

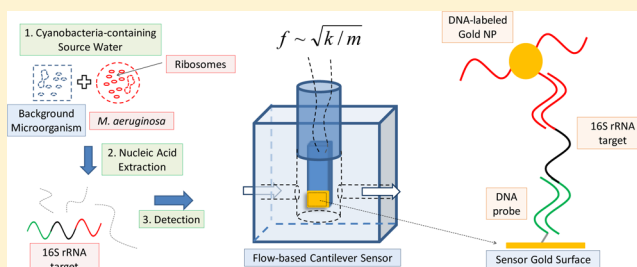
A Cantilever Biosensor-Based Assay for Toxin-Producing Cyanobacteria *Microcystis aeruginosa* using 16S rRNA

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S Supporting Information

ABSTRACT: Monitoring of cyanotoxins in source waters is currently done through toxin-targeting assays which suffer from low sensitivity due to poor antibody avidity. We present a biosensor-based method as an alternative for detecting toxin-producing cyanobacteria *M. aeruginosa* via species-selective region of 16S rRNA at concentrations as low as 50 cells/mL, and over a five-log dynamic range. The cantilever biosensor was immobilized with a 27-base DNA strand that is complementary to the target variable region of 16S rRNA of *M. aeruginosa*. The cantilever sensor detects mass-changes through shifts in its resonant frequency. Increase in the biosensor's effective mass, caused by hybridization of target strand with the biosensor-immobilized complementary strand, showed consistent and proportional frequency shift to *M. aeruginosa* concentrations. The sensor hybridization response was verified in situ by two techniques: (a) presence of duplex DNA structure postdetection via fluorescence measurements, and (b) secondary hybridization of nanogold-labeled DNA strands to the captured 16S rRNA strands. The biosensor-based assay, conducted in a flow format (~ 0.5 mL/min), is relatively short, and requires a postextraction analysis time of less than two hours. The two-step detection protocol (primary and secondary hybridization) is less prone to false negatives, and the technique as a whole can potentially provide an early warning for toxin presence in source waters.



1. INTRODUCTION

Environmental water monitoring is important to human health and requires detecting hazardous contaminants and toxins in source water. Of late, monitoring of toxins produced by cyanobacteria has received increased attention due to their increased abundance and toxicity.^{1–5} Demand for assays to monitor toxin-producing cyanobacteria has grown as their frequency and size of algal blooms have increased. Such blooms are associated with water pollution-induced eutrophication and undesirably provide conditions associated with toxin expression.^{6,7} As a consequence, *The Harmful Algal Bloom (HAB) and Hypoxia Amendments Act of 2004* was created, and it reauthorized the mandate to the National Oceanic and Atmospheric Administration (NOAA) to advance the ability to detect, monitor, and predict HAB and hypoxia events in both coastal waters and the Great Lakes.⁸

Monitoring of the species *Microcystis aeruginosa* (*M. aeruginosa*) is of particular importance as it produces one of the strongest cyanotoxins, microcystin-LR. Microcystin-LR is a hepatotoxin whose consumption has been linked to pathology in mammals.^{9–12} Conventional detection of cyanotoxin uses enzyme-linked immunosorbent assay (ELISA),^{13,14} high performance liquid chromatography (HPLC),^{15,16} protein phosphatase assay,^{17,18} or integration of these assays with complementary techniques, such as mass spectrometry. Various excellent reviews on their application to cyanotoxin monitoring are available.^{19–21} Although ELISA-based methods are

commonly used due to their high selectivity offered by antibody-based recognition, their detection sensitivity is limited to nanogram/mL (ng/mL) levels due to low avidity of the available antibodies. However, this has been slightly overcome by use of sensitive sensors. For example, cantilever biosensors using the same antibody exhibited sensitivity at a much lower concentration of picogram/mL (pg/mL), due to their intrinsically high mass-change sensitivity.²²

Sensitive assays for cyanotoxin provide very useful data on the presence of the toxin, but they do not provide predictive capabilities or information on the presence of the toxin-producing species itself. Thus, the use of a nucleic acid (NA)-based assay which detects the presence of the toxin-producing species itself would be desirable, and useful from a public health perspective, as such measures could potentially provide early warning of an impending algal bloom. NA-based detection of toxin-producing species occurs through targeting genes of the species associated with toxin production or the 16S ribosomal subunit.²³ Although quantitative polymerase chain reaction (qPCR)-based detection approaches using the former nuclear genes have provided sensitivity for detection of *M. aeruginosa* ranging as low as $\sim 10^2 - 10^3$ cells/mL,^{24–26} the use of 16S

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rRNA provides a valuable identifying sequence which may enhance sensitivity of detection, as it is present in higher copy numbers than nuclear toxin-associated genes.^{27–35} In fact, molecular approaches of qPCR,^{36,37} microarray,³⁸ and flow-based immunoassay³⁹ using rRNA target have provided an even higher sensitivity ranging from $10 - 10^4$ cells/mL. However, such techniques have associated drawbacks of long time-to-result (TTR), typically many hours, and high cost.⁴⁰ Thus, as biosensor-based assays require minimal sample preparation and short TTR, they are potentially attractive from an environmental monitoring perspective. In fact, biosensors have facilitated detection of toxin-producing and pathogenic species via nuclear genes and 16S rRNA alike. For example, surface plasmon resonance (SPR)-based biosensors have led to detection of *E. coli* at 10 cfu/mL in less than one hour via 16S rRNA targeting.⁴¹ Similarly, electrochemical-based biosensors have achieved detection directly in bacterial lysate at 250 cfu/mL in approximately 30 min.⁴² Such are the advantages of biosensors over the traditional molecular techniques.

