Use of real-time PCR with propidium monoazide for enumeration of viable *Escherichia coli* in anaerobic digestion

Wataru Ruike, Atsushi Higashimori, Junichi Yaguchi and Yu-you Li

**ABSTRACT**

A combination of propidium monoazide (PMA) with real-time quantitative polymerase chain reaction (PMA-qPCR) was optimized to enumerate only viable *Escherichia coli* in anaerobic digestion processes. Repeating the PMA treatment twice and a final concentration of 100 μM resulted in an effective exclusion of DNA from heat-treated *E. coli* cells. In three anaerobic digestion processes, real-time PCR, PMA-qPCR, and the most probable number method (MPN) were used to estimate the numbers of total, viable, and culturable *E. coli* cells, respectively. Culturable concentrations of fecal coliforms were also measured by the membrane filter method. For thermophilic digestion, the reductions in total and viable *E. coli* cells from the digester influent to the effluent were significantly lower than those in culturable cells and fecal coliforms by two to four orders of magnitude. For mesophilic digestion, the differences in the reductions in *E. coli* and fecal coliforms counts were less than two orders of magnitude. Based on the measurements of viable *E. coli* determined by the PMA-qPCR method, the microbial quality of digester effluents was discussed for agricultural application, and pasteurization after anaerobic digestion was suggested for the destruction of viable pathogens.

**Key words** | anaerobic digestion, DNA, *Escherichia coli*, propidium monoazide, viable but nonculturable

**INTRODUCTION**

For centuries, anaerobic digestion has been applied and developed to stabilize municipal organic waste, manure from livestock, and various industrial wastes. Methane produced by anaerobic digestion can replace fossil fuel sources and reduce greenhouse gas emissions. Furthermore, anaerobic digestion has long been known to reduce pathogens and has a beneficial use as ‘biosolids’ for land application. In the 21st century, the number of anaerobic digestion facilities in Japan has increased to approximately 600.

However, recent researchers have reported a sudden increase in *Escherichia coli* or fecal coliform concentrations in anaerobically digested sludges immediately after dewatering (Qi *et al.* 2007; Higgins *et al.* 2007; Chen *et al.* 2011). Storing these sludges results in a further increase in bacterial concentrations (Higgins *et al.* 2007; Qi *et al.* 2008), which poses a potential human health hazard after land application (Sidhu & Toze 2009). It was hypothesized that indicator bacteria enter a viable but nonculturable (VBNC) state during anaerobic digestion and regain their culturability after dewatering and storage (Higgins *et al.* 2007). A number of bacteria, including some pathogens and enteric bacteria, were reported to enter a VBNC state under stressful conditions (Xu & Colwell 1982; Colwell *et al.* 1985; Roszak & Colwell 1987), and some evidence suggested that they retained their virulence in the VBNC state (Rahman *et al.* 1996; Kahla-Nakbi *et al.* 2007). In addition, the reactivation and regrowth of VBNC bacteria were frequently observed when suitable conditions were provided.

Conventional enumeration based on current cultivation methods cannot detect bacteria in the VBNC state, and this results in an underestimation of the actual number of viable pathogens or fecal indicator bacteria in anaerobic digestion. In recent years, molecular enumeration methods based on polymerase chain reaction (PCR) amplification of DNA, such as real-time PCR, have been widely and intensively developed. PCR-based methods are capable of rapid, sensitive, and specific DNA detection from target microorganisms. These
methods, however, lead to an overestimation of the total number of target organisms because they amplify target DNA from dead cells and extracellular DNA due to the persistence of DNA after cell death (Sheridan et al. 1999; Nielsen et al. 2007). Several studies on biosolids have demonstrated that PCR-based methods result in significantly higher counts than culture methods (Higgins et al. 2007; Novinscak et al. 2003; Viau & Peccia 2003). Moreover, m-RNA detection by reverse transcription-PCR has been developed for monitoring the viability of VBNC bacteria (Del Mar Lleo et al. 2003; Coutard et al. 2008). However, detection of VBNC Salmonella sp. utilizing an m-RNA application in anaerobic digestion still requires RNA isolation from biosolids (Dunaev et al. 2008).

