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Cellular and Transcriptional Response of *Pseudomonas stutzeri* to Quantum Dots under Aerobic and Denitrifying Conditions

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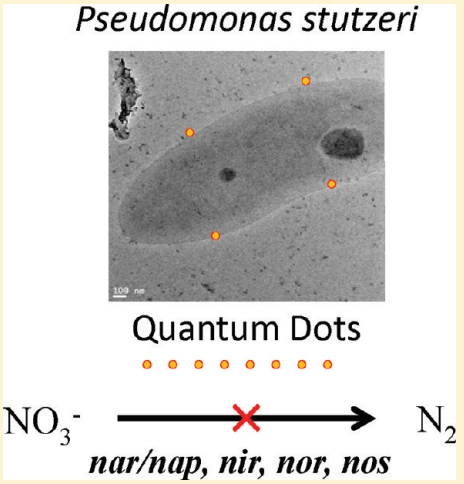
Supporting Information

ABSTRACT: *Pseudomonas stutzeri* was exposed to quantum dots (QDs) with three different surface coatings (anionic polymaleic anhydride-alt-1-octadecene (PMAO), cationic polyethylenimine (PEI), and carboxyl QDs) under both aerobic and anaerobic (denitrifying) conditions. Under aerobic conditions, toxicity (assessed per growth inhibition) increased from PMAO to carboxyl to PEI QDs. The positive charge of PEI facilitated direct contact with negatively charged bacteria, which was verified by TEM analysis. Both PMAO and PEI QDs hindered energy transduction (indicated by a decrease in cell membrane potential), and this effect was most pronounced with PEI QDs under denitrifying conditions. Up-regulation of denitrification genes (i.e., nitrate reductase *narG*, periplasmic nitrate reductase *napB*, nitrite reductase *nirH*, and NO reductase *norB*) occurred upon exposure to subinhibitory PEI QD concentrations (1 nM). Accordingly, denitrification activity (assessed per respiratory nitrate consumption in the presence of ammonia) increased during sublethal PEI QD exposure. However, cell viability (including denitrification) was hindered at 10 nM or higher PEI QD concentrations. Efflux pump genes *czcB* and *czcC* were induced by PEI QDs under denitrifying conditions, even though Cd and Se dissolution from QDs did not reach toxic levels (exposure was at pH 7 to minimize hydrolysis of QD coatings and the associated release of metal constituents). Up-regulation of the superoxide dismutase (stress) gene *sodB* occurred only under aerobic conditions, likely due to intracellular production of reactive oxygen species (ROS). The absence of ROS under denitrifying conditions suggests that the antibacterial activity of QDs was not due to ROS production alone. Overall, this work forewarns about unintended potential impacts to denitrification as a result of disposal and incidental releases of QDs, especially those with positively charged coatings (e.g., PEI QDs).



INTRODUCTION

More than one-half century has passed since Richard Feynman presented the perspective of nanomaterial manipulation and nanotechnology application.[1] Nanotechnology is now leading a creative and epochal revolution covering various subjects, such as pharmaceutics, electronics, physics, chemistry, and biology.[2−4] With the increasing worldwide usage of manufactured nanomaterials (MNMs), their accidental or incidental releases to the environmental seem inevitable. Accordingly, the potential adverse effect of MNMs to different types of biological molecules, cells, and organisms has received significant recent attention.[5−11] However, considerable uncertainty remains about the potential impacts to microbial ecosystem services (e.g., nutrient cycling) and the associated risks to ecosystem health.

The nitrogen cycle is one of the fundamental biogeochemical processes on the earth and denitrification is a key component that is critically implicated in water quality, agricultural productivity, and climate change. This form of anaerobic respiration (which is performed by a wide range of *Bacteria* and *Archaea*) involves the reduction of nitrate (NO_3^-) or nitrite (NO_2^-) (both common water pollutants) coupled with the production of dinitrogen gas (N_2) through intermediate nitrogen oxides (NO and N_2O).[12−14] Consequently, denitrification results in a loss of nitrogen (a common limiting nutrient) from aquatic and soil systems with concomitant production of a greenhouse gas (N_2O).[15] *Pseudomonas stutzeri* is one of the most studied denitrifying bacteria,[16,17] and its denitrification genes have been discerned and sequenced (Figure 1).[17] Thus, *P. stutzeri* is a convenient model bacterium to investigate the potential impact of MNMs to denitrification processes and associated bacterial response, which has not yet been addressed in the literature.

