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Optimization, validation, and application of a real-time PCR protocol for quantification of viable bacterial cells in municipal sewage sludge and biosolids using reporter genes and *Escherichia coli*

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Abstract Biosolids result from treatment of sewage sludge to meet jurisdictional standards, including pathogen reduction. Once government regulations are met, materials can be applied to agricultural lands. Culture-based methods are used to enumerate pathogen indicator microorganisms but may underestimate cell densities, which is partly due to bacteria existing in a viable but non-culturable physiological state. Viable indicators can also be quantified by real-time polymerase chain reaction (qPCR) used with propidium monoazide (PMA), a dye that inhibits amplification of DNA found extracellularly or in dead cells. The objectives of this study were to test an optimized PMA-qPCR method for viable pathogen detection in wastewater solids and to validate it by comparing results to data obtained by conventional plating. Reporter genes from genetically marked *Pseudomonas* sp. UG14Lr and *Agrobacterium tumefaciens* 542 cells were spiked into samples of primary sludge, and anaerobically digested and Lystek-treated biosolids as cell-free DNA, dead cells, viable cells, and mixtures of live and dead cells, followed by DNA extraction with and without PMA, and qPCR. The protocol was then used for *Escherichia coli* quantification in the three matrices, and results compared to plate counts. PMA-qPCR selectively detected viable cells, while inhibiting signals from cell-free DNA and DNA found in membrane-compromised cells. PMA-qPCR detected 0.5–1 log unit more viable *E. coli* cells in both primary solids and dewatered biosolids than plate counts. No viable *E. coli* was found in Lystek-treated biosolids. These data suggest PMA-qPCR may more accurately estimate pathogen cell numbers than traditional culture methods.

Keywords Biosolids · *Escherichia coli* · Propidium monoazide · Real-time PCR · Reporter gene · Viable pathogen

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Introduction

The solid, nutrient-rich, organic material remaining after wastewater treatment is called sludge [17]. Processes used to reduce organic matter and ammonia levels in wastewater during treatment tend to reduce the pathogen content in the treated wastewater but may serve to increase pathogen content in the remaining sludge [9]. Several sludge treatments such as aerobic or mesophilic anaerobic biological digestion can be used to digest and stabilize organic matter and to reduce the pathogen content in sewage sludge. In some cases, further treatment is done by heat-drying, composting, or addition of hydrated lime (calcium hydroxide) to increase pH [9]. The City of Guelph, Ontario, Canada, employs the "Lystek" process for further treatment of digested sludge. Briefly, this involves heating the material to 70 °C and raising the pH to 10 by adding KOH. The product is a stable high-solid and low-viscosity liquid that is rich in nutrients [24]. Once treated sludge passes

government regulations, including pathogen-reduction standards, it is designated "biosolids" and can be applied to agricultural lands according to nutrient management regulations [7].

When biosolids are used as fertilizer, there may be potential health risks for people who are exposed to any remaining pathogens, either directly (e.g., by ingesting pathogens via contaminated hands) or indirectly (e.g., by ingesting contaminated food crops grown in the amended soil) [2]. In Canada, there is some federal oversight and guidance on biosolids management [4, 5] but provincial governments, and in some cases municipalities, regulate the treatment and quality of biosolids, including setting numerical standards to control the levels of metals and pathogens in materials that are land-applied [4]. For example, in Ontario, Canada, the General Nutrient Management Regulation (Ontario Regulation 267/03, as amended from time to time) under the Nutrient Management Act 2002 regulates land-applied biosolids quality, which includes *Escherichia coli* testing standards as an indicator for pathogen reduction during treatment [18].

The current technique used to determine whether these standards are met is based on enumeration of *E. coli*, and this is typically done by plate counts, such as membrane filtration of the biosolids samples followed by culturing on media containing 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid cyclohexylammonium salt (BCIG) [13]. β-D-Glucuronidase, produced by *E. coli*, hydrolyzes BCIG, resulting in the formation of blue *E. coli* colonies [12]. Culturing indicator bacterial species on selective media is traditionally used for surrogate pathogen detection in biosolids. One problem with culture-based techniques is that indicators, like *E. coli*, and some human bacterial pathogens (e.g., *Campylobacter jejuni*, *Helicobacter pylori*, *Listeria monocytogenes*, *Salmonella typhimurium*, among others) may enter a "viable but non-culturable" (VBNC) physiological state in which they are living but cannot be easily grown on media [19, 21]. The VBNC state can be induced by stressful conditions such as fluctuating temperatures and oxygen levels during wastewater treatment [20] and leads to an under-estimate of viable pathogen numbers in samples.

Molecular tools, such as real-time PCR (qPCR), could eliminate the VBNC challenge, but may overestimate living cell densities due to amplification of DNA from non-viable cells and extracellular DNA persisting in the environment [11, 23]. Propidium monoazide (PMA) covalently binds to DNA bases every 4–5 nucleotides upon exposure to light, forming a carbon–nitrogen bond that inhibits further PCR amplification [15]. It is excluded from cells with intact cytoplasmic membranes, thus inactivating extracellular DNA or DNA contained in dead cells and allowing the PCR amplification of DNA from only the viable cells present in the sample.

PMA-qPCR has been used for enumeration of viable pathogens in environmental samples [1, 16, 27], but has never been validated using samples spiked with a reporter gene. For a review on the use of PMA-qPCR for viable cell detection in environmental matrices, see van Frankenhuyzen et al. [26]. Spiking with a unique gene marker would enable novel studies in complex matrices, such as optimization of the PMA-qPCR protocol in biosolids, and experimentation with the DNA-binding ability of, and cell permeability to, PMA.