In the last decade, a combination of ethidium monoazide (EMA) and propidium monoazide (PMA) with real-time PCR has been developed to distinguish between viable and dead bacterial cells (Nogva et al. 2003; Rudi et al. 2005; Nocker et al. 2006). These dyes selectively penetrate dead cells with compromised membranes and covalently bind to DNA after photoactivation. In conjunction with real-time PCR, they can prevent PCR amplification of extracellular DNA and DNA derived from dead cells. However, EMA was observed to partly penetrate both dead and viable cells of some bacteria (Nocker et al. 2006; Flekna et al. 2007; Cawthorn & Witthuhn 2008). In contrast, the use of PMA combined with real-time quantitative PCR (PMA-qPCR) has been successfully shown to enumerate viable numbers in pure cultures (Nocker et al. 2006; Pan & Breidt 2007) and water samples (Bae & Wuertz 2009; Varma et al. 2009; Yokomachi & Yaguchi 2012; Gensberger et al. 2014). The use of PMA-qPCR was proposed as a suitable approach for viable microbial enumeration in biosolids (van Frankenhuyzen et al. 2011; Taskin et al. 2011). To our knowledge, no studies have successfully determined the real proportion of viable pathogens or indicator bacteria, including VBNC cells, in anaerobic digestion.

The objectives of this research were to utilize PMA-qPCR to determine the actual proportion of viable E. coli in sludge and to understand the behaviors of pathogens in the anaerobic digestion process.

METHODS

Bacterial strain and culture condition

E. coli JCM1649T was purchased from the RIKEN Bio-Source Center (Saitama, Japan). Cultivations of E. coli on Luria–Bertani (LB) medium and the enumerations using epifluorescence microscopy and plate counting were carried out as described by Yokomachi & Yaguchi (2012). When experiments required dead cells, heat-killed E. coli cell suspensions were prepared by exposure at 80°C for 10 min. The absence of viable cells was examined by culturing the cell suspension on LB agar plates.

Sampling overview

Samples were collected from three anaerobic digestion facilities in Aomori and Iwate Prefecture, Japan. Of these facilities, two plants (digesters S and K) treated cattle wastes and food-processing residues through mesophilic digestion, whereas thermophilic digestion was used at the third sewage treatment plant (digester T). Detailed specifications of these anaerobic digestion processes are provided in Table 1. After anaerobic digestion, thermophilically digested sludges were stored and dewatered by the screw press. Organic wastes mesophilically digested at plant S were heated at 65°C for one and a half hours and stored for 2 or 3 months before land application. Other wastes digested mesophilically at plant K were directly applied to land.

Sludge samples were collected before and after digestion. Stored and dewatered sludges were also taken from plant T, 

<table>
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<tr>
<th>Table 1</th>
<th>Characteristics of three anaerobic digestion processes sampled</th>
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<td>Plant</td>
<td>Digester</td>
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<tr>
<td>T sewage treatment plant</td>
<td>Digester T</td>
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<tr>
<td>Plant S in the dairy farm</td>
<td>Digester S1</td>
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<td>Plant K in the cattle ranch</td>
<td>Digester S2</td>
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<td>Plant K in the cattle ranch</td>
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*Food waste was not loaded into digester K in December 2012.
and stored wastes after heat treatment were collected from plant S. Digester samples were transported to our laboratory and enumerations by the culture method were conducted within 2 h of sampling. Samples collected for enumeration based on real-time PCR were kept at 4 °C, and DNA was extracted within 5 h of sampling. Dewatered sludges from digester T were resuspended with 1× phosphate-buffered saline (PBS). The total suspended solids (TSS) content of the samples was measured according to the Japanese Standard Methods for the Examination of Wastewater (JSWA 1997). All samples were then diluted with 1× PBS solution to the desired TSS concentration and homogenized by a homogenizer at 10,000 rpm for 5 min.

**PMA treatments**

A 50 mM solution of PMA (Biotium, Inc., CA, USA) was dissolved in 20% dimethyl sulfoxide (DMSO) and stored in the dark at 4 °C. A PMA stock solution was added to the samples in light transparent 0.5-mL microcentrifuge tubes at a given concentration. After incubation for 5 min in the dark at room temperature, the sample tubes were exposed to a 500-W halogen lamp (Arcland Sakamoto, Niigata, Japan; GTHT500S) for 5 min at a distance of 13 cm. To avoid excessive heating, the tubes were placed on ice during the light exposure.