Quantum dots (QDs) offer valuable functionality for bioimaging, solar cells, electronics, and drug (or gene) delivery, since these semiconductor nanocrystals possess uniform sizes and distinct optical and electrical properties.[2,18−20] Some QDs can

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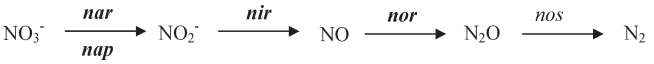

Figure 1. Genes coding for denitrification in *Pseudomonas stutzeri*. The effect of QDs on the genes depicted in bold was assessed by RT-qPCR.

be toxic to microorganisms and animals, and their toxicity depends on their physiochemical properties such as core composition, size, charge, and surface coatings,[6,8,21−24] which are critical determinants of QD stability and biosafety.[25,26] Previous research showed that the chemical composition and stability of the organic coating play a key role in cytotoxicity of QDs, influencing the release of toxic heavy metal ions and the potential uptake of QDs.[25,27,28]

In this study we address the effect of three types of QDs on *P. stutzeri*. Exposure was conducted in minimal mineral medium under neutral pH conditions to minimize extensive weathering (e.g., hydrolysis) of the QD coating and avoid confounding effects on bacterial toxicity associated with the release of Cd and Se ions.[26] Bacterial response is assessed at cellular and molecular levels, under both anaerobic (denitrifying) and aerobic conditions. Anaerobic conditions accentuated the susceptibility of *P. stutzeri* to the toxic effect of the positively charged PEI QDs, indicated by decreases in cell viability, cell membrane potential, and reductase activity. We also demonstrated the up-regulation of denitrification genes as a result of sublethal exposure, which is a novel finding with implications on nitrogen cycling.

MATERIALS AND METHODS

QD Preparation and Characterization. Three different types of QDs were used in this work. CdSe/CdZnS quantum dots were synthesized as described earlier.[29] Briefly, a mixture of purified CdSe nanocrystal chloroform solution, octadecylamine (ODA), zinc/cadmium precursor, and thiourea/ethanol solution was stirred and heated to 150 °C, cooled to 60 °C, and separated by precipitation. Polyanionic polymaleic anhydride-alt-1-octadecene (PMAO, M_n = 30 000−50 000) and polycationic polyethylenimine (PEI, M_n = 10 000) were used to transfer the as-synthesized QDs into aqueous phase (Figure S1).[30,31] Qdot 655 ITK carboxyl QDs were purchased from Invitrogen Inc. (Carlsbad, CA). Their size distribution and zeta-potential were measured in the exposure medium using a Zetasizer Nano (Malvern Instruments, U.K.), and concentrations were calculated per the method of Peng et al.[32] QD size increased from carboxyl (7.4 ± 1.1 nm) to PMAO (13.0 ± 0.2 nm) to PEI QDs (19.8 ± 1.3 nm) (Figure S1, Supporting Information). The zeta-potential of PEI QDs was positive (3.3 ± 0.5 mV), while that of carboxyl QDS (−10.2 ± 1.2 mV) and PMAO QDs (−36.6 ± 2.3 mV) was negative.

Bacterial Strain and Reagents. *P. stutzeri* (ATCC 17588) was purchased from the American Type Culture Collection (Manassas, VA) and was grown aerobically or anaerobically at 37 °C in Difco nutrient broth from BD (Franklin Lakes, NJ), containing 1 g/L $NaNO_3$.[33] Ammonium (0.8 g/L NH_4NO_3) was provided as N source to preclude assimilatory (anabolic) nitrate reduction and ensure that nitrate consumption was due to respiration.[34] Anaerobic exposure experiments were conducted inside an anaerobic chamber using the same growth medium, except that it was previously deoxygenated by bubbling N_2 gas for 15 min. Reagents purchased from Invitrogen include 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), RNaseOUT, Random primers, dNTP set, Superscript II reverse transcriptase, Bacteria Counting Kit, BacLight Bacterial Membrane Potential Kit, and BacLight RedoxSensor Green Vitality Kit. RNAprotect bacteria reagent, QIAquick PCR Purification Kit, and RNeasy Mini Kit were obtained from Qiagen Inc. (Valencia, CA). SYBR Green Master Mix was purchased from Applied Biosystems (Carlsbad, CA), and PCR primers were synthesized by Integrated DNA Technologies, Inc. (San Diego, CA). Lysozyme, ethylenediamine-tetraacetic acid (EDTA) buffer, Tris-EDTA (TE) buffer, phosphate buffered saline (PBS), multielement standard solution, and other chemicals were provided by Sigma-Aldrich (St. Louis, MO). CHEMets colorimetric dissolved oxygen test kit was purchased from CheMetrics, Inc. (Calverton, VA).