The objectives of this research were to design and optimize a PMA-qPCR method in biosolids to selectively amplify DNA from viable cells, to systematically test the optimized protocol using unique gene markers, and to apply the optimized PMA-qPCR method to enumerate naturally occurring *E. coli* cells in three sewage sludge matrices and compare it to the traditional viable plate counting technique. To design the optimized protocol, samples of sewage sludge matrices, including biosolids, were spiked with genomic *Pseudomonas* UG14Lr DNA (containing *luxAB*), dead UG14Lr cells, live UG14Lr cells, and a mixture of live UG14Lr and dead *Agrobacterium tumefaciens* strain 542 cells (containing *gfp*), followed by extraction with and without PMA, and qPCR analysis. The *luxAB* and *gfp* genes are unique DNA sequences not naturally found in sewage sludge matrices, thus allowing for specific detection of this DNA in spiking experiments. To validate the protocol, *E. coli* cells were enumerated by plating and PMA-qPCR in each of the three sewage sludge matrices.

Materials and methods

Municipal sewage sludge collection, processing, and storage

Three municipal sewage sludge matrices were used in this research, primary (untreated) solids, dewatered mesophilic anaerobically digested biosolids, and Lystek-treated biosolids, collected from the City of Guelph wastewater treatment plant (WWTP). Primary solids refers to the raw sludge solids collected from the settling basins prior to digestion and stabilization (3–6 % total solids on a dry weight (dw) basis). Dewatered biosolids were produced by digestion for 14–15 days at 36 °C in a single-phase mesophilic anaerobic digester followed by mechanical dewatering on a belt filter press to 24 % total solids (dw). Lystek-treated biosolids were dewatered biosolids that were further treated with KOH at 70 °C to further reduce

pathogen numbers, producing a low viscosity material (15–17 % solids dw) [24]. Samples were collected periodically during the study in sterile jars or bags, and stored at 4 °C until use. Dry weights were determined in triplicate by drying 1.5-g wet weight samples at 105 °C for 48 h.

Bacterial cultures

Cells with unique reporter genes absent from sewage sludge matrices were used for spiking experiments. *Agrobacterium tumefaciens* strain 542 (kindly provided by Dr. C. Hall, University of Guelph) carrying the *gfp* gene on a plasmid [8] and *Pseudomonas* sp. UG14Lr, a derivative of UG14 [22], which was modified to contain the *luxAB* genes from *Vibrio harveyi* [29], were used as test microorganisms. *Pseudomonas* sp. UG14Lr and *A. tumefaciens* strain 542 were grown overnight in UG14 medium [22] and LB, respectively, at 28 °C with shaking at 180 rpm. When experiments required spiking with *E. coli* cells, a laboratory strain (ATCC 11775) was grown at 37 °C in LB broth with shaking at 180 rpm (duration of growth depended on intended use).

When dead cells from a culture were required, a mixture of 5 µl of toluene and 5 µl of 95 % (v/v) ethanol was added to 10^8 cells suspended in 500 µl of 0.85 % (w/v) NaCl. After 3 min of continuous mixing by inversion, the tubes were centrifuged (7 min at 6,000 × *g*) and the supernatant decanted. Cells were resuspended in 0.85 % (w/v) NaCl. No bacterial colonies were observed when toluene/ethanol-treated cell samples were spread on UG14 medium (UG14Lr cells) or LB (*A. tumefaciens* and *E. coli* cells) agar plates.

DNA extraction

DNA extraction from living and dead UG14Lr, *A. tumefaciens*, and *E. coli* cells in sewage sludge matrices was performed using a PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) as per the manufacturer's instructions, with the exception of a 5-min centrifugation after the 10-min vortex, and a 3-min spin (to dry the spin filter membrane) after decanting the C5 (wash) solution. DNA extraction from sewage sludge matrices spiked with genomic DNA was done using the same protocol, amended further by adding 200 µl of bead solution and omitting beads and C1 (lysis) solution and all vortexing. For all mixing steps, samples were inverted ten times instead of vortexing. For all DNA extractions from pure culture, a DNeasy Blood and Tissue Kit (QIAGEN, Germantown, MD, USA) was used as per the manufacturer's instructions.

Real-time PCR

Two oligonucleotide primers ("*lux*F" 5′-AGGTGGTGCT CCTGTTTATGTC-3′ and "*lux*R" 5′-CTCGTGAGTGTT-GATGATCCAG-3′) were designed to target a 106-bp section spanning part of both the *luxA* and *luxB* genes. A temperature gradient curve was completed from 55 to 65 °C to determine the optimal annealing temperature (62 °C). To amplify a 96-bp fragment of the *gfp* gene, two primers were designed ("*gfp*F" 5′-GCTCGCCGACCACT ACCAGC-3′ and "*gfp*R" 5′-TTGCTCAGGGCGGACTGG GT-3′) and run in a temperature gradient curve from 62 to 67 °C to determine the optimal annealing temperature (67 °C). Primers used for amplification of an 82-bp section of the glucuronidase gene (*uidA)* were "*uid*AF" 5′-GTGTG ATATCTACCCGCTTCGC-3′ and "*uid*AR" 5′-AGAACG GTTTGTGGTTAATCAGGA-3′ [6]. A temperature gradient curve was run from 60 to 67 °C using primer concentrations of 300 and 500 nM to determine the optimal annealing temperature and primer concentration. With each of the primer sets a melt curve was generated to illustrate primer specificity. For all experiments, two-step qPCR assays were conducted with an initial 3-min denaturation at 95 °C, followed by 40 cycles of 95 °C for 15 s and 30 s at the determined annealing temperature. A 25-µl assay volume was used with iQ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Standard curves were completed in triplicate for each qPCR assay. Two template-free controls were also included in each assay to ensure reagents were not contaminated. Template DNA from samples was assayed in triplicate. When qPCR results of sub-samples averaged less than three DNA copies, the sample was considered free of the target DNA as per the minimum information for publication of quantitative real-time PCR experiments guidelines [3]. Threshold values were calculated by the thermocycler (Bio-Rad, IQ5).