**DNA extraction and real-time PCR**

A 500 μl sample of *E. coli* cell suspension treated with or without PMA was harvested by centrifugation at 13,500 rpm for 2 min. Genomic DNA was extracted from the cell pellets using an Instagene Matrix (Bio-Rad Laboratories, Tokyo, Japan) following the manufacturer’s instructions. Cell lysis of samples from digesters was achieved by bead beating using a mini-beat beader (Ieda Trading Co., Tokyo, Japan; Model 3110BX) at 4,800 rpm for 30 s. DNA was extracted from the 500 μl sample using the FastDNA® SPIN Kit for soil (MP Biomedicals, CA, USA) according to the manufacturer’s instructions.

Real-time PCR was conducted using a Mini Opticon System (Bio-Rad). An existing primer set and a TaqMan probe were used to amplify the *E. coli* uidA gene coding for the β-D-glucuronidase enzyme (Frahm & Obst 2003). PCR reagents (25 μl) and conditions were described previously (Yokomachi & Yaguchi 2012). Template DNA (2.0 μl) from the samples was assayed in triplicate. Negative controls containing no template DNA were included in each run. Unlike previous reports (Frahm & Obst 2003; Taskin et al. 2011), there was little amplification in the no template controls. Threshold cycle (Ct) values were obtained using the Opticon Monitor Software v.3.1 (Bio-Rad).

**Construction of standard curve**

To construct the standard calibration curve, DNA from *E. coli* cell suspensions was extracted using the FastDNA® SPIN Kit for soil. DNA quantity and purity were analyzed by a UV-visible light spectrophotometer (Nipponbunko, Tokyo, Japan; V-630BIO). The gene copy number was determined on the basis of DNA concentration by the Whelan formula (Whelan et al. 2003). A standard curve was generated using 10-fold serial dilutions of the DNA. Three replicates for each standard point were used. Since *E. coli* has a single copy of the *uidA* gene (Taskin et al. 2011), the *uidA* copy number is equal to the cell number.

**Experimental design**

An initial experiment was performed to optimize the PMA treatments. The effects of PMA concentration and the repeated PMA treatments on the differentiation between viable and dead *E. coli* cells were examined. The optimal conditions of PMA-qPCR were evaluated at different concentrations of dead cells and solids. Dilution of the sludge samples obtained from three anaerobic digestion processes was necessary for successful application of the PMA-qPCR method. Enumeration of viable *E. coli* based on the PMA-qPCR method and comparison with traditional methods were carried out to determine the actual proportion of viable *E. coli* cells in anaerobic digestion.

**Optimization of the PMA treatment**

In order to investigate the effects of PMA concentration on the differentiation between viable and dead cells, PMA was added to the mixtures with defined ratios of viable and heat-treated *E. coli* cells as shown in Table 2. The mixtures of viable and heat-treated *E. coli* cells were subjected to

<table>
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<tr>
<th>Sample (<em>E. coli</em>)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
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<tr>
<td>Viable cells (μL)</td>
<td>1,000</td>
<td>100</td>
<td>10</td>
<td>1</td>
<td>0.1</td>
<td>0</td>
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<tr>
<td>Heat-treated cells (μL)</td>
<td>0</td>
<td>900</td>
<td>990</td>
<td>999</td>
<td>999.9</td>
<td>1,000</td>
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PMA treatment in final concentrations of 50, 100, and 200 μM. Incubation in the dark for 5 min and light exposure for 5 min took place prior to real-time PCR. The effect of repeated treatments of 50 μM PMA at 5-min intervals was also examined using the mixtures of viable and heat-treated E. coli cells in Table 2. One, two and three PMA treatments were carried out and resulted in a final concentration of approximately 50, 100, and 150 μM, respectively. Each sample treatment was replicated.

To determine the effect of dead E. coli cells on the quantification of viable cells, mixtures of viable and heat-treated E. coli cells with a constant number of viable cells were prepared. Each mixture was subjected to a double treatment of 50 μM PMA prior to real-time PCR in replicate. The effects of PMA treatments on the mixtures containing low (5.1 × 10^4 cells/mL) and high (3.1 × 10^6 cells/mL) concentrations of viable cells were explored at different ratios of heat-treated to viable cells.