Effect of QDs on Bacterial Growth. *P. stutzeri* was exposed to PEI QDs (1−80 nM) or PMAO QDs (50−800 nM) under either aerobic or anaerobic conditions for 24 h at 37 °C. Higher concentrations (0.5 and 1 μM) were used for carboxyl QDs, which were less toxic; these tests were conducted under aerobic conditions. Bacterial growth was monitored using flow cytometry, according to the Bacteria Counting Kit instructions, using a BD LSRFortessa cell analyzer (Franklin Lakes, NJ). The experiments were performed at least in triplicate and calculation of half-maximal inhibitory concentration (IC_{50}) was described in Supporting Information (SI). Experiments were repeated for confirmation purposes.

To assess the role of released heavy metal ions in microbial toxicity, the supernatant was collected by ultracentrifugation (35 000 rpm for 3.5 h) and Cd and Zn concentrations were measured by inductively coupled plasma-mass spectrometry (ICP-MS) Elan 9000 (Perkin-Elmer, Waltham, MA). Then, additional growth inhibition tests were conducted by exposing *P. stutzeri* for 24 h to similar concentrations of heavy metal ions (without QDs). The heavy metals were added as $ZnCl_2$ and $Cd(NO_3)_2$ salts under both aerobic and anaerobic conditions. The tested concentrations were 2 ppm Zn^{2+} and 0.2 ppm Cd^{2+}, 1 ppm Zn^{2+} and 0.1 ppm Cd^{2+}, and 0.5 ppm Zn^{2+} and 0.05 ppm Cd^{2+}. Growth was monitored by measuring optical density at 600 nm with a SpectraMax plus spectrometer (Molecular Device, Sunnyvale, CA).

Assessment of Membrane Potential and Redox Activity. *P. stutzeri* was grown aerobically or anaerobically on Difco nutrient broth at 37 °C overnight, and diluted to an OD_{600} of 0.002 in PBS buffer.[35] The cells were exposed to 0.5 μM PEI or PMAO QDs for 15 min. The BacLight Bacterial Membrane Potential Kit and the BacLight RedoxSensor Green Vitality Kit were used, respectively, to determine the effect of exposure on membrane potential and reductase activity, according to the manufacturer's instructions. Triplicate samples were analyzed by flow cytometry using the LSRFortessa cell analyzer.

Measurement of ROS. *P. stutzeri* (grown under aerobic or anaerobic conditions at 37 °C overnight) was washed twice, suspended in PBS buffer, and 200 μL of the bacterial suspension was transferred to a 96-well plate. Blank controls were amended with the same volume of PBS buffer. The bacteria were then exposed to PEI or PMAO QDs (10 nM and 100 nM) for 1 h and a positive control was treated with H_2O_2 (100 μM). One microliter H_2DCFDA (4 mM in dimethyl sulfoxide) was subsequently added to each well, and fluorescence was measured after 30 min using an Infinite M1000 fluorometer (Tecan Systems Inc., San Jose, CA) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm. To ensure that H_2DCFDA did not yield false positives,[36] additional negative controls were

Table 1. Total Dissolved Cd and Zn Concentrations in PEI QD Suspensions after 24 h Exposure[a]

	aerobic		denitrifying	
	Cd (ppb)	Zn (ppb)	Cd (ppb)	Zn (ppb)
control	4.65 ± 1.11	51.17 ± 14.34	4.89 ± 0.26	43.07 ± 2.80
10 nM PEI	4.98 ± 0.58	94.69 ± 4.98*	6.35 ± 0.73*	171.53 ± 13.89*
20 nM PEI	8.16 ± 0.37*	116.86 ± 6.94*	25.27 ± 0.46*	349.55 ± 35.66*
40 nM PEI	37.65 ± 3.64*	171.39 ± 20.35*	53.82 ± 11.29*	390.62 ± 22.64*
80 nM PEI	177.97 ± 8.33*	884.67 ± 31.60*	108.87 ± 6.23*	1177.71 ± 23.06*

[a] * Indicates a statistically significant difference from control ($p < 0.05$).

prepared with QDs and H_2DCFDA alone (no cells). Background QD fluorescence was subtracted from the signal, and ROS-induced fluorescence increases was compared with the fluorescence of control samples. All the samples were replicated at least six times.