Preliminary tests to evaluate ways of reducing inhibition of the PCR assays by matrix components indicated bovine serum albumin (BSA) to be an effective agent. Thus, in subsequent experiments 1 µl of 10 mg/ml BSA was added to each PCR well.

PMA protocol optimization

PMA (phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride; Biotium Inc., Hayward, CA, USA) was stored as a 20 mM stock solution (1 mg PMA dissolved in 97.8 µl 20 % (v/v) dimethyl sulfoxide) at −20 °C, as per Nocker et al. [15]. To determine an appropriate PMA concentration, sample turbidity, and light exposure time to use, different amounts of PMA (final concentrations 100, 150, and 200 µM) were each added to 2-ml microcentrifuge tubes (Fisher Scientific Co.,

Ottawa, ON) containing 20, 100, and 40 mg (dw) primary solids, dewatered biosolids, and Lystek-treated biosolids, respectively, and matrix diluted tenfold with water, corresponding to a total of 2, 10, and 4 mg (dw) of primary solids, dewatered biosolids, and Lystek-treated biosolids, respectively. Each tube was spiked with about 10^5 or 10^7 copies of genomic UG14Lr DNA. After incubation in the dark for 5 min, the mixture was placed 15 cm from a broad-spectrum 500-W halogen lamp and mixed by frequent inversion for 10 or 20 min, followed by DNA extraction and amplification. To assess if PMA was entering viable cells, about 10^8 UG14Lr cells were added to saline solution (no wastewater material) and mixed with different amounts of PMA added to final concentrations of 50, 100, 150, 200, 300, 400, 500, and 600 μM. Solutions were exposed to light for 5, 10, or 20 min. Positive controls (containing cells but no PMA) were set up for each light exposure time. DNA was then extracted from each sample for qPCR analysis.

Optimal parameters were found to be a 200 μM PMA concentration with 20 min of light, using solids diluted tenfold with water, corresponding to a total of 2, 10, and 4 mg (dw) primary solids, dewatered biosolids, and Lystek-treated biosolids, respectively. These parameters were used for the following experiments. Extracted DNA was assayed in triplicate by qPCR using *luxAB* and *gfp* primers for UG14Lr and *A. tumefaciens* DNA, respectively. Percent of DNA inhibited from amplification was calculated by dividing the amount of amplifiable DNA in each PMA-treated sample by the amplifiable amount in the control, multiplying by 100, and subtracting from 100 %.

Experiments using unique reporter genes for PMA-qPCR protocol validation

To assess the effectiveness of PMA at inactivating cell-free DNA, about 10^5 copies of UG14Lr genomic DNA were spiked into triplicates of each matrix diluted tenfold with water, and mixed with PMA. In parallel, triplicates of each matrix dilution were used without PMA to serve as PMA-free controls.

A similar experiment was conducted to assess the ability of PMA to inhibit amplification of DNA inside membrane-compromised cells. About 10^6 toluene/ethanol-treated UG14Lr cells were spiked individually into two tubes of each matrix diluted tenfold with water, and mixed with PMA. Two tubes of each matrix dilution were used without PMA to serve as PMA-free controls. To check if PMA was effective on the DNA of other microorganisms used in this study, three broth culture samples of dead *A. tumefaciens* and dead *E. coli* (about 10^8 cells each), were also treated with PMA, while a second set of samples was left untreated.

To show that PMA does not affect viable cells, about 10^8 UG14Lr cells in exponential growth phase were spiked into triplicates of each matrix diluted tenfold with water, and mixed with PMA. Triplicates of each matrix dilution were left without PMA to serve as PMA-free controls. Six pure culture samples (three with PMA and three without) were also used to assess the effect of PMA on viable cells without sludge matrix.

To test the ability of PMA to selectively inactivate DNA from dead cells, extractions were performed on samples of each matrix spiked with both live UG14Lr cells and dead *A. tumefaciens* cells with and without PMA. Live and dead *A. tumefaciens* cells were plated on TSA to ascertain cell death. About 10^5 viable UG14Lr cells and 10^5 dead *A. tumefaciens* cells were spiked into triplicates of each matrix diluted tenfold with water, and mixed with PMA. Triplicates of each matrix dilution were used without PMA to serve as PMA-free controls. Extracted DNA was assayed by qPCR using *luxAB* primers first, and *gfp* primers second.

Experiments with *E. coli* cells for PMA-qPCR method application and comparison with plating technique

Bacterial plating method

The Ontario Ministry of the Environment (MOE) method E3433 was used as a guideline for isolation, detection, and enumeration of *E. coli* and content of the media and buffers used in these experiments, with some modifications [13]. 1 ± 0.25 g (ww) was added to 10 ml of a phosphate buffer each contained in 25-ml falcon tubes, which were shaken at 180 rpm for 10 min. Samples were left to settle for 2 min. Following this, 100 μl of the matrix-buffer was serially diluted and 50–100 μl of material were plated on mFC-BCIG agar (dilutions plated depended on matrix). Plates were incubated at 44.5 °C for 24 h as per MOE method E3433 and blue colonies were enumerated.

