Selective Quantification of Viable *Escherichia coli* Bacteria in Biosolids by Quantitative PCR with Propidium Monoazide Modification

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Received 10 December 2010/Accepted 10 May 2011

Quantitative differentiation of live cells in biosolids samples, without the use of culturing-based approaches, is highly critical from a public health risk perspective, as recent studies have shown significant regrowth and reactivation of indicator organisms. Persistence of DNA in the environment after cell death in the range of days to weeks limits the application of DNA-based approaches as a measure of live cell density. Using selective nucleic acid intercalating dyes like ethidium monoazide (EMA) and propidium monoazide (PMA) is one of the alternative approaches to detecting and quantifying viable cells by quantitative PCR. These compounds have the ability to penetrate only into dead cells with compromised membrane integrity and intercalate with DNA via their photoinducible azide groups and in turn inhibit DNA amplification during PCRs. PMA has been successfully used in different studies and microorganisms, but it has not been evaluated sufficiently for complex environmental samples such as biosolids. In this study, experiments were performed with *Escherichia coli* ATCC 25922 as the model organism and the uidA gene as the target sequence using real-time PCR via the absolute quantification method. Experiments with the known quantities of live and dead cell mixtures showed that PMA treatment inhibits PCR amplification from dead cells with over 99% efficiency. The results also indicated that PMA-modified quantitative PCR could be successfully applied to biosolids when the total suspended solids (TSS) concentration is at or below 2,000 mg · liter⁻¹.

Biosolids is a term used to describe the nutrient-rich organic materials resulting from biological treatment of sewage, and they have significant beneficial use potential through land application and direct public use. The density of fecal coliforms (FC) and/or *Escherichia coli* is the most commonly used indicator of public health risks associated with pathogen content of biosolids when biosolids are disposed of or used as a soil amendment. Density may be expressed as either the most probable number or CFU. A sudden increase in *E. coli* density in anaerobically digested biosolids immediately after high-speed centrifuge dewatering has been shown in a significant number of recent studies (10, 11, 13, 29). This sudden increase, also referred to as reactivation, is shown to be a phenomenon separate from growth during the storage of dewatered biosolids cake, referred to as regrowth.

Dewatering is the removal of water from biosolids, applied after biosolids treatment to achieve overall volume reduction by increasing the solids concentration. There are various dewatering processes in wastewater engineering, such as centrifugation, use of a belt filter press, vacuum filtering, and drying (19). These processes increase the solids contents of biosolids from 2 to 4% by mass to as high as 20 to 25%. It is an important wastewater management process since it helps treatment plants achieve nearly an order-of-magnitude decrease in the overall volume of biosolids, and thereby, it reduces significantly the cost of biosolids management.

Among the different dewatering technologies, reactivation and regrowth phenomena are observed only in high-speed centrifuge dewatering. For example, Erdal et al. (7) reported that FC density exceeds class B standards (greater than 2 × 10⁶ CFU per gram of total solids at the time of use) if biosolids, treated by a mesophilic digester, are dewatered using high-speed centrifugation. On the other hand, there was no change in FC density levels in the samples where a belt filter press was used for dewatering. A belt filter press is a mechanical dewatering device that applies mechanical pressure to biosolids slurry. When the sludge is compressed between two porous tensioned belts by passing those belts through decreasing diameter rolls, a significant amount of water is released (19). It has also been shown that the magnitude of increase in FC density is greater when biosolids are digested thermophilically (11). The increase in FC density in thermophilically digested biosolids reached 4 to 5 logs, compared to an increase of less than 1 log in biosolids digested mesophilically (1).