Effect of QDs on Gene Expression. *P. stutzeri* was exposed during 24 h to 10 nM PEI or 250 nM PMAO QDs under aerobic conditions, and to 1 nM PEI and 250 nM PMAO under anaerobic (denitrifying) conditions. Lower concentrations were used for conditions of higher susceptibility to QD toxicity. The expression of *czcB*, *czcC*, *sodB*, *narG*, *napB*, *nirH*, and *norB* genes was then quantified by reverse transcriptase quantitative PCE (RT-qPCR), using the housekeeping gene *gapA* (which codes for D-glyceraldehyde-3-phosphate dehydrogenase) as an internal standard. Genes *czcB* and *czcC*, located in the cobalt–zinc–cadmium efflux system *czcABC*, encode a resistance protein and an efflux transporter membrane fusion protein, respectively. Gene *nirH* encodes nitrite reductase H protein and is associated with denitrification, as well as *norB*, coding for NO reductase (Figure 1). Both *napB* and *narG* encode different nitrate reductase subunits, located in periplasm and membrane respectively. Superoxide dismutase gene *sodB* is protecting bacteria against oxidative stress caused by superoxide and hydroxyl radicals.[37] All treatments and RT-qPCR analyses for each sample were run in triplicate. Additional experimental details and gene sequences are described in the SI.

RESULTS AND DISCUSSION

Release of QD Constituents. Cd and Zn ions were released from QDs, and higher QD concentrations resulted in higher dissolved metal concentrations after 24 h exposure as illustrated for PEI (Table 1) and PMAO QDs (Table S1). For a given QD concentration, lower concentrations of total dissolved Cd and Zn were present in the growth medium under aerobic conditions, possibly due to more biosorption associated with higher biomass yield and faster growth under aerobic conditions.[38] The leaching of these heavy metals from QDs occurred to a much lesser extent than previously reported[26] due to differences in exposure conditions (e.g., pH 7 to minimize hydrolysis of the organic coating), and did not significantly contribute to bacterial toxicity. The fact that toxicity was primarily exerted by QDs rather than released metals is evident since (1) total dissolved Cd and Zn concentrations were less than 0.2 and 1.2 mg/L, respectively, and (2) these concentrations (when added as metal salts) were not toxic under aerobic or denitrifying conditions (Figure S2). Other studies have corroborated that *P. stutzeri* is quite resistant to even higher concentrations of Zn, Se, and Cd ions.[39−41]