Comparing results from plate counts, PMA-qPCR, and qPCR in primary solids, dewatered biosolids, and Lystek-treated biosolids

Fresh primary solids were collected during three sampling events (referred to as "trial 1, 2, and 3") and used to compare results from traditional plating, the PMA-qPCR protocol, and qPCR. Ten DNA extractions were performed immediately after collection, after incubation of the solids at 37 °C with shaking at 180 rpm for 24 h, and after 48 h. Each time, 610 mg (ww) of matrix were weighed into five 2-ml tubes, mixed with 1 ml water, and used for subsequent tenfold solids dilution (using water) and extraction. PMA was used in five of the samples, while the other five served as no-PMA controls. qPCR using *uidA* primers was

performed in triplicate on each extract. In addition to DNA extraction, 100 µl of the matrix was serially diluted and 50–100 µl of the 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions were plated on mFC-BCIG agar for *E. coli* cell quantification using the traditional plating method.

The above experiment was repeated using fresh dewatered biosolids collected during two sampling events. Eight and six DNA extractions were performed after sampling events 1 and 2, respectively, without sample incubation. An amount of 407 mg (ww) of material was mixed with 1 ml of water and used for subsequent tenfold solids dilution with water; 100 µl of matrix was serially diluted, and the 10^{-2}, 10^{-3}, and 10^{-4} dilutions were plated.

The above experiment was repeated using Lystek-treated biosolids with the following amendments: pH was brought to about 7 with HCl prior to sample incubation, and 270 mg (ww) were weighed into five tubes after each sampling event, mixed with 1 ml of water, and used for subsequent tenfold solids dilution with water, and extraction; 100 µl of matrix was plated directly.

Comparing theoretical and actual detection limit of E. coli in Lystek-treated biosolids using qPCR

This experiment was conducted to see if the detection limit for the PMA-qPCR protocol was above the Ontario CP 2 pathogen level standard [fewer than two million *E. coli* cells per 1 g (dw)]. In the 0.93 mg (dw) of biosolids samples extracted using this protocol, the detection limit must be 1,861 cells, and about 18 copies would be detected per 1 µl DNA template used in each PCR assay. To determine if this level was detectable, six 270-mg (ww) samples of a stored Lystek-treated biosolids sample were mixed with 1 ml of water and 25 µl of this was transferred to six tubes and spiked with 100 µl of about 1,800 *E. coli* cells. 0.85 % (w/v) NaCl was added to a final volume of 250 µl. As well, two Lystek-treated biosolids samples were subjected to DNA extraction without the addition of *E. coli*, and two DNA extractions were performed on 0.85 % (w/v) NaCl spiked with *E. coli* but lacking sewage sludge matrix. DNA extractions were performed, followed by qPCR using *uidA* primers. Each DNA extract was assayed in triplicate. Three spiked Lystek-treated biosolids were plated on mFC-BCIG agar, as well as two samples of the spiking solution. Plates were incubated at 44.5 °C for 24 h and blue colonies were counted. Lystek-treated biosolids were used for this experiment because they were nearly devoid of background *E. coli* cells.

Statistical analyses

All statistical analyses were done using SAS for Windows 9.2 (SAS Institute, Cary, NC, USA) as a two factorial complete randomized design (CRD) analysis of variance (ANOVA), with the factors being (1) matrix (primary solids, dewatered biosolids, Lystek-treated biosolids), and (2) treatment (PMA, control). Variances were partitioned into treatment, matrix, a treatment-matrix interaction, and error. Where interactions were significant at $\alpha = 0.05$, contrast statements were used to determine significant differences between controls and treatments within each matrix. Where interactions were insignificant, data were pooled across matrices to determine if significant difference existed between treatment and control.

Calculating detection limits

For qPCR assays and plate counts, detection limits were calculated by determining the amount of *E. coli* DNA required to be in the sample extracted or cells plated in order to be detectable, and back-calculating how much would therefore need to be in 1 g of the sewage sludge sample (dw). Factors taken into consideration for back-calculations included dry weight extracted or plated, dilutions carried out (via qPCR or for plating), and lowest detectable amount (three DNA copies per 1 µl template DNA for qPCR, 1 CFU/ml for plating).

Results and discussion

Protocol optimization

The purpose of this research was to optimize and validate a protocol for selective amplification of DNA from viable cells in sewage sludge matrices using unique reporter genes and to compare it to the traditional plating technique for *E. coli* enumeration. The first step was optimization of PMA concentration, light exposure time, and sample turbidity (diluted vs. undiluted matrices).

Undiluted matrices spiked with 10^7 genomic UG14Lr DNA copies and treated with 200 µM PMA resulted in amplifiable DNA near equal in quantity to PMA-free controls. In other words, in undiluted matrices, PMA showed no binding of cell-free DNA. When solids concentrations were diluted tenfold, PMA was effective at binding cell-free DNA. Regardless of the PMA concentration (100, 150, or 200 µM) and light exposure time (10 or 20 min), over 99 % of the UG14Lr DNA was inhibited from amplification in tenfold diluted matrices (equivalent to 2, 10, and 4 mg (dw), for primary solids, dewatered biosolids, and Lystek-treated biosolids, respectively). Using a higher PMA concentration did not consistently result in lower amounts of amplifiable DNA. Regardless of sludge matrix, when 10^7 genomic UG14Lr DNA copies spiked into dilutions of sludge matrices were treated with

PMA (200 μM), about 1,000 copies remained amplifiable, whereas the spiking of about 10^5 copies resulted in no amplification. Wagner et al. [28] found PMA to be ineffective at binding extracellular DNA in sewage sludge matrices, and suggested the turbidity of biosolids prevented photoactivated PMA from binding to DNA.