Effect of solids concentration in the PMA treatment

The digester effluent obtained from the sewage treatment plant T had a TSS concentration of 8,940 mg/L and was homogenized before autoclaving. Autoclaved effluent was diluted to make a TSS concentration ranging from 0 to 8,940 mg/L. These diluted effluents were mixed with a constant number (4.7 × 10^4 cells/mL) of heat-treated E. coli cells prior to the repeated treatments of 50 μM PMA. Viable E. coli cells in an equivalent concentration that did not include digester effluent were also prepared. Each sample was replicated to ensure statistical reliability.

Application of PMA-qPCR to quantify E. coli in samples from anaerobic digestion processes

Real-time PCR and PMA-qPCR were applied to three anaerobic digestion processes to estimate the total and viable number of E. coli cells, respectively. Each sludge sample, diluted to the desired TSS concentration, was assayed in triplicate. PCR inhibition was tested by spiking each sample with dead E. coli cells (1.3 × 10^8 cells/mL) and then comparing the CT value to that derived from only E. coli cells without samples from digesters.

The most probable number (MPN) method, using the EC-MUG medium (Difco) and membrane filter method on m-FC medium (Difco), was used to determine the numbers of culturable E. coli and fecal coliform bacteria, respectively (JSWA 1997; Becton Dickinson & Company 2009). Both media were incubated for 24 ± 2 h at 44.5 ± 0.2 °C.

Laboratory simulation of centrifugal dewatering and storage

A laboratory experiment simulating centrifugal dewatering and storage was achieved using digester S1 and S2 effluents collected on October 22, 2012 and digester T effluent collected on March 26, 2014. These effluents were centrifuged at 10,000 rpm for 30 min (Tomy Seiko Co., Ltd, Tokyo, Japan; CAX-370), and the supernatants were removed. The remaining solids were resuspended with PBS to the original volume. The effluents were stored at 20 °C for 10 days without centrifugation.

Statistical analysis

One-way analysis of variance (ANOVA) was used for statistical analysis. All bacterial numbers and gene copy numbers were log transformed to obtain constant variances. Two-way ANOVA was employed to analyse the effects of PMA treatments on the differentiation between viable and dead E. coli cells and the effects of screw press dewatering of the storage liquors from digester T. It was also used to show the difference between thermophilic and mesophilic digestion on the log reduction of indicator bacteria.

RESULTS AND DISCUSSION

Optimization of the PMA treatment

The effects of PMA concentration and the repeated PMA treatments on the differentiation between viable and dead cells are shown in Figure 1. The mixtures of viable and heat-treated E. coli cells were subjected to PMA treatment in final concentrations of 50, 100, and 200 μM (Figure 1(a)). At a PMA treatment of 100 μM, decreasing viable cell suspension volumes and increasing heat-treated cells led to a gradual decrease in copy numbers calculated from the CT values. However, at PMA treatments of 50 and 200 μM, there was no additional decrease in copy numbers when cell mixtures contained higher than 99% of heat-killed cells. The PMA treatment of 50 μM did not result in sufficient inhibition of the DNA amplification from heat-killed cells beyond 99%. Because both 50 and 200 μM PMA had little effect on the differentiation of viable from dead cells in mixtures containing less than 1% of viable cells, a concentration of 100 μM PMA was considered to be the optimal condition.
The effect of repeated treatments of 50 μM PMA on the differentiation between viable and dead cells is presented in Figure 1(b). Two and three PMA treatments resulted in final concentrations of approximately 100 and 150 μM, respectively, as shown in the parentheses of Figure 1(b). Very similar results to those shown in Figure 1(a) were observed. The copy numbers for one and triple PMA treatment mixtures containing viable cells below 1% did not decrease as the heat-treated cell suspension volumes increased. Conversely, the double PMA treatment indicated a gradual decrease in copy numbers as the ratios of dead to viable cells increased.