Although the exact mechanism of FC reactivation is not yet fully understood, a few extensive recent studies strongly suggest that FC go into a viable but nonculturable (VBNC) state during anaerobic digestion and resume culturability immediately after high-speed centrifugation (10, 11, 29). Unfortunately, currently available culturing-based tests for detection and enumeration of coliforms cannot differentiate live cells from those in the VBNC state, a fact which hampers efforts to study the exact mechanisms of coliform regrowth in biosolids. In addition, the inability to differentiate between live and VBNC coliforms and to accurately quantify cells in the VBNC state underestimates the potential public health risks associated with the beneficial use of biosolids. Thus, the current regulatory framework relying on culture-based tests must be revised to address this concern. Hence, there is a critical and
urgent need to develop culturing-independent analytical methods that can accurately estimate cells in the VBNC state in biosolids.

Recently, Chen et al. developed a conventional PCR method for more-accurate E. coli quantification in biosolids (6). However, prolonged persistence of DNA after cell death is a concern for the application of such methods, as DNA from dead cells can result in false-positive identification and overestimation of potential health risks. One of the most reliable criteria for differentiating viable and dead bacteria cells is membrane integrity. Our hypothesis is that when culturing-based tests are conducted concurrently with an analytical method that is based on cell membrane integrity, both live and viable cells would be quantified. The difference between those two quantities would be the portion of live cells—they meet the definition of live cell due to their intact cell membranes, and yet they are not culturable under the existing environmental conditions.

Live cells with intact membranes have the ability to exclude DNA-binding dyes that easily penetrate dead cells with compromised membranes (22). Molecular Probes (Eugene, OR) provides many dyes, such as propidium iodide (PI) and Sytox green, among which PI is one of the most commonly used (14). Exclusion of PI by live cells has been one of the most reliable methods, as PI-positive cells have not been shown to grow due to irreversible damage to the cell membrane. This supports the hypothesis that membrane integrity is still one of the most reliable indicators of cell viability (20, 32).

In the last few years, alternative DNA-based techniques have been developed. One of those methods includes removing free, extracellular DNA and DNA of dead bacterial cells during DNA extraction by using photoinducible nucleic acid staining dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA) (3, 21, 22, 31, 38). However, use of EMA prior to DNA extraction has been found to result in a significant loss of genomic DNA from viable cells in the cases of Escherichia coli O157:H7, Campylobacter jejuni, and Listeria monocytogenes (9, 21). On the other hand, PMA has been proposed as a more selective dye discriminating between dead bacterial cells and viable cells, ostensibly due to a higher positive charge (22).

PMA preferentially binds to double-stranded DNA with high affinity. Upon photolysis, the photoreactive azido group on the dye is converted to a highly reactive nitrene radical, which readily reacts with any hydrocarbon moiety at the binding site to form a stable covalent nitrogen-carbon bond, resulting in permanent DNA modification. The dye is nearly completely cell membrane impermeable and thus can be selectively used to modify exposed DNA only from dead cells while leaving DNA from viable cells intact. This results in blocking subsequent amplification of modified target DNA sequences by PCR. This feature makes the dye highly useful in the selective detection and quantification of viable cells by quantitative real-time PCR in the presence of dead cells whose DNA has been PMA modified and thus cannot be amplified (22).

Quantitative PCR with PMA modification has been successfully used in a number of studies for different microorganisms (5, 8, 15, 22, 27, 28, 30, 37). To our knowledge, the only E. coli strain tested in a PMA-modified quantitative PCR is E. coli O157:H7. In this study, we examined the suitability of PMA treatment prior to DNA extraction in quantitative PCR for selective absolute quantification of live E. coli ATCC 25922 bacteria from cultures with defined portions of live and dead cells. Evaluation of this application in complex environmental matrices is limited to a few studies (3, 16, 23, 36, 38), none of which focused on the effectiveness of PMA in biosolids, to the best of our knowledge.