When intact UG14Lr cells from exponential growth phase cultures were treated with varying PMA concentrations (50–200 μM) and different light exposure times (5, 10, 20 min) a similar amount of amplification was seen as with controls (containing cells but no PMA), regardless of PMA concentration and light exposure time. When viable cells were subjected to 300–600 μM PMA and 20 min of light, treatments still showed the same amount of amplifiable DNA as controls. Results showed that PMA did not react with DNA in the live intact cells (from exponential growth phase), even at 600 μM.

A PMA concentration of 200 μM was used for subsequent experiments because: (1) UG14Lr cytoplasmic membranes were shown to be impermeable up to at least 600 μM; (2) 200 μM is fourfold higher than the amount used for PMA experiments in pure culture by Nocker et al. [15]; the higher concentration may compensate for the suspended solids that interfered with light penetration (and therefore PMA-DNA binding); (3) other studies involving PMA and sludge matrices have used between 100 and 300 μM PMA [1, 27]. Though Varma et al. [27] reported some DNA loss from viable cells after treatment with 100 μM PMA in sludge matrices, this was hypothesized to be from a subpopulation of dead stationary phase cells. Since viable cells were not affected by PMA after a 20-min light exposure, this duration was used for subsequent experiments to allow for maximum mixing of the sample.

Testing the optimized protocol using reporter genes

This study was novel in that known quantities of cells and DNA containing unique gene markers were spiked into environmental samples to validate PMA-qPCR as a method for selective amplification of viable cells, while other studies have used in situ microorganisms or spiked using microorganisms indigenous to the matrices [1, 16, 27, 28]. Using unique gene markers meant that all signals obtained in PCR assays from matrix DNA extracts could be attributed to the spiked cells/DNA, enabling unambiguous comparison between results from PMA-treated samples and PMA-free controls.

Spiking with genomic UG14Lr DNA

Using the optimal PMA-qPCR protocol (i.e., 200 μM PMA and a light exposure time of 20 min), 10^5 copies of extracellular UG14Lr DNA added to each of the three tenfold diluted matrices were inhibited from amplification to amounts below the qPCR detection limit (Table 1). Samples of each matrix that served as no-PMA controls showed amplification of spiked UG14Lr DNA. Within each matrix, all differences between the amount of DNA amplified in controls and that amplified in samples containing PMA were statistically significant ($\alpha = 0.05$, $p < 0.0001$). The amount of DNA recovered varied depending on the matrix, but up to 2.5×10^5

Spiking with dead UG14Lr cells

To investigate the effect of PMA on detection of DNA from dead cells, UG14Lr cells were killed by a toluene/ethanol treatment, which resulted in cells with compromised membranes. No colonies were observed on TSA plates spread with toluene-treated UG14Lr cells, suggesting this method had a high killing efficiency. In a study using *Pseudomonas stutzeri* AG259, Van Dyke et al. [25] suggested that toluene did not completely destroy membrane integrity after finding that bioaccumulation of germanium was not affected by treating cells with toluene.

In the no-PMA added controls, qPCR detected up to 10^6 dead cells, while samples exposed to PMA and light did not give a detectable signal by qPCR (Table 1). Statistically significant differences were found between amounts of amplifiable DNA extracted from controls and treatments within each matrix ($\alpha = 0.05$, $p < 0.0001$). In addition, samples of dead *A. tumefaciens* and *E. coli* cells subjected to PMA treatment contained signals below detection limits following qPCR, whereas DNA in controls was detected at expected amounts (data not shown). These results supported the claim that PMA entered cells with compromised membranes and efficiently inhibited DNA amplification.

Spiking with viable UG14Lr cells

After spiking each matrix with 10^8 viable UG14Lr cells, DNA ranging from log 6.25 to log 6.97 cells was recovered, regardless of whether the sample was treated with PMA. There were no statistically significant differences between the average number of viable UG14Lr cells extracted from each matrix (and pure culture) with and without the addition of 200 μM PMA, as determined by qPCR ($\alpha = 0.05$, $p = 0.8665$). This illustrated that 200 μM of PMA did not affect viable cell detection, and therefore should not lead to false-negative results when used to enumerate viable cells.

Optimal quantity of matrix used for DNA extraction

For the remaining experiments, instead of 1,000-μl samples, a 250-μl sample of tenfold diluted solids was

Table 1 Mean amplifiable UG14Lr DNA extracted from sludge matrices spiked with cell-free UG14Lr DNA or dead UG14Lr cells, with and without propidium monoazide (PMA) treatment

Matrix	PMA treatment or control	Mean amplifiable DNA (log_{10} no. copies/1 mL extraction) spiked into sludge matrices[a]		
		Cell-free UG14Lr DNA ($n = 3$)	Trial 1: dead UG14Lr cells ($n = 2$)	Trial 2: dead UG14Lr cells ($n = 2$)
Primary solids	Control	4.04 ± 0.31[b]	3.74 ± 0.47	3.50 ± 0.38
	PMA added	BDL[c]	BDL	BDL
Dewatered biosolids	Control	4.58 ± 0.12	5.44 ± 0.18	5.62 ± 0.74
	PMA added	BDL	BDL	BDL
Lystek-treated biosolids	Control	5.32 ± 0.07	4.21 ± 0.41	5.11 ± 0.29
	PMA added	BDL	BDL	BDL

