PCR of RNA (2−5 μg) using random primers RNaseOUT, dNTPs, and Superscript II reverse transcriptase. Purification of cDNA was performed with a QIAquick PCR Purification Kit using the manufacturer's instructions. Primers (Table S1) were designed using PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/Default.aspx). Quantitative PCR was performed using a 7500 real time PCR system from Applied Biosystems (Carlsbad, CA) in 15 μL of reaction mixture composed of 1 ng cDNA, SYBR Green Master Mix (7.5 μL), and 0.3 μM of each primer and water. The C_t values (cycle threshold) were calculated with SDS 1.3.1, and the $2^{-\Delta\Delta CT}$ method was used to determine relative gene expression.[32,33] All treatments were run in triplicate and each sample was prepared in triplicate during PCR test.

RESULTS AND DISCUSSION

QD Stability and Metals Release in Different Growth Media. Different growth media were used in QD exposure experiments to satisfy the nutritional requirements of the tested bacteria. This requires consideration of how media composition affects QD stability and the speciation of released metals. QD-PEI generally exhibited higher stability than QD-PMAO in different broths, as reflected by smaller aggregates (Table 1) and lower metal release (Figure 2). Despite extensive QD aggregation, no QD-PEI precipitation was observed over the concentration range used in dose−response and transcriptomic experiments (shadowed domain in Figure 2); some QD-PMAO precipitation occurred at high concentrations (1000 nM). The largest QD-PEI aggregates occurred in broths with high concentration of complex carbon sources or soil extract (i.e., *A. vinelandii* and *R. etli* broths, respectively) (Table 1). This contrasts previous studies showing that dissolved natural organic matter stabilizes nanoparticles by acting as surfactants.[34,35] Apparently, high concentrations of large organic molecules in our broths (e.g., proteins and humic acids) enhanced flocculation through interparticle bridging.[36,37] The contribution of broth organic matter in QD aggregation is reflected by the significantly smaller aggregates observed in *Nitrosomonas* growth medium. This medium has the highest ionic strength (Table 1), which promotes coagulation, but lacks organic matter which is not needed for autotrophic growth, and exhibited the lowest extent of QD aggregation.

An important aspect of QD stability is the release of heavy metal constituents, which can be influenced by the composition of the exposure media (pH, ionic strength, and presence of ligands), coating stability, and exposure time.[12,38,39] Both QD-PEI and QD-PMAO released Cd and Zn in different broths, and their concentrations increased with increasing QD concentrations (Figure 2). The highest extent of release occurred in *N. europaea* broth, which had the highest ionic strength (Table 1). Increasing ionic strength through salt addition has been previously reported to destabilize QDs and promote metal release.[12] Visual MINTEQ was used to model the equilibrium speciation of Cd and Zn released in different broths (Table S1 and S2).[40,41] These simulations predict that most Cd^{2+} would be chelated by EDTA in *N. europaea* broth, and all Cd^{2+} associates with carbon sources in *P. stutzeri* and *A. vinelandii* broths. Zn ions would also primarily associate with organic compounds in both *P. stutzeri* and *A. vinelandii* broths, and $ZnEDTA^{2-}$ would prevail in *N. europaea* broth. No precipitation of Cd or Zn species was predicted for any of the three broths considered. Speciation modeling illustrates how media composition could influence the bioavailability of released metals. However, toxicity implications of metal speciation were not considered due to their low concentrations relative to applicable MIC values (Figure 2).