The objective of this study was to assess the suitability of a modified PCR method to exclude extracellular DNA and DNA from dead cells in the complex chemical and biological matrices of biosolids. The modified PCR method evaluated used PMA as a DNA-binding dye that penetrates cells with compromised membranes while being easily excluded by cells with intact membranes.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. E. coli cultures were grown either in Luria-Bertani (LB) broth (Difco, Detroit, MI) or on Luria agar plates at 37°C. In the case of plasmid-containing cultures, ampicillin (100 µg·mL⁻¹; Sigma-Aldrich, St. Louis, MO) was added to the medium. Five to 50 ml of LB broth was inoculated with E. coli from a fresh LB agar plate in a 250-ml flask and incubated overnight with shaking at 37°C to obtain a stationary-phase culture. Three hundred microliters from this seed culture was inoculated into a fresh flask containing 50 ml LB broth. The culture was incubated for 2 to 2.5 h at 37°C with vigorous shaking in an orbital shaker to obtain a log-phase culture with an optical density of 1 at 600 nm. At the same time, the number of CFU was determined in triplicate by plating 100-µl aliquots from serial dilutions on LB agar. After incubation for 18 h, CFU were counted for each plate, averaged, and expressed as CFU ml⁻¹.

Killing conditions. Initial killing of cells by heat and isopropanol was analyzed, and the same results were obtained with each method (data not shown). Isopropanol was preferred to kill the cells due to its ease of use. Five-hundred-microliter cell aliquots with an optical density of 1 at 600 nm were killed by exposure to isopropanol (final concentration, 70%) for 10 to 15 min. Isopropanol was removed by centrifugation at 7,000 × g for 10 min. Pellets of killed cells were resuspended in 500 µl of LB medium. Loss of viability was tested by spreading 100 µl of cell suspension on the LB agar plates.

Mixture of live and dead bacteria. Defined quantities of isopropanol-killed cell cultures were mixed with the untreated live cultures in three different concentrations (1:10³ to 1:10⁷ dilutions) to give a total volume of 500 µl. Undiluted live and dead cultures were also used. All dilutions were made using LB broth.

Spiking of a wastewater treatment plant dewatered cake (biosolids) sample with E. coli cells. The biosolids samples were obtained from the Phoenixville Sewage Treatment Plant, Phoenixville, PA. The samples were stored at 4°C in an autoclave to ensure removal of all native microbial populations. The total suspended solids (TSS) content of the biosolids was measured according to standard method 2540 D (2). Then, the biosolids sample was resuspended with 1× phosphate-buffered saline (PBS) solution. A kitchen blender was used to homogenize the biosolids sample as much as possible in PBS. To determine the safety of the method with respect to E. coli cell integrity, we applied a blender method to the pure culture of E. coli cells for 30, 60, and 120 s. Possible impacts of homogenization on cell culturability and cell membrane integrity were determined via CFU counts and PMA-modified PCR, respectively, just before and after homogenization. Finally, stock solutions of homogenized, autoclaved biosolids in 1× PBS solution were prepared. The pure culture of E. coli cells was grown as mentioned above to an optical density of 1 at 600 nm. Then, 100 µl of broth medium containing 4.8 × 10⁷ dead E. coli cells was mixed with 400 µl PBS buffer solution, resulting in 0, 1,000, 2,000, 4,000, 8,000, 10,000, 20,000, and 40,000 mg · liter⁻¹ TSS in the final mixture before PMA treatment.

PMA cross-linking. PMA (Biotium Inc., Hayward, CA) was dissolved in 20% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) to get a stock solution of 10 mM and stored at −20°C. The PMA stock solution was transferred to 500-µl culture mixtures or biosolids samples spiked with E. coli cells at a final concentration of 100 µM. Light-transparent 1.5-ml microcentrifuge tubes (USA Scientific Inc., Ocala, FL) were used. All manipulations of PMA solution were performed under minimal light to prevent any potential chemical change in PMA structure, as it is a light-sensitive molecule. Following 10 min of incubation in the dark, samples were exposed for 5 min to a 600-W halogen light source at a distance of 15 to 20 cm from the light source, as suggested by literature and the
manipulator. Microcentrifuge tubes were placed on ice during the light exposure to avoid excessive heating.