It was clarified statistically that the differentiation between viable and dead cells was dependent on the PMA concentration (P < 0.001) and the repeated PMA treatments (P < 0.001). These results might suggest that inhibition of PCR amplifications of DNA derived from heat-killed cells was more dependent on the PMA concentration than the repeated PMA treatments. According to these results, repeating the PMA treatment twice, which led to a final concentration of approximately 100 μM, was selected in further experiments.

To determine the effect of dead E. coli cells on the quantification of viable cells, mixtures of viable and heat-treated E. coli cells with a constant number of viable cells were prepared. The effects of PMA treatments on the mixtures containing low (5.1 × 10^4 cells/mL) and high (3.1 × 10^6 cells/mL) concentrations of viable cells are presented in Figure 2(a) and Figure 2(b), respectively. As shown in Figure 2(b), the copy numbers for the mixtures of high viable cell counts and heat-killed cells were similar to that for the mixture containing only viable cells (ratio 1:0). This was maintained until the ratio of dead to viable cells reached 1,000 (P < 0.001). Conversely, the copy numbers for mixtures with low viable cell counts increased when the ratio was 100 or higher (P < 0.001). Thus, two PMA treatments brought about an improvement over our previous study (Yokomachi & Yaguchi 2012) in the discrimination concentration of approximately 100 μM, was selected in further experiments.

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between viable and dead E. coli cells in the presence of viable cells as high as $10^6$ cells/mL. However, it gave rise to little improvement in the presence of viable cells in a low concentration.

**Effect of solids concentration in the PMA treatment**

The effect of TSS concentration of the sludge sample on PMA-qPCR efficiency is illustrated in Figure 3. Viable cells (in parentheses in Figure 3) shows the $C_T$ value for the viable cells in an equivalent concentration to the dead cells without digester sludges. The $C_T$ values for the sludge samples at a TSS concentration below 3,000 mg/L were as high as those for the only heat-treated cells. In contrast, a gradual decrease in $C_T$ values for sludge samples at or above 3,000 mg/L indicated that a PMA treatment was insufficient to inhibit the PCR amplification of DNA from dead cells ($P < 0.001$).

The PMA-qPCR method has some limitations and problems with its application to environmental samples containing highly complex matrices such as biosolids. Wagner et al. (2008) showed that the dark black appearance and particulate matter associated with digested sludges inhibited the cross-linking of EMA and PMA to DNA because of the lower light penetration. Bae & Wuertz (2009) developed the PMA-qPCR method to discriminate between viable and dead Bacteroides fragilis cells in water samples with various solid concentrations. A 100 μM PMA and 10-min light exposure time successfully resulted in selective quantification of viable Bacteroidales cells at 1,000 mg/L TSS. In addition, Taskin et al. (2011) indicated that PMA-qPCR could be successfully applied to dewatered sludges spiked with E. coli cells when the TSS concentration was up to 2,000 mg/L. According to these researchers, the dilution rate of suspended solids to an essential concentration for photoactivation of PMA needs to be defined as well as the PMA concentration and light exposure time, for the application of PMA-qPCR to environmental samples, such as biosolids (van Frankenhuyszen et al. 2011). Similar to the previous study by Taskin et al. (2011), the PMA treatment optimized in this study succeeded in the application of the PMA-qPCR method to digester effluents with TSS concentrations at or below 2,000 mg/L.

**Application of PMA-qPCR in anaerobic digestion**

To determine the actual proportion of viable E. coli cells in anaerobic digesters, PMA-qPCR was applied to the sludges obtained from the three anaerobic digestion processes. All sludge samples were diluted with a PBS solution to make a TSS concentration below 2,000 mg/L. The TSS concentrations of the diluted sludges were close to or less than 1,000 mg/L. Real-time PCR, the PMA-qPCR, and the MPN method using EC-MUG medium were used to estimate the numbers of total, viable, and culturable E. coli cells, respectively. A standard curve showed that the correlation coefficient, $R^2$ was 0.997, and the PCR amplification efficiency was 93.9%. The quantification limit of the real-time PCR assay was around $10^3$ gene copies/tube due to a clear departure from the linear relationship. The limits of E. coli quantification, based on real-time PCR and MPN methods, in the sludge samples were calculated to be $10^4$ gene copies/mL and 3 MPN/mL, respectively. Culturable concentrations of fecal coliform bacteria were also enumerated by the membrane filter method to a detection limit of 10 cells/mL. Most of the total E. coli counts based on real-time PCR were higher than viable counts determined by the PMA-qPCR method for the three anaerobic digestion processes. The maximum ratio of total to viable E. coli cells was 29.1, indicating that the PMA-qPCR method was applicable to all of the sludge samples. The inhibitory effect of sludge samples on real-time PCR was estimated by spiking diluted sludges with a defined number of heat-treated E. coli cells. The differences between the $C_T$ values of the spiked sludges and the control with only E. coli cells were less than 1.0, representing an absence of PCR inhibition (Stoekel et al. 2009; Cao et al. 2012). Only the dewatered sludges from the thermophilic digester T on September 30, 2013 inhibited PCR amplification.