[a] 10^5 cell-free UG14Lr DNA copies or 10^6 dead UG14Lr cells were spiked into 2, 10, and 4 mg (dw) of primary solids, and dewatered and Lystek-treated biosolids, respectively. DNA from each treatment and control was assayed by qPCR in triplicate and DNA quantity averaged across the three sub-samples

[b] The data are presented as mean ± SD

[c] *BDL* below detection limits (i.e., <300 target DNA copies in an extract of 1 mL). All values that were BDL were significantly different from amounts found in control samples ($\alpha = 0.05$, $p < 0.0001$)

extracted per PCR reaction because preliminary experimentation showed extractions to be more efficient for experiments using 250 µl of a 1/10 solids concentration of sludge matrix than for experiments where 1,000 µl of a 1/10 solids concentration was used. When 250-µl sample volumes were used, more DNA was extracted from controls, and replicates of controls were also less variable (e.g., SD = 0.44, 0.60, 1.46 for primary solids, dewatered biosolids, and Lystek-treated biosolids, respectively, for 1,000 µl, vs. SD = 0.06, 0.02, 0.07 for primary solids, dewatered biosolids, and Lystek-treated biosolids, respectively, with 250-µl samples; $n = 3$ for all experiments). It was hypothesized that the smaller quantity resulted in higher extraction efficiencies because a smaller matrix quantity would have fewer humic acids and cations to bind DNA and inhibit its extraction. Higher recovery and consistency between results reduced ambiguity in results showing the selectivity of PMA in inactivating DNA from dead cells only. Using 250-µl samples resulted in the extraction of 0.5, 2.5, and 1 mg (dw) primary solids, and dewatered and Lystek-treated biosolids, respectively. The previously described experiments where samples were spiked with viable cells, dead cells, and extracellular DNA involved 1,000-µl samples, whereas the following remaining experiments were done with 250-µl of each sample.

Spiking with a mix of live UG14Lr and dead *A. tumefaciens* cells

To test the ability of PMA to allow selective amplification of DNA from viable cells only, a mixture of live UG14Lr and dead *A. tumefaciens* cells were spiked into the sludge matrices and DNA was extracted following PMA treatment. There were no statistically significant differences between the amount of amplifiable UG14Lr DNA extracted from samples with PMA and samples without ($\alpha = 0.05$, $p = 0.0696$), indicating the exclusion of PMA from viable cells (Table 2). In contrast, the amounts of amplifiable *A. tumefaciens* DNA extracted from samples spiked with dead cells that were treated with PMA were statistically significantly lower (up to 3.5 log units less) than those from samples without PMA treatment ($\alpha = 0.05$, $p < 0.0001$), showing the ability of PMA to selectively prevent qPCR amplification of DNA from dead cells.

Experiments involving *E. coli* for comparison of optimized PMA-qPCR and traditional plating technique

Comparison of results from plate counts and PMA-qPCR: primary solids, dewatered biosolids, and Lystek-treated biosolids

During three sampling events (trials 1, 2, and 3) primary solids were collected from the Guelph WWTP and used to compare quantitative results from plate counts, and PMA-qPCR. Samples were extracted and plated immediately, and again after 24- and 48-h incubation at 37 °C. At each time point, plate counts were lower than results from PMA-qPCR, by about 0.5–1 log unit (Fig. 1). For trials 1 and 3, differences between number of *E. coli* cells detected by plate counts and PMA-qPCR were statistically significant at 0 and 24 h ($\alpha = 0.05$, $p < 0.0001$). For trial 2, all differences were statistically significant ($\alpha = 0.05$, $p < 0.0001$).

Table 2 Average amount of amplifiable DNA from viable UG14Lr and dead *A. tumefaciens* cells extracted from sludge matrices with and without propidium monoazide (PMA)

Matrix	Genes[b]	Mean ($n = 3$) amount of amplifiable DNA ($\log_{10}$ no. copies)[a]	
		Control	PMA (200 μM)
Primary solids	*luxAB*	5.07 ± 0.07[c]	5.10 ± 0.11
	gfp	5.22 ± 0.09	3.31 ± 0.15[d]
Dewatered biosolids	*luxAB*	5.75 ± 0.06	5.78 ± 0.02
	gfp	5.39 ± 0.03	1.96 ± 1.72[d]
Lystek-treated biosolids	*luxAB*	5.16 ± 0.11	5.31 ± 0.03
	gfp	5.40 ± 0.16	1.76 ± 1.53[d]

[a] 0.5, 2.5, and 1 mg of primary solids, and dewatered and Lystek-treated biosolids were used for extractions. qPCR was performed in triplicate per extract, and DNA quantity averaged across the three sub-samples

[b] *luxAB* and *gfp* genes are found in UG14Lr and *A. tumefaciens* cells, respectively

[c] The data are presented as mean ± SD ($n = 3$)

[d] Significant difference exists between amount of DNA amplified in control and that amplified in sample containing PMA ($\alpha = 0.05$, $p < 0.0001$)

Results from qPCR and PMA-qPCR were similar, suggesting that much of the *E. coli* DNA detected was from viable cells. All data points were above the detection limits for both plate counts and qPCR. Differences were generally smaller (less than half a log unit) by 48 h of incubation.