DNA extraction and quantification. For studying the effect of PMA on pure cultures, DNA from pure E. coli culture (ATCC 25922) was extracted using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. DNA from biosolids samples was extracted using a Mo Bio PowerSoil DNA kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer’s instructions with the following modifications. The incubation time with solutions C2 and C3 was increased to 10 min at 4°C, and the final DNA elution was performed by adding 100 μl of solution C6 heated to 60°C and incubated for 5 min at room temperature before the final centrifugation. These minor modifications of the manufacturer’s instructions increased the quality of extracted DNA and yield. The Mo Bio kit was chosen after it performed better than the SoilMaster DNA extraction kit (Epigence Biotechnologies, Madison, WI) in a parallel comparison of DNA extraction efficiencies. DNA concentrations were measured by a Nanodrop ND-1000 UV-visible light spectrophotometer (Nanodrop Technologies, Wilmington, DE).

To determine the DNA extraction efficiencies of these two kits, 400-μl sterilized biosolids samples with 40,000 mg/liter (Sigma-Aldrich, St. Louis, MO) just before it was used. DNase I treatment elutions were compared to that from the same plasmid copy number in the standard curve. A major problem in quantitative PCR with an E. coli gene is the amplification in the NTC due to the presence of bacterial DNA in commercially available PCR mixes (33, 35). Commercial Taq polymerase enzyme is synthesized in E. coli as a recombinant protein, and traces of bacterial DNA remain in the mix, even though high-purification methods are used. During the beginning of the research, significant amplifications (C_T value of around 31) were observed in NTC reactions. The issue was addressed by treating the master mix with DNase I enzyme (Sigma-Aldrich, St. Louis, MO) just before it was used. DNase I treatment increased the C_T value of NTC from 31 to 36. For each reaction, a 0.1-unit amplification grade enzyme was used, together with 1× of its reaction buffer. This solution was incubated for 15 min at room temperature and then 10 min at 70°C after addition of 1× stop solution.

Standard calibration curve preparation. Standard calibration curves were generated using 10-fold serial dilutions of a plasmid containing a segment of the uidA gene. A 1.4-kbp segment of the uidA gene was amplified from genomic E. coli DNA by PCR primers UAL-754 (5'-AAAAACGCGCAAGAAAGACAGC-3') and UAR-2105 (5'-TGTGGCTCCTCGTGTGCAGG-3') (4), ligated into a pGEM-T plasmid vector, and then transformed into chemically competent E. coli JM109 cells using a pGEM-T vector system II cloning kit (Promega, Madison, WI). Plasmids were extracted using QIAprep spin miniprep kit (Qiagen, Valencia, CA) and linearized with the restriction enzyme Scal (New England BioLabs, Beverly, MA) before quantification as described above.

RESULTS

Effects of PMA on defined ratios of viable and dead pure culture E. coli cells. To study the effective covalent binding of PMA to the DNA of the dead cell population, uidA gene copy numbers were used as indicators in mixtures of viable and isopropanol-killed E. coli pure cultures. The mixtures were prepared at predefined ratios; viable cells consisted of 0, 0.1, 1, 10, and 100% of the total bacterial cell concentration. For example, the mixture designated 100% (Fig. 1) contained 500...
μl of live-cell stock solution (2.8 × 10^8 cells), while the mixture designated 10% was prepared by mixing 50 μl of live-cell stock solution with 450 μl of dead-cell stock solution. The results of PMA-modified and conventional qPCR on live-dead cell mixtures are presented in Fig. 1. The calibration run for the PCRs presented in Fig. 1 was highly accurate, with a linearity (R²) of 0.985 and an efficiency of 96%. When we compared the qPCR results of PMA-treated mixtures containing only live cells (100% viable cell content) with those containing only dead cells (0% viable cell content), treatment of the mixtures containing only dead cells caused a 4-log reduction in gene copy numbers (from ~10^9 gene copies to ~10^5). Similarly, each order-of-magnitude decrease in the proportion of viable bacteria in the defined mixtures [10% and 1% PMA (+) samples in Fig. 1] led to a 1-log decrease in gene copy numbers in each reduction due to PMA treatment, as expected. There were no significant differences in gene copy numbers between the PMA-treated live cells and five PMA-untreated mixtures (Fig. 1). These results indicate that the amplification of target DNA from the dead cells is inhibited by PMA with over 99% efficiency and amplification from the live cells is not affected by different concentrations of dead bacteria in the presence of PMA.