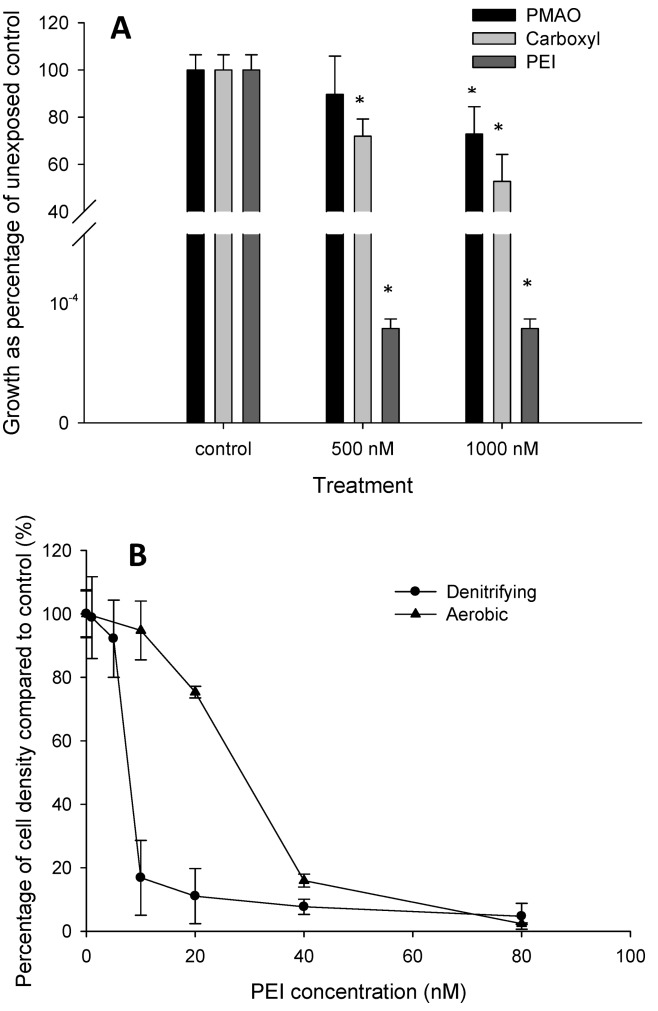

Figure 2. Toxicity of PEI, PMAO, and carboxyl QDs to *P. stutzeri* after 24-h exposure under aerobic or anaerobic (denitrifying) conditions. Panel A shows that aerobic toxicity to the bacteria increased from PMAO to carboxyl to PEI QDs. Panel B shows that bacteria were more vulnerable to PEI QDs under anaerobic (denitrifying) conditions. Error bars represent ± one standard deviation from the mean of triplicate measurements.

Toxicity of QDs to Bacteria. The antibacterial activity of the tested QDs (assessed as growth inhibition) increased from PMAO QDs to carboxyl QDs to PEI QDs (Figure 2A). Higher susceptibility to PEI QDs was observed under denitrifying than aerobic conditions (Figure 2B). The IC_{50} for growth inhibition by PEI QDs was 7.25 ± 0.43 nM under denitrifying conditions,

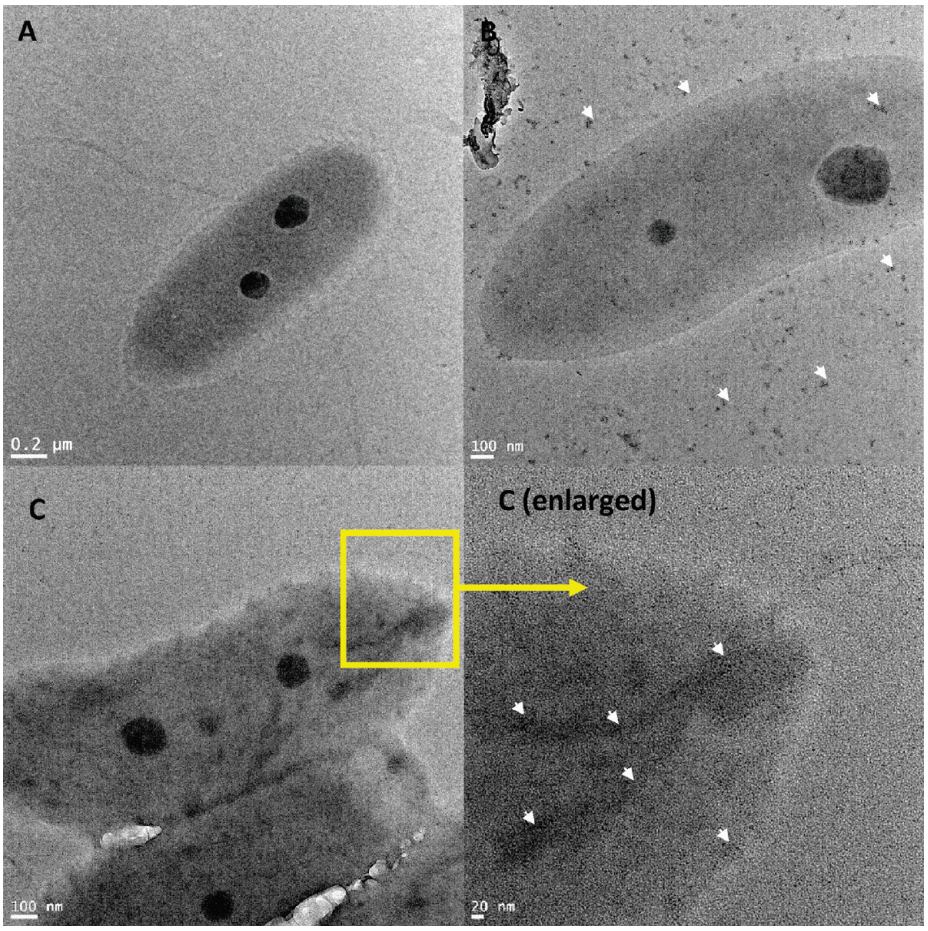

Figure 3. TEM images of (A) *P. stutzeri* unexposed control and those exposed to (B) PMAO and (C) PEI QDs. More PEI QDs were attached to bacteria than PMAO QDs. White arrows point to the QDs. Duplicate samples were used for TEM analysis.

compared to 26.45 ± 1.08 nM under aerobic conditions. No significant aerobic or anaerobic growth inhibition was observed when *P. stutzeri* was exposed to the least toxic PMAO QD up to 800 nM (Figure S3). Whether lower susceptibility under aerobic conditions was due to higher energy harvesting and faster growth capabilities remains to be determined.