Susceptibility of Different Bacteria to QDs. Similar to previous results with the denitrifier *P. stutzeri*,[1] anionic QD-PMAO (zeta potential, $\zeta = -29.4 \pm 7.3$ mV in the stock borate buffer) were relatively nontoxic, with MIC values generally higher than 1000 nM for all strains tested (Table 1). In contrast, cationic QD-PEI ($\zeta = 82.5 \pm 7.2$ mV) were relatively toxic with MIC values as low as 40 nM for the nitrifier *N. europaea* (Table 1). The higher toxicity of cationic QD-PEI likely reflects their higher affinity for negatively charged bacteria,[1] as well as the fact that anionic QD-PMAO aggregated to a higher extent (Table 1), possibly decreasing their bioavailability and toxicity. Unlike other tested bacteria, the nitrogen fixer *Rhizobium etli* was more vulnerable to QD-PMAO than QD-PEI. The growth medium for this strain includes soil extract, and negatively charged soil organic matter likely promoted the aggregation of positively charged QD-PEI, which decreased their bioavailability and toxicity (Table 1). Dynamic light scattering (DLS) measurements showed that the average size of QD-PEI increased in this medium to 812.1 ± 78.9 nm compared to 295.1 ± 9.8 nm for QD-PMAO ($p < 0.05$).

Previous work has shown that dissolution of QD core components under acidic (pH < 5) or basic conditions (pH > 9) can be a significant cause of toxicity.[12] Nevertheless, under the near-neutral pH selected for the exposure experiments (to minimize hydrolysis of the organic coating and the associated release of metal constituents), we can exclude released metals as principal contributors to the observed toxicity.[12] Specifically, within the QD concentration range used for dose−response and transcriptomic analyses, metals were released at lower levels than their corresponding minimum inhibitory concentrations (Figure 2). This is corroborated by further experiments with *N. europaea*, which is relatively sensitive to dissolved heavy metals.[42] Low (10%) mortality of *N. europaea* was observed following exposure to 4000 μg/L Zn^{2+} and 1000 μg/L Cd^{2+} (Figure S3C), compared to 90% mortality during exposure to 100 nM QD-PEI, when 450.6 ± 66.3 μg/L of Zn^{2+} and 39.1 ± 4.9 μg/L of Cd^{2+} were released, indicating the role of QD-PEI as critical effector of toxicity. Furthermore, no microbial

inhibition was observed when *P. stutzeri* or *A. vinelandii* were exposed to the highest observed concentrations of released metals when these were added as salts (Figure S3).

The coating material PEI can exert antibacterial activity at relatively high concentrations (e.g., 3000 μg/L, Figure S4), and its potential toxicity to eukaryotic cells has also been reported.[43] However, similar to the metal constituents, the potential release of PEI did not play a major factor in the observed toxicity. Separate experiments showed that the tested bacteria were not susceptible to dissolved PEI concentrations as high as 1000 μg/L (Figure S4). Considering that the PEI content in our QDs was 163 ± 3 μg/L/nM-QD (as determined by total organic carbon analysis described in the SI), the maximum PEI concentration that could be released to the broth by 4.7 nM of QD-PEI (i.e., the IC_{50} for *N. europaea*, Figure 3) would be 770 μg/L, which is too low to cause bacterial inhibition. This is a conservative estimate since PEI binds strongly to the QDs and is unlikely to be completely released. Thus, QD-PEI rather than released metals or potentially inhibitory coatings were the principal toxicants in these experiments.

Overall, the denitrifier *P. stutzeri* and the nitrogen fixers *A. vinelandii* and *R. etli* were the most tolerant to QD-PEI, and the nitrifier *N. europaea* was the most susceptible (Table 1). Nitrifiers are generally quite susceptible to the presence of xenobiotics and other MNMs.[44−46] However, other factors may have contributed to their lower tolerance, including the smaller extent of QD aggregation in *Nitrosomonas* medium (Table 1), which is conducive to higher surface area for potential exposure, as well as higher release of metals (Figure 2), and longer exposure time in experiments with *N. europaea* (7 days to compensate for the slower growth of these autotrophic nitrifying bacteria, compared to 2-day exposure for *A. vinelandii* and 1-day exposure for *P. stutzeri*).