One possible reason for this is that some cells may have been in a VBNC state when plated at 0 h and regained culturability over time at 37 °C. Several studies comparing molecular and culture techniques for bacterial enumeration in environmental samples have found qPCR to result in higher counts [10, 16, 29]. When comparing conventional culturing with qPCR to quantify *Salmonella* spp., *E. coli,* and *Clostridium perfringens* in wastewater and composted biosolids matrices, Weir et al. [29] found qPCR resulted in up to 5 log higher numbers than plate counts. The authors suggested the discrepancy in quantities may in part be due to cells in a VBNC state. Similarly, Higgins et al. [10] found indicator fecal coliform quantities detected by plate counts and qPCR to be similar prior to anaerobic thermophilic digestion of sewage sludge, but saw a 5 log unit difference after digestion, with qPCR resulting in the higher quantity. They proposed that thermophilic digestion causes the entrance of cells into a VBNC state, rendering them undetectable by plating methods.

As with the primary solids, results of *E. coli* numbers from PMA-qPCR were higher, by less than half a log unit, than plate counts in samples of dewatered biosolids (Fig. 2), although these differences were only statistically significant for analyses done with matrix collected at the first sampling event ($\alpha = 0.05$, $p < 0.0001$). Biosolids collected the second time contained an *E. coli* quantity that was at the threshold of detection using qPCR, but this detection limit was above the Ontario Ministry of the Environment regulation that stipulates there must be fewer than two million *E. coli* cells per 1 g (dw) sample in order to be land-applied.

No indigenous *E. coli* colonies grew on mFC-BCIG plates spread with Lystek-treated biosolids, even after 48 h of incubation at 37 °C. No detectable amounts of *E. coli* were found in samples treated with PMA. Prior to incubation, qPCR signal was detected in four of the five unspiked samples extracted without PMA (about 5.9–6.0 log units), but after 24 and 48 h qPCR signal was found in only one sample (5.7 and 6.2 log units after 24 and 48 h, respectively). This qPCR signal was probably from indigenous dead cells or extracellular DNA, which can persist in an environmental matrix for varying amounts of time, depending on matrix physical and chemical properties [11, 14, 23]. To ensure that negative samples were not the result of qPCR inhibition, 1 μl of extract was added to genomic UG14Lr DNA and assayed by qPCR using *luxAB* primers. Assays of UG14Lr DNA without extract (positive control) had the same amplification curves as UG14Lr with extract. These results suggested that materials in the extract did not inhibit PCR amplification.

Comparison of theoretical and actual detection limit of E. coli in Lystek-treated biosolids using qPCR

Samples of Lystek-treated biosolids were spiked with viable *E. coli* cells, followed by DNA extraction and qPCR to determine if the CP 2 pathogen level regulation of fewer than two million *E. coli* cells per 1 g (dw) was above the threshold of detection using the optimized PMA-qPCR method. If two million *E. coli* cells were in 1 g (dw) of Lystek-treated biosolids, 1,800 cells would be found in

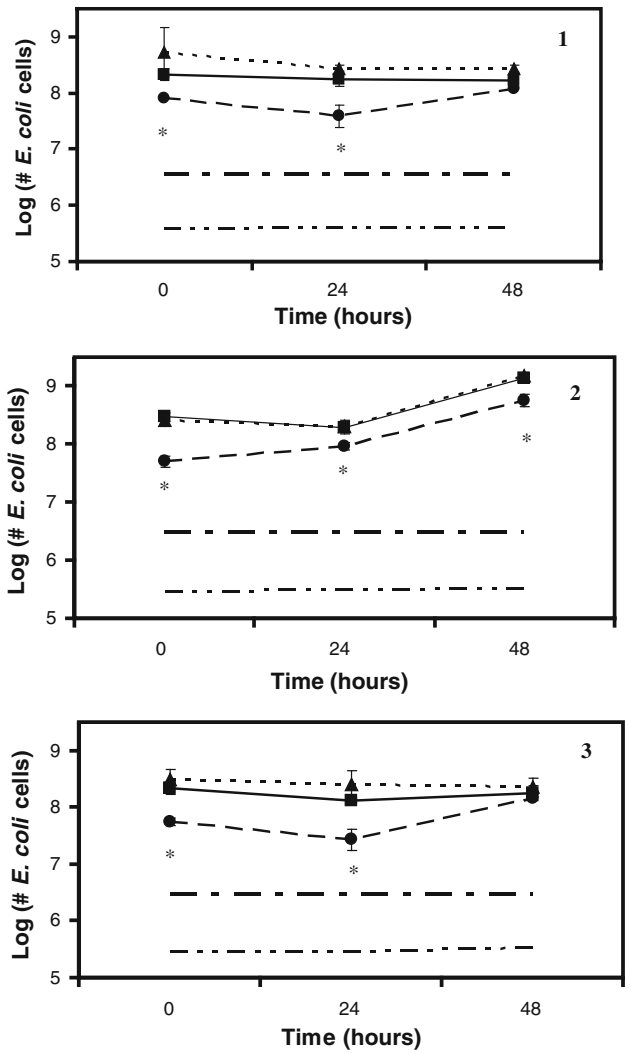

Fig. 1 Number of *E. coli* cells in 1 g (dw) primary solids during three trials (1, 2, and 3) determined by plate counts (*filled circles*), qPCR (*filled triangle*), and PMA-qPCR (*filled square*) carried out immediately after sample collection (0 h), and 24 and 48 h after incubation at 37 °C. qPCR was performed in triplicate per extract, and DNA quantity averaged across the three sub-samples. *Error bars* represent standard deviations, which were calculated using $n = 3$. Plate count detection limits (- - — - -) and qPCR detection limits (- — -) were calculated for each time point and define the minimum amount of *E. coli* required in 1 g (dw) in order to be detected. Significant differences between amount of DNA amplified and plate counts are marked with an *asterisk* ($\alpha = 0.05$, $p < 0.0001$)