The higher toxicity of PEI QDs is probably due to their positive charge (zeta-potential = 3.3 ± 0.5 mV), which enhances direct contact by electrostatic attraction to the negatively charged bacteria (zeta-potential = −0.03 ± 0.01 mV). TEM analysis (method details are included in the Supporting Information (SI)) confirmed that more PEI QDs associated with *P. stutzeri* than PMAO QDs (Figure 3), which predominantly remained suspended without attaching to the cells. The relatively small amount of PMAO QDs observed on the bacteria (Figure 3B) was likely deposited when the solution was dried during the sample preparation.

ROS Production. ROS—whether produced exogenously (by redox or photocatalytic reactions) or endogenously (by the immune response)—is commonly considered as a potential mechanism of MNM toxicity (i.e., oxidative stress).[42] ROS were detected in aerobic incubations with PEI QDs at 100 nM, but not at 10 nM (Table 1). No ROS were detected under denitrifying conditions due to the absence of O_2, which is a required ROS precursor.[43] Thus, the observed anaerobic toxicity of PEI QDs cannot be attributed to ROS production. No ROS was detected either upon aerobic or denitrifying exposure to (relatively non toxic) PMAO QDs.

Reductase Activity and Membrane Potential. Exposure to both PEI and PMAO QDs decreased the percentage of actively respiring cells under both aerobic and anaerobic conditions (Figure 4A), indicating the potential for QDs to hinder electron transport phosphorylation.[44] However, aerobic reductase activity was inhibited to a greater extent in the presence of PMAO QDs (100 nM) than with PEI QDs (10 nM), implying that the higher toxicity of positively charged PEI QDs was not solely due to interference with respiration. Lower reductase activities were generally observed under denitrifying conditions (Figure 4A), especially in the presence of the more toxic PEI QDs. This probably reflects that *P. stutzeri* uses a shorter nitrate-based electron transport chain with fewer reductases compared to the aerobic respiration mode.[15]

Cell membrane potential was affected by exposure to some QDs, but not by the respiration (aerobic versus denitrifying) mode. Exposure to PEI QDs (but not PMAO QDs) resulted in a significant decrease in cell membrane potential (Figure 4B), which is indicative of inhibition of the generation of the proton motive force (PMF) and associated energy transduction.[36,44] This effect is consistent with the high degree of microbial growth inhibition exerted by PEI QDs (Figure 2A). Changes in membrane potential could not be reliably assessed in treatments with PMAO QDs because their aggregation increased background