**Thermophilic digestion**

The anaerobic digester T in the sewage treatment plant treated the mixtures of primary and secondary sludges through thermophilic digestion at 55°C. After digestion, digested

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**Figure 3** | Effect of TSS concentration of sludge sample on the efficiency of the PMA-qPCR method. The error bars represent standard deviations for two samples.
sludges were stored and dewatered by the screw press. A small portion of primary sludges flowed directly into the storage tank and mixed with the digested sludges. Figure 4 shows the concentrations of E. coli and fecal coliform bacteria at the three stages of thermophilic digestion. On August 6, 2012 (Figure 4(a)), all of the measurements estimated by the four methods ranged from $10^6$ to $10^7$ cells/mL in the digester influent. The number of culturable E. coli based on the MPN method and fecal coliforms estimated by the membrane filter method decreased from the digester influent to the effluent by five to six orders of magnitude. However, $1.1 \times 10^6$ cells/mL of viable E. coli determined by the PMA-qPCR method was found in the effluent, resulting in a small decrease of one to two orders of magnitude. These results demonstrated that a great number of VBNC E. coli were present in the digester effluent. Little difference between total E. coli numbers in the digested sludges and those in the stored sludges was observed, indicating no effect from the mixture of primary sludges. In the storage tank of the digested sludges, the concentration of viable E. coli decreased, whereas comparable numbers of culturable E. coli and fecal coliforms increased. As shown in Figure 4(b), similar results were observed on September 30, 2013. Viable E. coli cells decreased below $10^5$ cells/mL, and culturable ones were found above $10^4$ cells/mL in the digester effluent. The number of VBNC E. coli was nearly as high as that on August 6, 2012. All of the measurements increased from the digested sludges to the stored ones, suggesting that mixing the primary sludges might have caused these increases. On January 9, 2014 (Figure 4(c)), approximately $10^2$ cells/mL of culturable E. coli and fecal coliforms were found in the digester effluent. The number of VBNC E. coli was lower than on the other 2 days.

No statistical difference in E. coli concentrations based on the PMA-qPCR and MPN method was observed in the thermophilic digester influents ($P > 0.5$), while there was significant difference in the digester effluents ($P < 0.005$).

No significant increase in the numbers of culturable E. coli and fecal coliforms before and after the screw press dewatering of the storage liquors was found on August 6, 2012 and January 9, 2014 ($P = 0.50$, data not shown). On September 30, 2013, total and viable E. coli counts were not measured because of PCR inhibition.

**Mesophilic digestion**

The anaerobic digestion plant S, located in a dairy farm, treated cattle wastes (digester S1) and food-processing residues (digester S2) at 37 °C. After mesophilic digestion, S1 and S2 effluents were mixed and heated at 65 °C for 90 min. Pasteurized wastes were stored for 2 or 3 months before land application. Wastewater samples were collected from the digester S1 influent, digester S1 effluent, digester S2 effluent, and the storage liquor. Unfortunately, we could not gather digester S2 influent due to the difficulty of sampling. Figure 5 illustrates the concentrations of
E. coli and fecal coliforms at four stages of mesophilic digestion. On October 22, 2012, the number of viable E. coli determined by the PMA-qPCR method was close to $10^8$ cells/mL, while the numbers of culturable E. coli and fecal coliforms were approximately $10^6$ cells/mL in digester S1 influent (Figure 5(a)). The digester S1 effluent had approximately $10^4$ cells/mL of culturable E. coli and fecal coliforms. Small decreases of only two orders of magnitude in culturable E. coli and fecal coliform counts from digester S1 influent to the effluent were observed. The reductions in total and viable E. coli cells were almost as low as that in culturable ones. In comparison with S1 effluent, the digester S2 effluent had only $10^4$ cells/mL of culturable E. coli. These results indicated that digester S1 effluent had a higher proportion of culturable cells than the S2 effluent. No viable E. coli estimated by the PMA-qPCR method was detectable in the storage liquor because of the excessive dilution. Despite the pasteurization by heat treatment at 65 °C, the number of culturable E. coli and fecal coliforms increased to $10^2$ cells/mL in the storage tank. The open tank design to reserve the pasteurized effluents allowed E. coli and fecal coliforms to invade from the air and grow for 2 or 3 months.