N. europaea, *P. stutzeri*, and *A. vinelandii*, whose genomes are sequenced, were respectively chosen as model nitrifying, denitrifying, and nitrogen fixing bacteria for dose−response tests (Figure 3) and transcriptomic analyses (Figure 5). QD-PEI concentrations that resulted in 50% bacterial inactivation (IC_{50}) were 4.7 ± 2.9 nM for *N. europaea*, 26.5 ± 1.1 nM for *P. stutzeri*, and 41.9 ± 7.1 nM for *A. vinelandii*. QD-PMAO IC_{50} values were 12.1 ± 0.8 nM for *N. europaea*, 500−700 nM for *A. vinelandii*, and more than 1000 nM for *P. stutzeri*. These data corroborate that *N. europaea* was the most sensitive of the tested species. The high susceptibility of nitrifying bacteria could have important environmental implications. The inhibition of ammonium oxidation is conducive to enhanced nutrient retention in soil (e.g., NH_4^+ is adsorbed to negatively charged soil particles and retained to greater extent than the main products of nitrification, NO_2^- and NO_3^-) and thus decreased water pollution by agricultural drainages.[47] Furthermore, decreased NO_2^- and NO_3^- production by nitrifiers would decrease the potential for subsequent denitrification and associated atmospheric emissions of N_2O (a green house gas) (Figure 1). However, such effects are unlikely to be felt beyond the local scale since it is improbable that the release of QDs to the environment would occur at sufficiently high frequencies and concentrations to significantly affect global nitrogen cycling and climate change.

Assessment of Oxidative Stress As Toxicity Mechanism. Intracellular ROS, which is a main cause for oxidative stress and has been suggested as a toxicity mechanism for QDs,[48−50] was produced in positive controls (bacteria without QDs, exposed to 100 μM H_2O_2), but was generally not

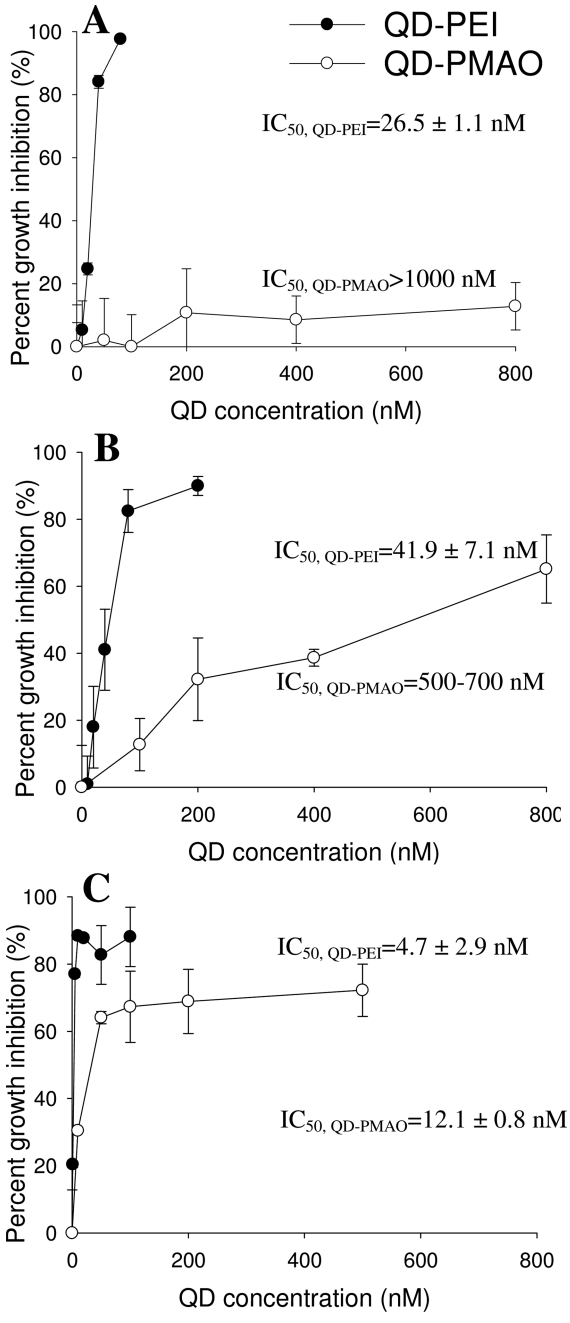

Figure 3. Growth inhibition of *P. stutzeri* (A), *A.vinelandii* (B), and *N. europaea* (C) exposed to QD-PEI or QD-PMAO. Error bars represent ± one standard deviation from the mean of triplicate measurements. QD concentrations resulting in 50% growth inhibition (IC_{50}) are depicted.