Thus, a biosensor-based assay using 16S rRNA identifying sequences provides a potentially useful strategy for sensitive detection of toxin-producing *M. aeruginosa*. In this paper, we show a piezoelectric cantilever-based assay provides rapid and sensitive detection of toxin-producing *M. aeruginosa* with a detection limit of 50 cells/mL. Sensor response was affirmed by complementary fluorescence measurements due to surface bound hybridized double strands. Use of a second hybridization of nanogold-labeled DNA strands to captured RNA simultaneously enhanced sensor response and provided measurement verification. Experiments with spiked river water samples show minimal loss of sensitivity, and thus the assay has practical significance in source water monitoring.

2. MATERIALS AND METHODS

2.1. Reagents. *Microcystis aeruginosa* (*M. aeruginosa*) mother culture (UTEX LB 2385) and Bold 3N Medium were purchased from University of Texas-Austin (UTEX) culture collection (Austin, TX). Phosphate buffered saline, ethylenediaminetetraacetic acid (EDTA), tris-hydrochloride (Tris-HCl), and chloroform were from Sigma-Aldrich. Thiolated DNA probes for 16S rRNA capture and nanoparticle enhancement, as well as nonthiolated random RNA oligos (22 bases) used for control studies were from Integrated DNA Technologies (IDT). Bond Breaker TCEP (tris(2-carboxyethyl)phosphine) solution, sodium azide, glass pipettes, nuclease-free water, and sodium chloride were from Fisher. Filler molecule, 6-mercapto-1-hexanol, was from Fluka Corporation. Nucleic acid (NA) extraction kit was from Fermentas. Ethanol (EtOH, 200 proof) was from Decon Laboratories, Inc. (King of Prussia, PA). Quant-iT PicoGreen dye was from Invitrogen (Grand Island, NY). Gold nanoparticles (10 nm) were from BBInternational (Cardiff, U.K.). Nickel electroded type-5A lead zirconate titanate (PZT-5A) and solder kit were from PiezoSystems (Woburn, MA). Epoxy adhesive (Loctite 1C-LV Hysol) used for embedding the PZT was from McMaster-Carr (Robbinsville, NJ). Parylene-c dimer was from Specialty Coating Systems, Inc. (Indianapolis, IN). Polyurethane was from Wasser Corporation (Auburn, WA). Gold targets were from Denton Vacuum (Moorestown, NJ). Solder (63% tin, 37% lead, rosin core) was from Kester.

2.2. Sensor Fabrication. Piezoelectric millimeter-scale cantilever biosensors were fabricated from diced Nickel (Ni)-

electroded PZT chips ($5 \times 1 \times 0.127$ mm³, American Dicing, Liverpool, NY). Electrical leads were attached to top and bottom electroded faces of the chip via soldering near the end region. The chip's base region to which electrodes were attached was embedded into a glass cylinder (diameter ~ 3 mm) via epoxy, creating a conventionally anchored piezoelectric cantilever sensor. Additional epoxy was added on one face of the cantilever base to facilitate the desired anchor asymmetry. Details were reported in earlier publications.^{43,44} The sensors were electrically insulated by spin-coated polyurethane layer (~ 2 day cure at room temperature), followed by subsequent chemical vapor-deposited parylene-c layer (10 μ m thick). Sensors were cured at 80 °C post-CVD for ~ 24 h. A 100 nm thick gold layer was sputtered (DeskIV, Denton Vacuum) at the cantilever tip which provided ~ 1 mm² Au <111> sites for anchoring DNA probes. Use of asymmetric-anchoring in self-excited piezoelectric cantilever sensors has been previously shown to manifest picogram (pg) to femtogram (fg) sensitive bending modes.³³

2.2. Culturing of *M. aeruginosa*. The cyanobacteria *M. aeruginosa* (UTEX LB 2385) was purchased from UTEX culture collection (University of Texas-Austin, Austin, Texas) as a starter culture. *M. aeruginosa* was cultured in volumes of 30 mL by inoculating 30 mL of sterile Bold 3N Medium with 200 μ L of starter culture ($\sim 3 \times 10^7$ cells/mL). During growth, the culture was continuously purged with filtered air ($\sim 3.5\%$ carbon dioxide, CO₂). The culture was grown under continuous illumination (cool-white fluorescent light, 100 W, ~ 5200 lx) for seven days at room temperature to reach high cell density, $\sim 2 \times 10^7$ cells/mL. A schematic of the experimental arrangement used for culturing is shown in Figure S.1 of the Supporting Information (SI). The culture was centrifuged (2,500 rpm, 10 min, Clay Adams, DYNAC II Centrifuge) and resuspended in 10 mM phosphate buffered saline (PBS, pH 7.4) with 0.01% w/w sodium azide to final sample concentrations of 5×10^6 cells/mL which provided the cyanobacteria-containing water sample for the stock nucleic acid (NA)-extract used in detection assays. NA-extract resulting from this stock was serially diluted to in 10-fold steps to provide NA-extract samples corresponding to samples containing 5×10^1 to 5×10^5 *M. aeruginosa* cells/mL.

2.3. 16S rRNA Extraction for Biosensor Experiments. Nucleic acid extraction was done by adapting vendor-supplied protocol. Detailed schematic of the extraction protocol is in Figure S.2 of the SI. Briefly, 100 μ L of stock cyanobacteria culture was added to 100 μ L TE buffer and 400 μ L Fermentas lysis solution followed by incubation at 80 °C for 30 min. The cell suspension was repeatedly sheared (20 gauge syringe) to assist disruption of cell walls. Chloroform was then gently added to the sample (~ 600 μ L) followed by gentle mixing and centrifugation at 14 K rpm for 2 min (Beckman Coulter, Microfuge 18 Centrifuge). Fermentas precipitation solution (80 μ L) was added to aqueous phase extract (200 μ L); then, 800 μ L cold EtOH was added and incubated overnight at 4 °C. Samples were then centrifuged at 14 K rpm for 30 min which formed a NA-pellet containing 16S rRNA and background genomic DNA. The pellet was then gently rinsed in cold EtOH twice and resuspended in 300 μ L 1 M TE buffer. The NA-extract was sheared through a sterile 30 gauge needle 25 times at ~ 80 °C to reduce the average size distribution of NA strands to ~ 300 nucleotides in length,⁴⁵ and then cooled to assay temperature for detection experiments.