The measurements of two indicators estimated by a culture assay on December 17, 2013 were relatively high compared with other indicators (Figure 5(b)). Some measurement were more than the viable E. coli counts determined by the PMA-qPCR method in digester S1 influent and effluent. According to these results, viable E. coli was almost in a culturable state in digester S1 effluent. In the S2 effluent, the concentration of viable E. coli was approximately $10^4$ cells/mL, whereas those of culturable E. coli and fecal coliforms were approximately $10^3$ cells/mL. The difference between viable and culturable cells showed that most of the viable E. coli was in the VBNC state.

There was no significant difference in E. coli concentrations based on the PMA-qPCR and MPN method in digester S1 influents ($P > 0.5$) and effluents ($P > 0.1$), while a statistical difference in digester S2 effluents was observed ($P = 0.076$).

The anaerobic digester K treated mixtures of cattle wastes and food-processing residues in a cattle ranch at 37 °C. Food-processing residues were not loaded into digester K in December 2012. The number of E. coli and fecal coliforms in the mesophilic digester K is shown in Figure 6. The concentration of viable E. coli was approximately $10^6$ cells/mL, while that of culturable E. coli was just $10^5$ cells/mL in the digester influent on December 25, 2012 (Figure 6(a)). Mesophilic digestion resulted in lower reductions in total and viable E. coli cells than those in culturable cells and fecal coliforms by one to two orders of magnitude. Most E. coli cells remained in a VBNC state in the digester effluent. On November 16, 2013, small amounts of food wastes were loaded (Figure 6(b)). The number of E. coli estimated by the three methods was close to $10^6$ cells/g dry weight (dw) in the cattle waste. Small decreases in E. coli and fecal coliforms counts after anaerobic digestion was observed. The digester K effluent had above $10^5$ cells/gdw of culturable E. coli and fecal coliforms, indicating that the number of culturable E. coli cells was almost as high as that of VBNC cells. There was a significant difference in E. coli concentrations based on the PMA-qPCR and MPN methods in digester K effluents ($P < 0.05$).

Comparison of thermophilic with mesophilic digestion

Sludge samples were investigated using the PMA-qPCR method to determine the actual numbers of viable E. coli
containing VBNC cells in anaerobic digestion. For comparison of viable cells with total target DNA and culturable cells, *E. coli* was also enumerated by the PCR and MPN method. Table 3 shows a log reduction of *E. coli* and fecal coliforms from the digester in influent to the effluent in this study. van Frankenhuyzen et al. (2013) indicated that PMA-qPCR detected 0.5–1 log units more viable *E. coli* cells in both primary and dewatered mesophilic digested solids than plate counts. However, our measurements of viable cells based on the PMA-qPCR method are not necessarily consistent with their results. For thermophilic digestion, the reductions in total and viable *E. coli* cells from the digester influent to the effluent were significantly (*P* < 0.05) lower than those in culturable cells and fecal coliforms by two to four orders of magnitude. For mesophilic digestion, the differences in the reductions in *E. coli* and fecal coliforms counts estimated by the four methods were less than two orders of magnitude. There was no significant difference (*P* > 0.1) among them. A significant difference between thermophilic and mesophilic digestion was statistically found in this manner (*P* < 0.001). These results demonstrated that a great number of *E. coli* remained in a VBNC state in thermophilic digester effluents. In contrast, mesophilic digester effluents had a higher proportion of culturable *E. coli* cells. The differences between thermophilic and mesophilic digestion in VBNC *E. coli* counts closely agreed with the estimation reported by Higgins et al. (2007). Thus, the PMA-qPCR method proved that indicator bacteria can enter a VBNC state during anaerobic digestion.