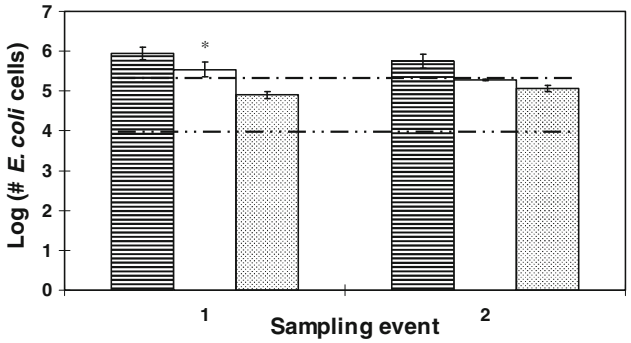

Fig. 2 Number of *E. coli* cells in 1 g (dw) dewatered biosolids using plate counts (*dotted columns*), DNA extraction and qPCR (*striped columns*), and DNA extraction with PMA followed by qPCR (*white columns*), performed immediately after each of the two sampling times (referred to as a "sampling event"). qPCR was run in triplicate per extract, and DNA quantity averaged across the three sub-samples. *Error bars* represent standard deviations, which were calculated using $n = 4$ (qPCR, sampling event 1), and $n = 3$ (plate counts, sampling event 1, and qPCR and plate counts, sampling event 2). Plate count detection limits (- - — - -) and qPCR detection limits (- — -) were calculated for each time point and define the minimum amount of *E. coli* required in 1 g (dw) in order to be detected. Significant differences between amount of DNA amplified and plate counts are marked with an *asterisk* ($\alpha = 0.05$, $p < 0.0001$)

0.9 mg (dw), which was the weight of biosolids used in the extraction. Based on plate counts from culture used for spiking, 960–1,200 cells were spiked into the sample instead of the desired 1,800. However, a detectable amount of DNA was extracted from five of the six samples of Lystek-treated biosolids samples (0.93 mg, dw) (Table 3). Plate counts yielded similar results. In one qPCR assay 2,040 *E. coli* cells were detected, possibly due to pipetting error. Though all qPCR analyses and plate counts resulted in lower *E. coli* counts than expected, this experiment did show that if 1,200 cells were in the biosolids sample being extracted, they would be detected by the optimized PMA-qPCR protocol. The necessity for replicates was also demonstrated since one sample was negative, even though the other five contained DNA in amounts that exceeded 3.3×10^5 cells in 1 g (dw). The detection limit in Lystek noted above may be inadequate for select regulatory standards, because, for example, the standard for CP 1 pathogen category biosolids in Ontario's General Nutrient Management Regulation (O. Reg. 267/03) is <1,000 *E. coli* cells per 1 g (dw), which is too small of a quantity to be detected by the current PMA-qPCR method. To increase the sensitivity of the PMA-qPCR method a greater biosolids weight would have to be extracted. This would be difficult due to the capacity of the extraction kit and potential increase in qPCR inhibitors.

Summary of key results and conclusions

PMA performance was adequate and similarly effective for all three sewage sludge matrices. When applied to *E. coli* found in the environmental matrices, PMA-qPCR consistently resulted in a higher number of viable *E. coli* cells than plate counts for both primary solids and dewatered biosolids, but not higher by more than half a log unit in dewatered biosolids. It was also shown that the PMA-qPCR method could potentially be used to monitor for regulatory

Table 3 Enumeration of *E. coli* based on plate counts and qPCR of DNA, extracted after propidium monoazide treatment, in Lystek-treated biosolids spiked with about 1,800 *E. coli* cells

Sample	Amount of *E. coli* detected (CFU or #DNA copies)[a]	Extrapolation of *E. coli* quantity in 1 g (dw) (log_{10}# DNA copies or CFU)
Lystek DNA extraction 1	0	0
Lystek DNA extraction 2	717	5.89
Lystek DNA extraction 3	2,040	6.34
Lystek DNA extraction 4	306	5.52
Lystek DNA extraction 5	725	5.89
Lystek DNA extraction 6	437	5.67
Lystek plate count 1	795	5.93
Lystek plate count 2	765	5.91
Lystek plate count 3	810	5.94
Plate count of spiking solution 1	960	6.01
Plate count of spiking solution 2	1,200	6.11

[a] Each extract was assayed in triplicate by qPCR and DNA quantity averaged across the three sub-samples

compliance (e.g., Ontario's CP 2 standard of 2 million *E. coli*/g dw), particularly in cases where a rapid result is needed. However, the detection limit was not sufficiently low to be considered for use with Ontario's CP 1 category materials (i.e., pathogen standard <1,000 *E. coli*/g dw). To further validate this method, PMA also needs to be tested on other pathogens and pathogen indicators that would specifically be of interest to monitor in biosolids, such as *Salmonella* and *Campylobacter*. Data from these studies suggest that PMA-qPCR may yield a more accurate estimation of pathogen cell numbers than traditional culture methods, and thus may be a suitable method for viable *E. coli* enumeration in biosolids intended for land application.

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