Table 1. Single Strand DNA Used in Cantilever-Based Assay

name	sequence	MW(kDa)	ref
DNA probe (probe strand A)	HS(CH ₂) ₆ T ₆ CCC TGA GTG TCA GAT ACA GCC CAG TAG	10.4	46
16S rRNA (target strand B)	UGGGAAGAACAUCCGUGGCG... ...AAAGCGAGCUACUGGGCUGUAUCUGACACUCAGGG	~97.5	47
NP-DNA probe (enhancement strand C)	CGCCACCGATGTTCTTCCCAT ₆ (CH ₂) ₃ SH	8.1	
random RNA (random strand D)	N ₂₂	7.0	

Practicality of the assay for direct use in source water was examined using a slightly modified protocol as described below. We first suspended a 500 cell *M. aeruginosa* pellet (from the laboratory grown culture, enumerated, and diluted) in 1 mL river water obtained from the Schuylkill River (Philadelphia, PA, USA) to make a test river water sample containing *M. aeruginosa* at 500 cells/mL. The spiked river water sample was centrifuged at 14 K rpm for 30 min. Supernatant was discarded and the cell pellet was resuspended in 200 μ L TE buffer to begin the NA extraction protocol. For river water-based detection, NA-extract was melted and sheared at slightly higher temperature (98 °C) and chilled to 0 °C rapidly prior to injecting into the flow loop.

2.4. Sensor Probe Immobilization and Gold Nanoparticle Labeling. A DNA probe (strand A, see Table 1) was synthesized based on previously investigated PCR primers for targeting 16S rRNA of *Microcystis* genera.⁴⁶ We verified selectivity of the probe for *M. aeruginosa* species using basic local alignment search tool (BLAST) of the National Institute of Health's (NIH) GenBank.⁴⁷ Synthetic thiolated DNA probe was reconstituted in Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 7.9, 1 M NaCl) and stored at -22 °C. Strand A (Table 1) contains a thiol group at the 5' end for immobilization on the gold-coated sensor. Prior to immobilization, the disulfide form of the DNA probe (1.4 μ M) was reduced by adding 1 μ L 500 mM TCEP to 300 μ L DNA probe and incubating at room temperature (~25 °C) for ~45 min.⁴⁸ For labeling of gold nanoparticles with DNA strands, we first washed stock Au NPs in TE buffer by centrifugation at 14 K rpm for 45 min and resuspension in TE. Immobilization of DNA on Au NPs was done by mixing 200 μ L of TE-washed Au nanoparticles ($c = 5.7 \times 10^{12}$ particles/mL; vendor provided protocol) with 300 μ L of TCEP-reduced strand C probe (Table 1) for ~1.5 h. The labeled-NPs were washed twice to remove unbound ssDNA (14K rpm for 45 min).

2.5. Fluorescence Assays. Hybridization between the probe (strand A) and the target 16S rRNA strand (strand B) present in the NA-extract was verified by PicoGreen fluorescence. Sensors, posthybridization experiment, were incubated in the dye-containing cuvette for 5 min in dark. Emission spectra were obtained with the sensor surface positioned at a 45° angle with respect to incident radiation (schematic in Figure S.3 of the SI). Fluorescence spectra over 500–600 nm were obtained at 490 nm excitation with a 1 nm slit width (Spectrofluorometer, PTL, Birmingham, NJ).

2.6. Sensor Experiments. A typical experiment began by cleaning the freshly Au-sputtered sensor with room temperature piranha solution for ~30 s followed by copious DI water rinse. The sensor was then installed in the flow cell and the resonant frequency was allowed to stabilize under continuously flowing buffer under an AC driving voltage of 100 mV and 0 DC bias (Agilent 4294A). Mass sensing was done by sweeping a frequency range near resonance of the applied AC voltage and continually tracking the resonant frequency, the frequency

where maximum phase angle of PZT electrical impedance (Z) occurs. After steady state was reached, the flow was switched to reduced-DNA probe solution (strand A) and the flow format was switched to a once-through mode to completely replace the loop volume with DNA probe. After ~4 mL probe solution was allowed to flow through the flow loop, the flow format was returned to the recirculation mode. When thiolated probe strands chemisorbed onto sensor Au <111> sites, mass of the sensor increased causing resonant frequency decrease. Subsequently, 1 mL 100 nM MCH was injected directly into the DNA probe reservoir and the flow format switched to a once-through mode until 1 mL was removed from the loop, a time at which the flow format was returned to the recirculation mode. MCH filling of unoccupied Au <111> caused a further small resonant frequency decrease. This procedure enabled maintenance of a near constant flow loop volume. The experimental arrangement facilitated in situ buffer rinse post-DNA probe immobilization and MCH passivation in all our experiments. Rinse step was done by switching the flow from the MCH to TE buffer while at the same time switching the flow format to the once-through mode until MCH and DNA probe were completely removed from the loop. Minimal rinse time of ~ flow cell time constant ($[\text{loop volume} + \text{cell volume}]/\text{flow rate} = [3 \text{ mL} + 0.3 \text{ mL}]/(0.5 \text{ mL/min}) \sim 6.5 \text{ min}$) was used in all cases. Buffer rinse removed loosely bound strands and caused only small sensor response. We note that sensor surface preparation time from start to finish takes ~90 min post-TCEP reduction. Detection of cyanobacteria was facilitated by recording sensor response to solutions containing various different concentrations of *M. aeruginosa* NA-extract. Hybridization between strand A (immobilized DNA probe) and strand B (the 16S rRNA target) over a concentration range of 5×10^1 to 5×10^5 cells/mL caused various levels of sensor mass increase, and thus, different levels of decrease in sensor resonant frequency. A schematic of the sensing principle and assay is given in Figure 1. We note that the sensor detection time postsensor preparation and -NA extraction was less than one hour and is governed by the probe-target hybridization rate. Following target detection, the flow loop was subsequently rinsed and sensor enhancement and verification steps were facilitated by introducing the secondary Au NP-labeled DNA probe. We note that the time period for simultaneous signal enhancement and verification was also governed by hybridization rate and required on an average ~45 min. Thus, the assay has a TTR of ~90 min, after target nucleic acid extraction.