**Determination of the effect of homogenization on cell viability and integrity of E. coli cells.** It was important to make sure that the nonhomogenous nature of the biosolids cake did not skew the results as to the effectiveness of PMA in the biosolids sample. A homogenizer was used for homogenization of biosolids samples in PBS buffer as much as possible. Before the homogenizer was adopted for homogenization of biosolids samples, the effect of shear stress on the pure E. coli cells was tested. E. coli density was determined via plating 100-μl aliquots from serial dilutions in triplicate, when the pure E. coli cells growing at logarithmic phase were subjected to shear stress of the blender for 0, 30, 60, and 120 s. Results indicated that such shear stress did not affect the cell viability (data not shown). In addition, the cell wall integrity of the E. coli cells was analyzed by qPCR after PMA treatment of the cells just before and after the blender effect. Any damage to cell wall integrity would cause PMA to enter into the cells, leading to inhibition of PCRs. The qPCR results indicated that there was no damaging effect of shear stress on cell wall integrity (data not shown). The calibration run for this particular set of PMA-modified qPCRs was within an acceptable range, with an R² value of 0.999 and an efficiency of 92%. The results clearly showed that a home blender could be safely used for homogenization of biosolids samples prior to applications without damaging the cells.

**Comparison of two different soil DNA extraction kits.** There is no common soil DNA extraction kit or method for all environmental samples, due to their complex nature. Their effectiveness may change from one sample to another. In a search for the most effective extraction kit, two different commercially available extraction kits were compared in terms of their extraction efficiencies, that is, the mass of DNA extracted from each of three samples: pure culture E. coli, biosolids, and biosolids spiked with the pure culture E. coli. Both kits were used to extract DNA from samples in triplicate from each sample. The yields were 2,727 (±197), 1,867 (±72), and 453 (±12) ng, respectively, when the PowerSoil DNA kit (Mo Bio Laboratories, Carlsbad, CA) was used. On the other hand, yields were significantly lower when the SoilMaster DNA extraction kit (Epitcentre Biotechnologies, Madison, WI) was used; 1,580 (±20), 1,120 (±98), and 213 (±15) ng, respectively. The results clearly indicated higher DNA extraction efficiency for the Mo Bio PowerSoil kit and reflected the effectiveness of combining mechanical (bead beading) and chemical processes for cell lysis when extracting DNA from biosolids samples.

**PMA treatment of biosolids samples spiked with E. coli cells.** We assessed the ability of PMA-modified qPCR to eliminate PCR amplification from dead cells at increasing solids concentrations. Known amounts of dead E. coli cells were spiked into PBS buffer solutions containing 0, 1,000, 2,000, 4,000, 8,000, 10,000, 20,000, and 40,000 mg·liter⁻¹ TSS just before PMA treatment. Cₚ values of the PMA-modified qPCR at TSS concentrations of 1,000 and 2,000 mg·liter⁻¹ were nearly identical to those of the control (no biosolids), indicating that a TSS concentration at or below 2,000 mg·liter⁻¹ did not inhibit the efficiency of PMA treatment on dead cells. Solids concentrations at or above 4,000 mg·liter⁻¹, on the other hand, caused interference with PMA treatment, as indicated by increased copy numbers being detected by PMA-modified qPCR (Fig. 2A).