detected in cells exposed to QDs (Figure 4). The exception was *A. vinelandii* and *P. stutzeri* exposed to 100 nM QD-PEI, which exceeded the corresponding IC_{50} values. Although H_2DCFA can yield false positives for ROS production in bacteria,[30] no false positive signals were detected in control tests without cells containing H_2DCFA and QDs. For *A. vinelandii*, the ROS signal was less than twice that from the unexposed control, although the difference was statistically significant ($p < 0.05$). In *A. vinelandii*, the respiratory activity of cytochrome bd-type oxidases protects oxygen-labile nitrogen-fixing nitrogenases by consuming oxygen rapidly, and this mechanism was postulated to also mitigate against ROS formation.[51] Oxidative stress can

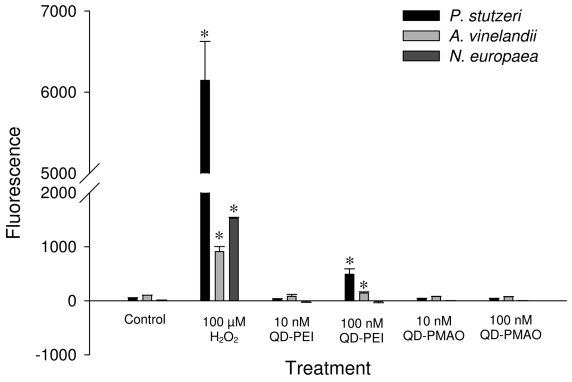

Figure 4. ROS measurement for *P. stutzeri*, *A. vinelandii*, and *N. europaea* exposed to 10 nM and 100 nM QD-PEI or QD-PMAO. Bacteria were treated with H_2O_2 (100 μM) as positive control. Asterisks (*) indicate significant increases compared to control samples ($p < 0.05$). Error bars represent ± one standard deviation from the mean of triplicate measurements.

also impact nitrification by inactivating hydroxylamine oxidoreductase in *Nitrosomonas*,[52] although no ROS induction was detected in *N. europaea*.

Some enzymes, such as superoxide dismutase (coded as *sod*), can sense oxidative stress and protect bacteria from ROS, and *sod* expression may be induced by H_2O_2 and intracellular ROS production.[53,54] In *A. vinelandii*, *sodA* was induced by both QD-PEI (10 nM) and QD-PMAO (250 nM), and its up-regulation (2.9−3.1-fold) was probably related to ROS induction by QDs. The sensitivity of quantitative PCR exceeds that of the H_2DCFDA assay used to quantify ROS production.[55] This could explain the apparent dichotomy of *sod* up-regulation with a lack of ROS detection. However, the lack of ROS detection in the most sensitive species (*N. europaea*) and our previous observations of QD toxicity to *P. stutzeri* under anaerobic conditions (which preclude ROS formation)[1] suggest that ROS-induced oxidative stress was not the principal cause of the observed toxicity of QDs.

Microbial Transcriptional Response to QDs. We previously reported that sublethal exposure to QD-PEI induced denitrifying genes in *P. stutzeri* only under anaerobic conditions.[1] Here, we extend this study to the transcriptional response of model nitrogen fixing (*A. vinelandii*) and nitrifying bacteria (*N. europaea*) to sublethal exposure to QDs of different surface charges.

Cationic QD-PEI had a higher impact on gene expression than anionic QD-PMAO (Figure 5), probably because of electrostatic attraction between QD-PEI and bacteria.[1] For *A. vinelandii*, we investigated the expression of a Cd transcriptional regulatory gene (*cadR*), superoxide dismutase (*sodA*), and nitrogenase genes associated with the reduction of N_2 to ammonia. Three classes of nitrogenases were considered (Mo-, V-, and Fe-only nitrogenases), all of which comprise two metalloproteins: component 1 (MoFe, VFe, or FeFe protein) and component 2 (Fe protein).[56] The targeted nitrogenase genes were *nifD* and *nifH* (encoding MoFe protein α chain and Fe protein subunit, respectively), *vnfD* and *vnfH* (encoding subunits of V-nitrogenase), and *anfD* and *anfK* (coding for subunits of Fe-only nitrogenase).