2.7. Sensor Control Experiments. Various control experiments were conducted on the same batch of sensors used in detection studies for gaining confidence that exponential decrease in sensor resonant frequency response was indeed caused by hybridization of the DNA probe and the fragmented 16S rRNA target. The following four controls were done routinely: (1) examination of sensor response in the absence of binding analyte, (2) examination of sensor response to injections of buffer which lacked a binding analyte, (3)

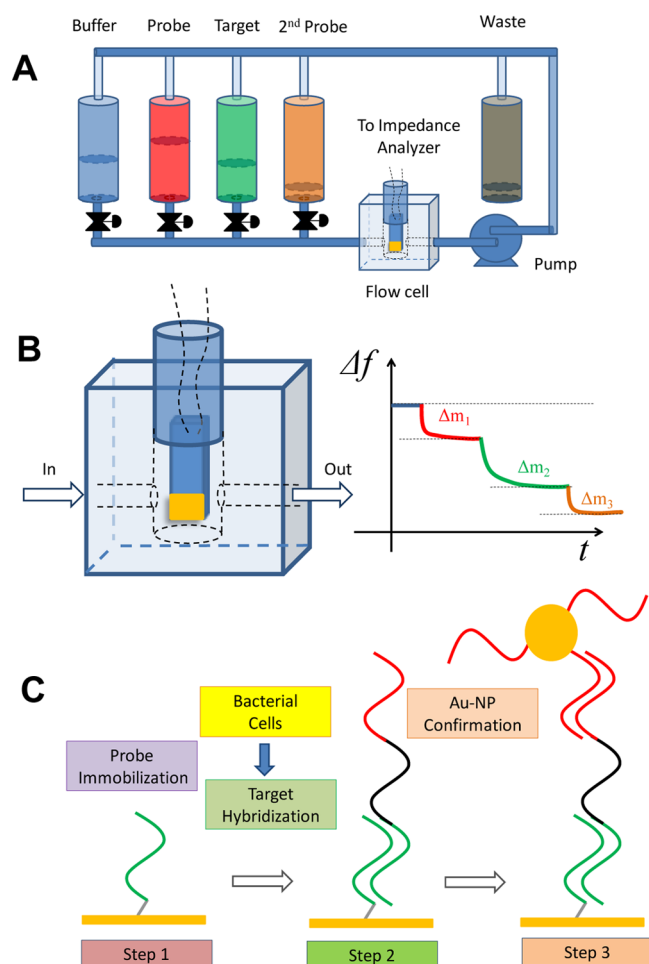


Figure 1. Schematics of (A) apparatus used for continuous sensing, (B) biosensor mass-sensing transduction principle, and (C) protocol for detection of 16S rRNA from target cyanobacteria.

examination of prepared-sensor response to injection of random RNA ($c = 5$ nM), and (4) examination of prepared-sensor response to extract of unspiked river water. Controls (1) and (2) address potential false signals which may arise from fabrication or apparatus abnormalities, such as defects in device coating or pressure effects, respectively, while controls (3) and (4) address potential false signal caused by nonspecific binding between the sensor and background RNA and organic material present in river water. The first control was done by first allowing the sensor to stabilize in the flow cell under continuously flowing buffer while tracking the resonant frequency over 2–3 h time periods (typical full assay length including preparation). The second control was done by allowing the sensor to stabilize in flowing buffer and subsequently making injections of buffer which lacked binding analyte to the flow cell in the same fashion as done for addition of DNA probe, MCH, and 16S rRNA target samples while at the same time monitoring resonant frequency. The third and fourth controls were done by making an injection of sample containing either random RNA oligos or river water extract, respectively, subsequent to TE buffer rinse and DNA probe and MCH immobilization while at the same time monitoring resonant frequency shift. For purposes of clarity, control (1) is shown throughout the manuscript given the qualitative similarity observed for the four controls. This agreement is discussed in further detail in the following sections.