In addition, the possible inhibitory effect of biosolids TSS content on PCR was evaluated. A plasmid carrying the uidA segment was spiked into biosolids DNA extracts at a predefined amount per reaction as described above. No significant difference between mean Cₚ values of the samples and the control itself was observed, indicating an absence of PCR inhibition (Fig. 2B). The calibration runs for these particular sets of PMA-modified qPCRs were within an acceptable range, with an R² value of 0.999 and an efficiency of 99.86% (Fig. 2A) and an R² value of 0.999 and an efficiency of 100.5% (Fig. 2B).

**DISCUSSION**

Although no single group of microorganisms can meet all criteria as effective indicators, FC, including E. coli, are by far the most widely used indicators for assessing the microbial public health risks associated with treated wastewater and biosolids generated during wastewater treatment. Quantitative microbial risk assessment and thus regulations on beneficial use or final disposal of anaerobically digested biosolids are based largely on the density of FC determined via culture-dependent detection and enumeration methods. Nevertheless, the sudden increase in E. coli density after high-speed centrifugation shown in many studies underlines the significant drawbacks of using culture-based methods for microbial risk assessment and points to an urgent need to develop alternative analytical methods for accurate quantification of E. coli cells in biosolids (7, 10, 11, 13, 29). Such alternative methods include nucleic acid-based approaches, such as targeting DNA, quantitative PCR, or RNA reverse transcription followed by PCR (RT-PCR). However, the persistence of DNA molecules in the environment after cell death and the short turnover of RNA molecules makes these techniques unreliable to discriminate between dead cells and viable cells.

Using impermeable nucleic acid binding dyes before extraction and qPCR has been an important tool to accurately differentiate between dead and viable cells. Nogva et al. (25) and
Nocker et al. (22) described EMA PCR and PMA PCR, respectively, as discriminating between viable cells and dead cells. It has been shown that PMA is excluded by live cells more effectively than EMA (22). A few recent studies tested nucleic acid binding dyes on environmental samples. For example, Nocker et al. (23) evaluated PMA-modified PCR to differentiate between live and dead pure culture cells spiked into wastewater treatment plant effluent and water sediments. Wagner et al. (38) tested the effectiveness of EMA and PMA in excluding extracellular DNA from the effluent of a thermophilic anaerobic fermentor treating wastewater. Bae and Wuertz (3) developed a similar PMA-modified PCR protocol to differentiate between live and dead Bacteroidales cells in human feces as well as in the influent and effluent of wastewater treatment plants. These studies have all concluded that PMA-modified PCR is promising to a large extent for quantifying the live portion of cells. However, the authors of the studies noted above also pointed out the important challenges remaining in the application of PMA to environmental samples with highly complex chemical and biological matrices. Such challenges include inhibition of dye penetration into membrane-compromised cells and/or cross-linkage of PMA to double-stranded DNA and the presence of PCR-inhibiting constitutions in environmental samples. The high solids contents of biosolids, ranging from 2% by mass in digested biosolids to 25% in dewatered biosolids, would be expected to magnify such challenges. For example, color and particulate matter associated with biosolids samples may reduce radiation of light in the medium and lead to inhibition of the cross-linking of the PMA molecules to DNA in the cells.

**FIG. 2.** Effect of different solids concentrations (TSS) of the biosolids on PMA-modified qPCR efficiency. (A) uidA gene copies of extracts of biosolids spiked with $4.8 \times 10^8$ dead cells measured by qPCR with or without PMA treatment. (B) $C_T$ values of PMA-treated samples spiked with the pGEM-T uidA plasmid as an exogenous control.
In this study, the applicability and effectiveness of our PMA conditions were first investigated on defined mixtures of live and dead pure *E. coli* ATCC 25922 cultures, before the protocol was applied to complex biosolids samples. We showed that increasing the final PMA concentration to 100 μM, incubation in the dark for 10 min, and exposing the samples to light for 5 min for cross-linking of PMA molecules were effective in eliminating PCR amplification of genomic DNA from dead cells (Fig. 1). One possible explanation for the failure to inhibit the PCR signals completely from the DNA from dead cells is that the target gene might have been too short. It is possible that the amplicon’s being too short prevented complete cleavage by PMA and the non-cross-linked DNA was amplified.