Up-regulation of gene *cadR*, which can sense the released Cd^{2+} and regulate some metal resistance genes, was observed following sublethal exposure to QD-PEI (10 nM) and (to a lesser extent) QD-PMAO (250 nM) (Figure 5). Exposure to QD-PEI (but not by QD-PMAO) up-regulated genes coding all three types of nitrogenases, including *nifD* (1.9−4.0-fold), *nifH* (10.5−19.4-fold), *anfD* (8.1−12.5-fold), *anfK* (8.1−9.0-fold),

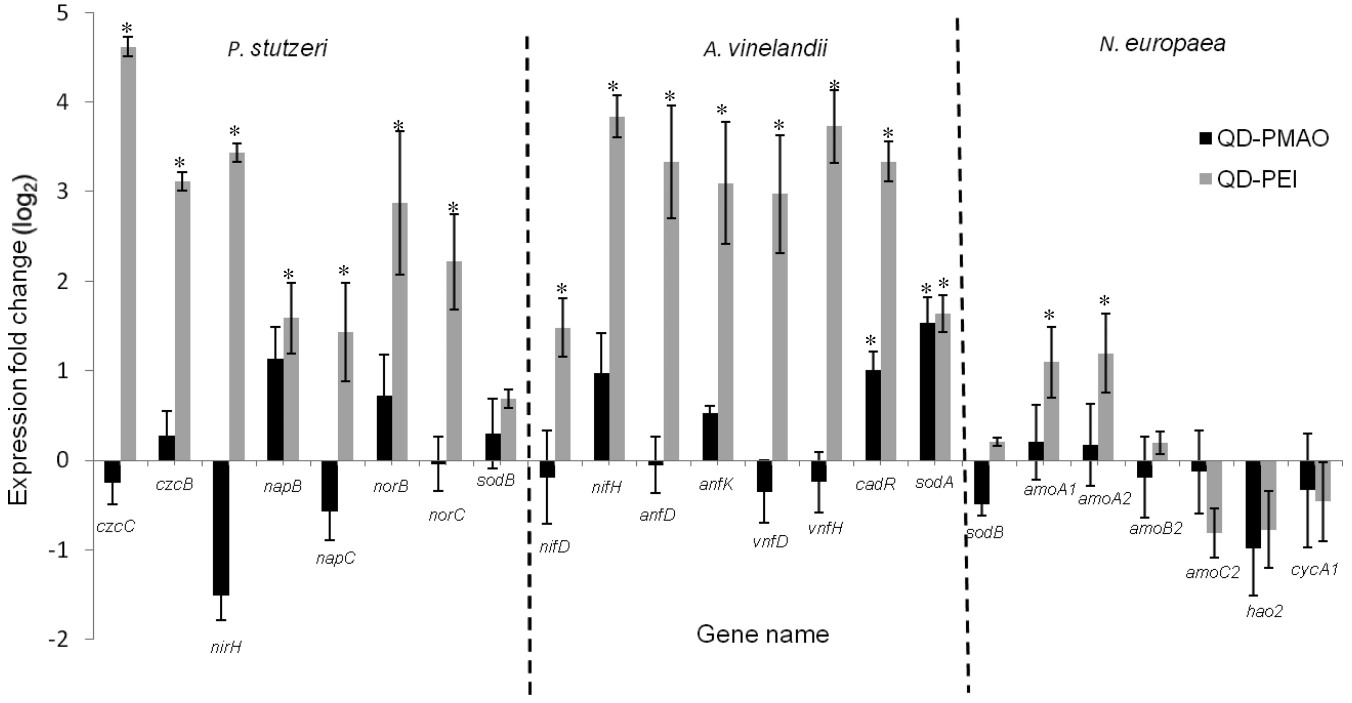

Figure 5. Transcriptomic analysis for *P. stutzeri*, *A. vinelandii*, and *N. europaea* exposed to sublethal concentrations of QD-PEI or QD-PMAO. *P. stutzeri*[1] and *A. vinelandii* were exposed to 10 nM QD-PEI and 250 nM QD-PMAO under anaerobic and aerobic conditions, respectively, when the exposure concentration to QD-PEI or QD-PMAO is 1 nM for *N. europaea*. Asterisks (*) indicate significant induction compared to unexposed controls ($p < 0.05$). Error bars represent ± one standard deviation from the mean of triplicate measurements.

vnfD (7.2−8.6-fold), and *vnfH* (12.9−13.7-fold). This suggests that short-term exposure to sublethal concentration of QD-PEI may stimulate nitrogen fixation. Whether up-regulation of nitrogen fixation genes was a response to repair or replenish damaged DNA and/or proteins, which would require more fixed (organic) nitrogen, remains to be determined. Enhanced nitrogen fixation, confirmed by the acetylene reduction assay[57] (Figure S5), may also be an indirect consequence of the induction of antioxidant pathways, which would protect against ROS-induced nitrogenase inhibition.[53,58]

The genes targeted in *N. europaea* include *amoA1*, *amoA2*, *amoB2*, and *amoC2* (coding for ammonia monooxygenases (AMO), which catalyze the oxidation of ammonia (NH_3) to hydroxylamine (NH_2OH)), as well as *hao2* and *cycA1* (respectively coding for hydroxylamine oxidoreductases (HAO), and its associated c-type cytochromes c554, which catalyze the oxidation of hydroxylamine to nitrite[59,60]). QD-PEI (1 nM) up-regulated *amoA1* (1.7−2.5-fold) and *amoA2* (1.9−2.7-fold) (Figure 5) and slightly down-regulated *amoB2*, *amoC2* *hao2*, and *cycA1* (0.6−0.7-fold). Induction of *amoA* by Cd^{2+} was previously reported despite concomitant inhibition of nitrification, indicated by a decrease in ammonia-monooxygenase-specific oxygen uptake rate (AMO-SOUR); this was attributed to the *de novo* synthesis of AMO to compensate for damaged enzymes during Cd^{2+} exposure.