3. RESULTS AND DISCUSSION

3.1. Sensor Characteristics. Several piezoelectric-excited millimeter-cantilever (PEMC) sensors ($n \sim 10$) with similar spectral and liquid stabilization properties were used in detection studies. In Figure 2A and B, we show photographs

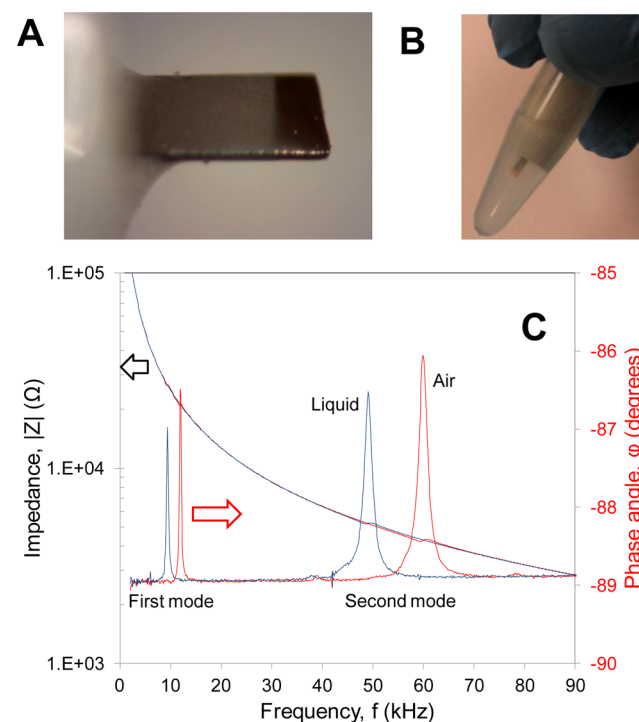


Figure 2. (A and B) Photographs of piezoelectric-excited millimeter cantilever (PEMC) sensors. (C) Sensor frequency response over 0–90 kHz shows two mass-sensitive resonant modes at ~12 and 60 kHz in air which undergo shift in resonant frequency upon liquid immersion.

of the typical electrically insulated asymmetric-anchored PEMC sensors with sputtered Au at the distal tip for sensing. The PZT impedance response over 0 – 250 kHz exhibits various resonant modes corresponding to impedance-coupled transverse, torsional, lateral, and longitudinal modes. As shown in Figure 2C, the PEMC sensors exhibited transverse modes at 11.9 and 60.1 kHz which strongly couple to high impedance change in the PZT layer. Immersion of the PEMC sensor in TE buffer caused 2.5 and 11.0 kHz decrease in resonant frequency of the first and second modes, respectively, due to added mass effect of the surrounding liquid. We note that immersion in liquid only caused a minor reduction in Q-value (a measure of peak sharpness), suggesting negligible viscous damping, and thus, measured shift is primarily due to inertial effects. PEMC sensors have been shown to be effective in liquids of viscosity 1000 times more viscous than water.⁴⁹

3.2. Sequential Addition of 16S rRNA Probe. The cantilever-based detection assay was begun by installing the PEMC sensor in a custom flow cell, shown schematically in Figure 1, and allowing the resonant frequency to reach steady state in TE buffer with the flow in a recirculation mode. As shown schematically in Figure 1B, detection occurs through measurable and proportional resonant frequency shifts caused by added mass of sensor-binding analytes. The resonant frequency remained constant in the absence of a binding analyte and showed a ~ 3 Hz noise level over a three hour period. When the flow was changed from TE buffer to DNA

probe (described in Section 2.6), the resonant frequency decreased. As shown in Figure 3A, introduction of 1 mL DNA

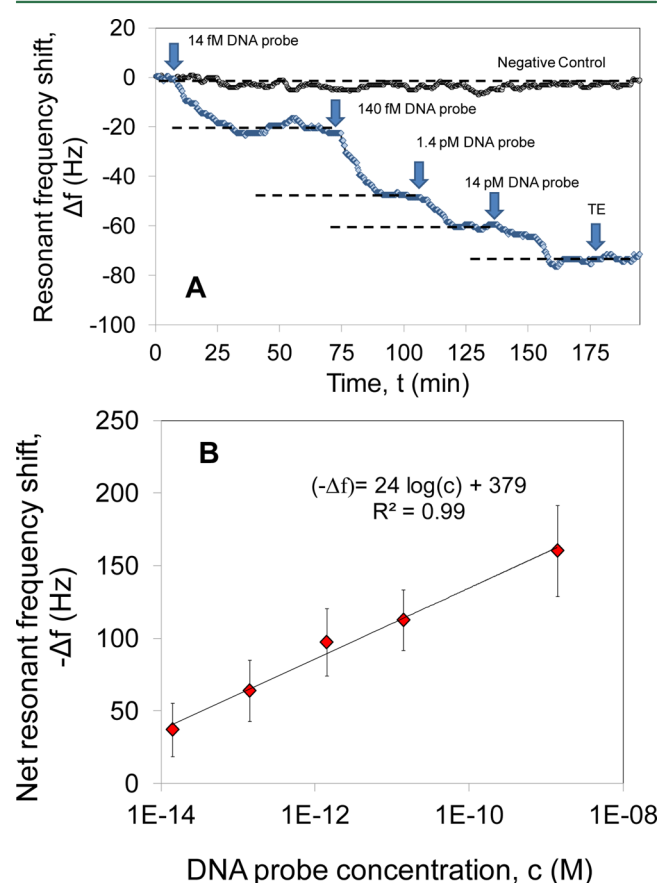


Figure 3. (A) Biosensor response to immobilization of DNA probe (strand A) via sequential concentration-dependent response. (B) Sensor response to DNA probe immobilization over a dynamic range of five log units. Sensor response is semilog linear. Error bars represent average experimental error gathered from $n \sim 3$ similar sensors.