Soejima et al. (34) indicated that EMA could not completely inhibit DNA amplification when the target DNA was short (113 bp), while PCR amplification of long DNA (894 bp in the 23S rRNA gene) was suppressed by EMA more than that of short DNA (113 bp in the *hly* gene). Also, Luo et al. (17) recently showed that PMA treatment cannot efficiently suppress dead cells from PCR amplification when the targeted gene is as short as 190 bp. To increase the sensitivity of the detection limit of qPCR for viable cell quantification, longer amplicons may be designed for future studies. Nevertheless, a 1-log decrease in gene copy numbers as a result of a 10% decrease in the proportion of viable bacteria in the defined mixtures showed the sensitivity of the treatment (Fig. 1).

Successful application of PMA treatment to a biosolids sample with 2,000 mg · liter⁻¹ TSS content without PCR inhibition is reported for the first time in this study (Fig. 2A and B). Considering that typical TSS in dewatered biosolids might be around 20 to 25% by weight, the findings indicate that a 10⁻² dilution would be sufficient for successful application of PMA-modified PCR to actual field samples.

In conclusion, this study has shown that qPCR with PMA can accurately quantify viable *E. coli* cells with no interference caused by the presence of dead cells and sample matrix in biosolids samples with a TSS concentration of 2,000 mg · liter⁻¹ or less. This approach, combined with a standard culture-based method, might quantitatively assess the number of cells that are alive but cannot be cultured. When we compared uidA gene copy numbers revealed by qPCR in *E. coli* cells and numbers of CFU, the cell density quantified as CFU and the uidA copy numbers were nearly identical when pure culture *E. coli* cells growing at late log stage were used (data not shown). Our findings is supported by a database search using GenBank. The database search, covering 23 different strains of *E. coli* with complete genome sequencing, showed that these *E. coli* strains contain a single copy of the uidA gene. Although the actual relationship between the copy number of a particular gene and CFU depends on the growth stage of the cells, our findings suggest that an accurate correlation of gene copy number to CFU is possible. In addition, several previous studies have developed that correlation for different matrices (12, 18, 24).

Oliver et al. (26) emphasized the need for a program of cross-validation of techniques before a complete revamp. It is likely that molecular techniques such as qPCR will gain an industry standard and revolutionize sample analysis times. However, cross-comparison of the results of traditional approaches (i.e., CFU) and units of DNA (equivalent to a total count) of appropriate indicators would offer more comprehensive, trusted, and validated data. The recent emergence of cross-validation literature has a strong potential to change future detection and quantification approaches (26). Thus, PMA-modified qPCR combined with culturing-based methods has a strong potential to enhance our understanding of pathogen behavior in biosolids, as well as a wide range of applications in other fields, including microbial source tracking, food safety, and medicine. More applications of PMA treatment on complex environmental samples will help us to overcome the limitations of the technique and validate the method further.

ACKNOWLEDGMENTS

Bilgin Taskin was partially supported by the Faculty Development Program (OPY) of the Middle East Technical University (grant BAP-08-11-DPT-2002 K120510). We are also grateful for the support from TUBITAK (The Scientific and Technological Research Council of Turkey), namely, the BIDEB 2211 National Doctoral Scholarship Program. Partial support for the project was provided by the Villanova Center for the Advancement of Sustainability in Engineering (VCASE) and The Edward A. Daylor Chair in Environmental Engineering.

REFERENCES