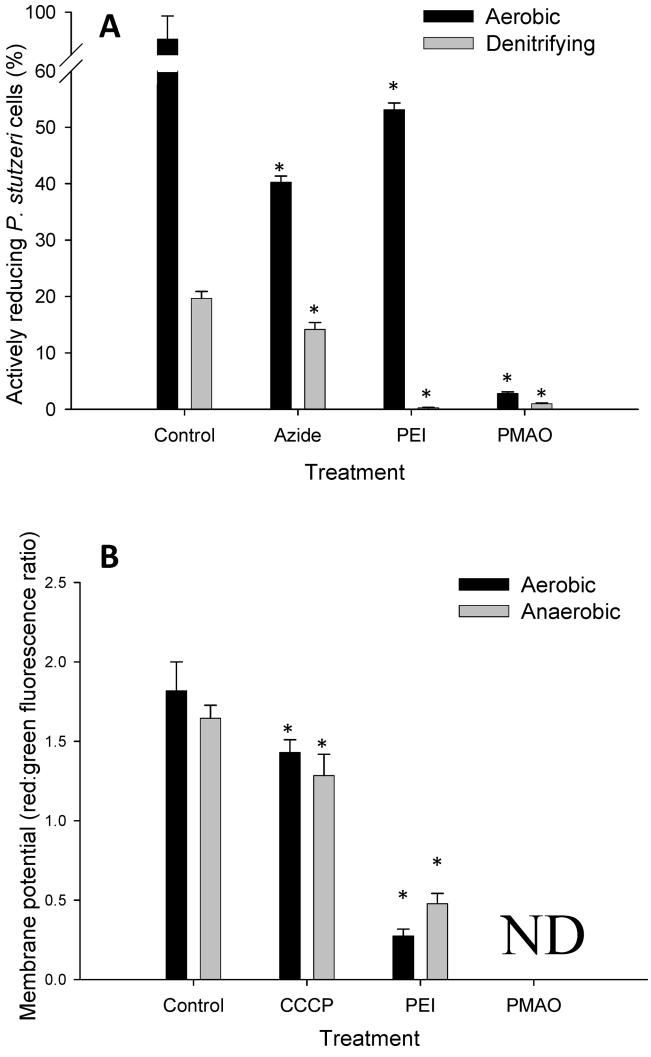

Figure 4. Effect of 0.5 μM PEI and 0.5 μM PMAO QDs on (A) reductase activity and (B) membrane potential under aerobic and denitrifying conditions. Azide and CCCP were used as positive controls to reduce reductase activity and membrane potential, respectively. Both PEI and PMAO QDs hindered reductase activity, and this effect was more pronounced under anaerobic conditions. PEI QDs decreased the membrane potential. Asterisks indicate significant reduction compared to control samples ($p < 0.05$). ND means not determined due to interference from fluorescence background noise emitted by aggregated PMAO QDs. Error bars represent ± one standard deviation from the mean of triplicate measurements.

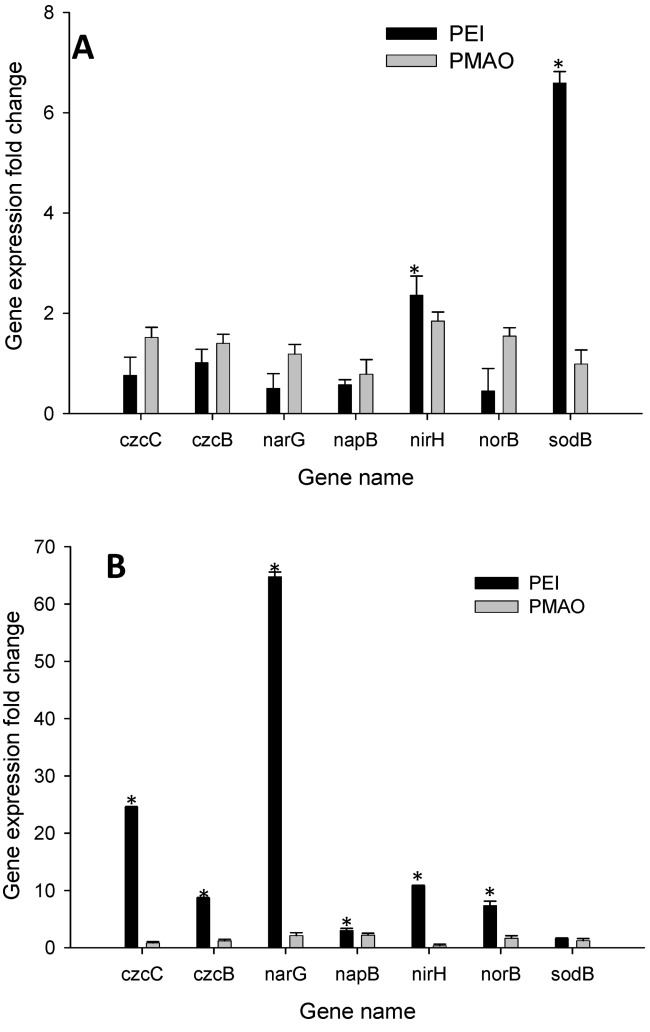

Figure 5. Effect of PEI or PMAO QDs on gene expression under (A) aerobic and (B) denitrifying conditions. Exposure duration was 24 h. Under anaerobic conditions, PEI QDs (1 nM) induced *czcB*, *czcC*, *narG*, *napB*, *norB*, and *nirH*, while PMAO QDs (100 nM) repressed *nirH*. Under aerobic conditions, the expression of *nirH* and *sodB* was induced by PEI QDs and PMAO QDs had no significant effect. Asterisk indicates up-regulation compared to housekeeping gene *gapA* ($p < 0.05$). Error bars represent ± one standard deviation from the mean of triplicate measurements.

fluorescence and confounded the signal emitted from the BacLight RedoxSensor Green. Membrane potential plays an important role in bacterial cell division,[45] and previous studies have reported that exposure to QDs can damage the cell membrane.[46] However, it is unclear whether the collapse in cell membrane potential was the main cause for microbial toxicity rather than another symptom.