sample (14 fM; strand A in Table 1) caused exponential resonant frequency decrease over the next 20 min, ultimately reaching a steady state value 22 Hz lower than the initial value. The net response is over nine times the noise level in the measurement. Decrease in resonant frequency is consistent with net increase in PEMC mass due to immobilized DNA probe on the Au-surface through chemisorption of the thiol end group to Au <111> sites. The 1 mm^2 Au sensing area contains $\sim 10^{12}$ Au <111> sites,⁵⁰ and the 1 mL of 14 fM DNA sample contains $\sim 8.4 \times 10^6$ strands. Therefore, we expect less than 10 ppm coverage of the available adsorption sites at equilibrium. Realistically, we expect only a fraction of the probe become bound to the sensor, and thus the actual surface coverage would be even lower. Injection of DNA probe at sequentially higher concentrations should cause further added mass response, and thus, such further response could both verify initial sensor immobilization response and allows us to characterize the sensitivity to DNA probe chemisorption over a range of concentration. As shown in Figure 3A, introduction of a 10-fold higher concentration caused a further decrease in resonant frequency with a transient period similar to the initial response. Sensor response to further step increases in concentration was examined up to 14 pM. Responses at 1.4 nM were obtained from *M. aeruginosa* detection studies discussed in the following

sections. Ultimately, in situ rinsing of the sensor was done by returning the flow to TE buffer in a once-through mode, and it caused negligible change in the resonant frequency indicating that DNA probe remained strongly bound to the sensor surface. In Figure 3B, cumulative sensor response ($n = 3$ similar sensors) is plotted as a function of concentration (strand A in Table 1) and shows a semilog linear empirical relationship given by $(-\Delta f) = A + B \log(c)$, where A and B are empirical characteristic sensor constants corresponding to concentration given on a molar basis.^{51,52} Sensor response to probe immobilization yielded sensor constants $A = 378.9$ Hz and $B = 24.4$ Hz. The semilog linear sensor response obtained in this study is similar to earlier studies with microRNA and DNA detection.^{22,48} From the sensor response correlation $(-\Delta f) = A + B \log(c)$, one can obtain a sensitivity parameter, such as $d(-\Delta f)/dc = B/c$ which has the units of resonant frequency shift per molar unit (Hz/M). The log-linear correlation suggests that the device is more sensitive at lower concentrations than at higher concentrations, and is an attractive property of the sensor. Therefore, discerning sensor response at low concentration would be dictated by acceptable signal-to-noise ratio and is further discussed in a later section.

3.3. Concentration-Dependent Response to 16S rRNA. Response to various concentrations of *M. aeruginosa* NA-extract was obtained for determining detection limit and dynamic range. A typical assay began with immobilization of DNA probe (strand A; Table 1), passivation by MCH, and in situ rinsing described in Section 3.2, followed by exposure to sheared NA-extract. The probe was immobilized by injection of 1 mL 1.4 nM to ensure good surface coverage. Such a concentration was experimentally found to give good surface packing density that yielded sensitive detection of target hybridization. As shown in Figures 3B and 4A, chemisorption of probe at 1.4 nM caused a 195 Hz shift in resonant frequency over a typical binding period of ~ 1 h. The empty Au <111> sites were filled by mercaptohexanol injection (MCH, 100 nM; 1 mL). MCH displaces nonoriented strands bound to Au via the nitrogeneous bases,⁵³ and caused a further resonant frequency shift of ~ 10 Hz. In situ rinsing of the sensor surface was achieved by switching the flow to TE buffer and the flow changed to a once-through mode. The change to buffer caused negligible shift in resonant frequency indicating the probe remained bound to the sensor. At this stage, the sensor was ready to be exposed to NA sample containing the melted and sheared 16S rRNA target.

NA-extract of *M. aeruginosa* samples was prepared at 5×10^6 cell/mL and serially diluted in 10-fold steps to provide samples ranging from 5×10^1 to 5×10^5 cells/mL. Since a single cell contains many ribosomes, it is useful to estimate the number of target strands one expects to be present in a given NA-extract sample. Although there is lack of information on the number of ribosomes per *M. aeruginosa*, one can estimate the amount of 16S rRNA per cell equivalent as follows. Given that rapidly growing *E. coli* (doubling time ~ 2 h) has $\sim 48\,000$ ribosomes,^{54,55} and *M. aeruginosa* has a much lower doubling time of ~ 24 h, the estimated number of ribosomes per *M. aeruginosa* cell is approximately $(2\text{ h}/24\text{ h}) \times 48\,000$ ribosomes = 4000 ribosomes. Since the sheared NA is ~ 300 bases [MW ~ 97.5 kDa ($=300$ bases $\times 325$ Da/base)], the concentration range tested (5×10^1 to 5×10^5 cells/mL) corresponds to ~ 33 fg/mL to 330 pg/mL NA of 16S rRNA origin. Since the extraction procedure also yields ~ 1 fg genomic DNA per cell,⁵⁶ the target strand represents $\sim 40\%$ of total NA

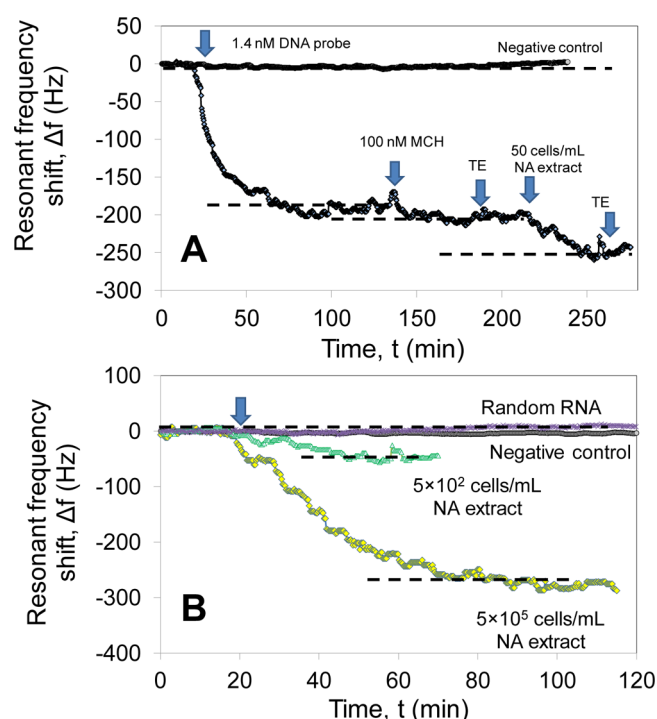


Figure 4. (A) Typical cantilever biosensor response to NA-extracts from ~50 cells/mL of *M. aeruginosa*. Shown is initial immobilization of DNA probe (strand A) followed by MCH chemisorption and TE buffer rinse. One mL *M. aeruginosa* NA-extract was injected post-rinse step resulting in ~45 Hz resonant frequency decrease. (B) Time-shifted responses at low and high concentrations of NA-extract.

present. We recognize that this value could be significantly different in microorganisms with higher growth rates which will contain higher number of ribosomes per cell.