[61,62] Inhibition of nitrification by other metal-based NPs and heavy metal ions was previously reported.[45,63] However, no inhibition of nitrification (assessed by ammonia removal) was observed in the present study during sublethal exposure to 1 nM QDs relative to unexposed controls (Figure S6). The dissolved Cd^{2+} concentration in this treatment (0.07−0.08 mg/L) was below the inhibitory threshold for nitrification, corroborating previous studies that estimated this threshold at around 1.1 mg/L.[61]

Overall, sublethal exposure to positively charged QDs induced the expression of several bacterial genes associated with nitrogen cycling. Consistent with this finding, higher denitrification and nitrogen fixing bacteria with no inhibition of nitrification were observed. Further research is needed to determine whether this novel finding is a response to overcome QD toxicity by generating energy (in the case of nitrification and denitrification) or to obtain more organic nitrogen (in the case of N_2 fixation) to repair or replenish damaged proteins. The relatively high susceptibility of nitrifying bacteria suggests that inhibition of ammonia oxidation would be the first nitrogen cycling process that would be impacted in wastewater treatment plants receiving cationic QDs and in soils amended with associated biosolids that concentrate QDs. Whereas this study underscores the importance to mitigate incidental and accidental releases of QDs, longer-term studies with mixed cultures exposed in their indigenous matrices are needed to assess the potential impacts of QDs and other MNMs on N cycling, and its implications on water quality, soil fertility, and ecosystem productivity.

■ ASSOCIATED CONTENT

Supporting Information

Detailed description of QD spectra, growth conditions for the bacteria, acetylene reduction assay, toxicity data for PEI coating and heavy metal ions, and component release from PMAO QDs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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