Influence of QDs on Gene Expression. PEI QDs had a higher impact on gene expression than PMAO QDs, and denitrifying conditions exacerbated this effect (Figure 5). Under denitrifying conditions, PEI QDs induced *czcB* (8.7 ± 0.1 fold), and *czcC* (24.5 ± 0.1 fold) at very low concentrations (1 nM) (Figure 5B), possibly as a defense mechanisms to enhance excretion of potentially toxic agents (e.g., metals released from QDs).[47] Interestingly, at this concentration which is below levels that inhibited growth (Figure 2B), PEI QDs also induced denitrifying genes *narG* (64.8 ± 0.8 fold), *napB* (3.0 ± 0.4 fold), *nirH* (10.8 ± 0.1 fold), and *norB* (7.3 ± 0.8 fold) under anaerobic conditions. Thus, exposure to QDs at subinhibitory concentrations increased denitrifying activity, which was corroborated by faster respiratory nitrate consumption after 4 h exposure to 1 nM PEI QDs (0.91 ± 0.04 mg/mg protein) compared to unexposed controls (0.76 ± 0.02 mg/mg protein) ($p < 0.05$). Recently, TiO_2 NPs were reported to increase the activity of nitrate reductase and accelerate nitrogen uptake in spinach.[48,49] Faster denitrification has also been previously observed after exposure to trace metals,[50] although the physiological or metabolic benefits of this sublethal response remain to be determined (e.g., whether it is a compensation for greater energy requirements from nitrate-based respiration to overcome stress, or if it is related to the regulation of other nitrogen metabolism pathways

Table 2. ROS Production (Indicated by % Increase in Fluorescence Relative to Unexposed Control) under Aerobic and Denitrifying Conditions

QD concentration	aerobic conditions (%)	denitrifying conditions (%)
PEI 10 nM	58 ± 7	
PEI 100 nM	916 ± 188	
PMAO 10 nM	82 ± 5	32 ± 7
PMAO 100 nM	79 ± 4	27 ± 7

such as the need for aminoacid synthesis to repair damaged proteins). Gene *nar* and *nor* code for membrane-associated proteins whereas gene *nap* and *nir* code for periplasmic proteins.[15,17] Bacterial cell membranes are susceptible to direct contact with QDs (Figure 3) and other nanomaterials,[36,44] which was corroborated by the collapse in membrane potential (Figure 4B). Thus, membrane-bound proteins might be more susceptible to damage, and genes associated with their synthesis may be expressed more frequently to restore regular activity. However, higher PEI QD concentrations (5 nM, which is close to the IC_{50}) were toxic (Figure 2B) and did not significantly upregulate denitrification genes (Figure S4).

Under aerobic conditions, *sodB* was induced (6.6 ± 0.2 fold) by 10 nm PEI QDs and (consistent with previous studies[37]) its expression was likely induced by intracellular production of ROS (Table 2). Thus, the lack of *sodB* expression following anaerobic exposure to PEI or PMAO QDs (Figure 4B) is consistent with the absence of ROS (Table 2). Similarly, significant up-regulation of *sodB* following aerobic exposure to PEI QDs (Figure 5A) agrees with the observed high ROS levels (Table 2). None of the tested genes were expressed differentially upon aerobic exposure to PMAO QDs at 100 nM, consistent with their lack of inhibition of microbial growth at this concentration.

Overall, these results demonstrate the potential for sublethal exposure to QDs to influence microbial gene expression and associated metabolic pathways. Potential negative effects include microbial growth inhibition, collapse of the cell membrane potential, and hindered energy transduction, which could be aggravated under anaerobic conditions (e.g., in anaerobic sediments containing precipitated QDs). Although molecular and biochemical details on the toxicity mechanisms remain elusive, heavy metal dissolution and ROS production were ruled out as principal modes of action. It is unlikely that microbial exposure to QDs in the environment would occur at sufficiently high frequencies and concentrations to significantly affect nitrogen cycling and associated impacts on water quality (i.e., nitrate and nitrite concentrations) and climate change (i.e., N_2O emissions). However, discarded or inadvertently released QDs could concentrate in wastewater treatment plants or associated biosolids applied to land, and in turn affect microbial processes associated with N cycling and other ecosystem services. This may be particularly pertinent for positively charged QDs, which have a higher propensity to adhere to the cells and impact microbial viability at nanomolar concentrations.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Detailed description of reverse-transcriptase quantitative PCR, size distribution of QDs, toxicity data for PMAO and carboxyl QDs, 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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The first sentence of the Introduction section had Richard Feynman's name spelled incorrectly in the version of this paper published April 28, 2011. The correct version published May 2, 2011.