Assay sensitivity for 16S rRNA detection was examined by switching the flow to 50 cell/mL NA-extract (33 fg rRNA/mL) after the flow had restabilized in TE post-DNA probe and -MCH immobilization. As shown in Figure 4A, sensor response decreased by 47 Hz over the next 40 min due to target hybridization to surface-bound probe. Such a result is consistent with rates of chemisorption and hybridization observed previously on resonating cantilever biosensors.⁴⁸ In Figure 4B we compare sensor response to hybridization at two extreme concentrations: low and high concentrations. The results suggest two main points: (1) time scale of sensor response decreases with decreasing concentration of *M. aeruginosa*, and (2) detection at the higher concentration occurs in less than two hours. It should be noted sensor detection response differed from that of the controls discussed in the Materials and Methods section. For example, each of the control responses (sensor response in absence of a binding analyte, buffer injection, random RNA (5 nM), and unspiked river water extract) caused no exponential binding-like sensor response suggesting that response was not an experimental artifact. The control responses in which random RNA or unspiked river water extract were introduced to the prepared sensor caused no change in resonant frequency greater than experimental error indicating that random background nucleic acid present in sample did not cause false sensor responses. Sensor response to NA-extract sample derived from 5×10^1 to 5×10^5 cells/mL showed a semilog-linear relationship with $A = -41.4$ Hz and $B = 29.5$ Hz (Figure 5A). Semilog linear

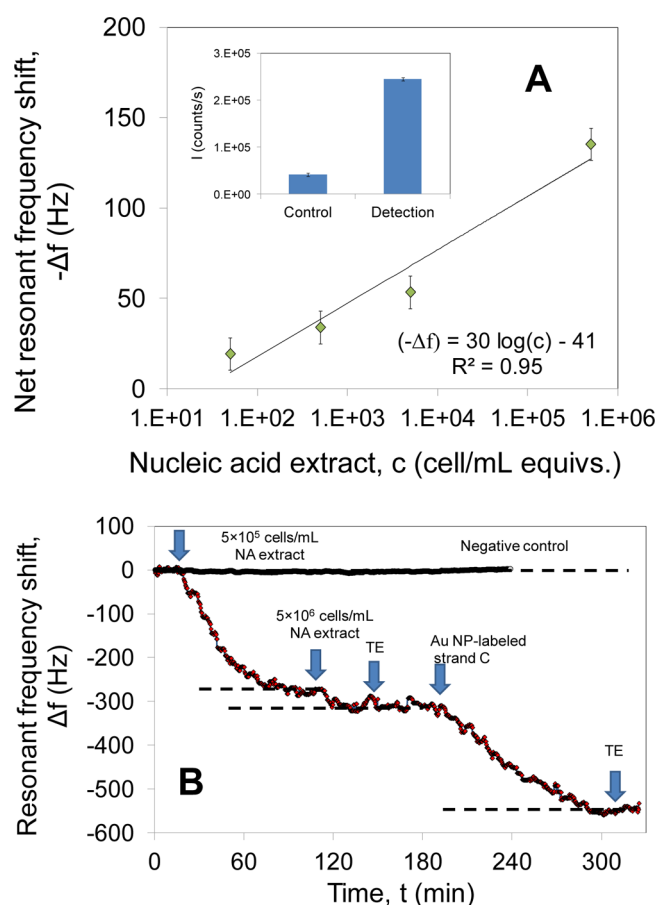


Figure 5. (A) Sensor response to *M. aeruginosa* NA-extract over a dynamic range of five log units caused semilog linear sensor response similar to DNA probe response. (B) Verification of sensor response by saturation response and secondary binding of DNA probe-labeled gold nanoparticles.

relationship for NA hybridization is similar to earlier work on DNA hybridization.⁵⁷ At low concentration, sensor response is low, as is seen in Figure 4. For low sensor response, it is prudent to affirm that the response is indeed due to the target hybridization by second and third measures. We examined the feasibility of such approaches in the next section.

3.4. Simultaneous Enhancement and Verification of PEMC Sensor Response. The sensor response caused by hybridization of NA-extract to immobilized DNA probe was verified for the presence of double stranded NA postdetection response by measuring PicoGreen fluorescence. PicoGreen fluorescence is indicative of DNA-RNA double strands. As shown in Figure 5A inset, fluorescence signal of the sensor surface was significantly higher than the signal obtained on a sensor that was not exposed to 16S rRNA (measuring only single stranded DNA probe). Both target-hybridized and control sensors showed maximum fluorescence intensity near 525–530 nm which agrees with PicoGreen properties (see Figure 5A).

3.5. Verification and enhancement of PEMC sensor response. PicoGreen measurement verified sensor response was indeed caused by hybridization of 16S rRNA strands in the sample. We sought to further confirm hybridization and amplify sensor response using a secondary hybridization with Au nanoparticle (NP)-labeled DNA designed to hybridize at the distal end of the capture probe-hybridized region as illustrated

schematically in Figure 1C. We note that many NP-based techniques have been examined for enhancing signal in both sensors and molecular approaches, but it is important to note that the mechanism by which the NP affects signal enhancement vary and often differ in practicality for application to detection assays. For example, some NP enhancement techniques require direct conjugation to the target itself,^{58,59} which constitutes a label and is less desirable to those approaches which incorporate NP enhancement into selectively binding secondary probes, such as linear DNA molecules. We also note that the enhancement mechanism associated with the NP also highly varies from optical^{59–61} to electrical-based^{58,62} and often occurs through different reaction chemistry than that which governed the initial probe-target response.^{58,62,63} Therefore, the fact that the Au-NP enhancement response used here shares the same mass-based transduction principle and the species selective binding chemistry which generated initial target response makes it highly attractive for detection, where the goal is to eliminate false responses and improve measurement confidence. Thus, after detection of NA sample, the flow cell was rinsed with TE buffer to remove unbound strands from the flow loop and the sensor response was subsequently allowed to restabilize. Flow was then switched to a solution containing Au NP labeled strand C. As shown in Figure 5B, the second hybridization between labeled-Au NPs and the distal single strand region of the target (Figure 1C) caused a significant further increase in sensor response. We examined Au NP enhancement of sensor response at low and high *M. aeruginosa* concentrations (5×10^1 and 5×10^5 cells/mL) and found it caused similar relative enhancement at each level, more than doubling the initial response signal at both concentrations examined. The average Au NP hybridization response was slower, yet comparable, to the target hybridization rate. Such enhancement was similar to that found in earlier studies on microRNA.⁴⁸

3.6. Feasibility for Application to River Water. Given the ability to detect *M. aeruginosa* down to 50 cell/mL in buffer samples, we investigated the practicality of the assay for detection of *M. aeruginosa* in source waters by spiking river water samples with known number of *M. aeruginosa*, 500 cells/mL. In these samples the NA-extract contained not only background genomic DNA of *M. aeruginosa*, but also NA derived from background microorganisms. Upon repeating the experiments described in Section 3.3 but using model river water samples in place of buffer samples, we found sensor response to 500 cells/mL produced both measurable and verifiable response (26 ± 23 Hz). DNA labeled Au-NP hybridization postdetection gave an additional shift of 22 ± 11 Hz, representing a $\sim 85\%$ signal enhancement, similar to levels observed in experiments lacking additional NA material from background microorganism. We note that slight reduction in sensor response was observed due to presence of background microorganisms, and the reduction is likely be due to losses associated with NA extraction.

3.7. Analysis of Theoretical Detection Limits with and without Nanoparticle Enhancement. The empirical sensor response relationship $(-\Delta f) = A + B \log(c)$ for 16S rRNA target hybridization is useful for estimating the assay limit of detection with and without Au NP enhancement. From the data in Figure 6, one can estimate the limit of detection by extrapolating the semilog linear correlation using concentration data. In the low concentration range, sensor constants are $A = -10.5$ and $B = 17.8$ Hz, and were determined by finding the best fit at low

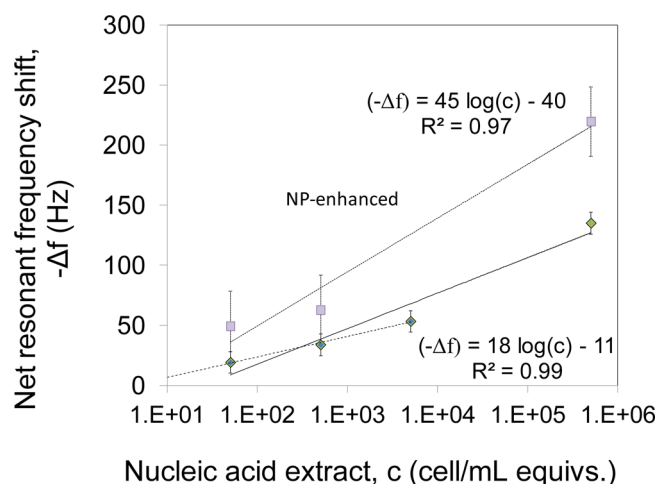


Figure 6. Comparison of sensor response with and without nanoparticle enhancement. Also shown are empirical correlation and sensor constants for low concentration region.

concentration. If a minimum acceptable signal-to-noise ratio of ~ 3 is assumed, the limit of detection predicted by the response correlation is ~ 14 cells/mL. The nanoparticle labeled DNA strand amplified the sensor response. From the data in Figure 6, the NP-enhanced sensor constants are $A = -40.2$ and $B = 44.9$ Hz. Because the correlation for NP-enhanced and first-step sensor response were done on the same concentration basis, it is possible to compare their values for estimating the sensitivity enhancement due to NPs over the dynamic range used. Values of B for case of first step and NP-enhanced were over 50% (44.9 Hz vs 28.6 Hz), suggesting that NP-enhancement improved detection limit by about 50%. Thus we estimate detection limit of the two step hybridization method to be less than 10 cells/mL.

4. CONCLUSIONS

A biosensor-based assay using piezoelectric cantilever sensors was examined for detecting toxin-producing cyanobacteria *M. aeruginosa* at concentrations ranging from 5×10^1 to 5×10^5 cells/mL with minimal sample preparation. Rapid detection based on species-specific 16S rRNA region was experimentally shown to be feasible. The limit of detection of 50 cell/mL (~ 33 fg 16S rRNA/mL) shown experimentally is comparable to detection limit of molecular techniques. The two step hybridization method not only amplified the sensor response, but it also verified detection response. The correlation of sensor response to various cell concentration NA-extract suggests detection of *M. aeruginosa* at less than 10 cells/mL is feasible. The biosensor method provides an attractive alternative for monitoring toxin-producing cyanobacteria in source waters.

■ ASSOCIATED CONTENT

● Supporting Information

Additional information as noted in the text. 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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