Temperature is suggested to play an important role in stimulating or inducing bacteria into a VBNC state. Bacterial species exhibit different behaviours in response to changes in temperature. In the aquatic environment, *Vibrio* strains became VBNC at a low temperature (4°C), while other bacteria entered a VBNC state at a high temperature (25–30°C) (Duncan et al. 1994). *E. coli* entered a VBNC state more rapidly at high temperatures above 25°C than at low temperatures of 10°C or lower (Xu & Colwell 1982). However, no studies of aquatic bacteria have been conducted at extremely high temperatures above 30°C, as seen in thermophilic digestion.

**Laboratory simulation of centrifugal dewatering and storage**

A laboratory experiment simulating centrifugal dewatering and storage was achieved using digester S1 and S2 effluents collected on October 22, 2012 and digester T effluent collected on March 26, 2014. Figure 7 presents the results of a laboratory dewatering and storage experiment of digester T effluent. There was little difference in the numbers of the four indicators before and after centrifugation (10,000 rpm, 30 min). Five-day storage at 20°C led to greater increases in the numbers of the two indicators based on a culture assay than that of the PMA-qPCR method. The remarkable regrowth of culturable *E. coli* was observed during the storage of thermophilic sludge with no centrifugation. However, the centrifuge dewatering and storage of digester S1 and S2 effluents did not show the reactivation and regrowth of culturable *E. coli* and fecal coliforms (data not shown).

Reactivation induced by centrifugal dewatering (Qi et al. 2007; Higgins et al. 2007; Chen et al. 2011) was not verified in this study, because there was no increase in the numbers of the two indicators based on a culture assay after laboratory
centrifugation. Although most of the centrifuged sludges after thermophilic digestion resulted in a significant increase in the numbers of fecal indicator bacteria, some results showed no reactivation during centrifugal dewatering (Qi et al. 2011; Higgins et al. 2011). It is not clear what reactivates fecal indicator bacteria into a culturable state.

**Microbial quality of digester effluents**

In Japan, digester effluents are increasingly applied to land as anaerobic digestion is widely used to stabilize various organic wastes. However, there is no current regulation on the microbial quality of biosolids for agricultural application in Japan. Although the hygienization of sludges through thermophilic digestion above 55 °C or through pasteurization at 70 °C for 60 min as well as the European Union directive 278/86/EEC (CEC 1986) is recommended, we have few data for pathogens or indicator bacteria concentrations of digested sludges. This research can provide the necessary viable indicator concentrations for the legislation regarding the microbial standards in Japan.

In the United States, the Environmental Protection Agency (EPA) classifies biosolids as Class A or Class B based on 40 CFR Part 503 (USEPA 1999). The directive 278/86/EEC is currently under revision and indicator bacteria concentration limits are proposed in the working documents (EC 2010). The results for thermophilic digestion showed that the culturable concentrations of fecal coliforms in digester effluents corresponded to Class A standard (10³ cells/dry g). Mesophilic digestion also led to a sufficient decrease in fecal coliforms concentration below the Class B limits (2 × 10⁶ cells/dry g). Furthermore, the results for *E. coli* counts estimated by the MPN method indicated all the digester effluents were good enough to reach the limit in the EU working document (5 × 10⁵ cells/wet g). However, the number of viable *E. coli* determined by the PMA-qPCR method was frequently higher than the suggested EU limit, because they contained a great number of VBNC organisms. Based on the results for viable *E. coli*, much higher concentrations of fecal coliforms might be present in the VBNC state and the thermophilic digester effluents would not meet the Class A standard. Consequently, this research suggests that pasteurization after anaerobic digestion and preservation in the closed tank are essential for the destruction of VBNC pathogens and prevention of microbial contamination.

**CONCLUSIONS**

We demonstrated that the indicator bacteria entered a VBNC state during anaerobic digestion by the application
of the PMA-qPCR method to three anaerobic digestion processes. For thermophilic digestion, the reductions in total and viable E. coli cells from the digester influent to the effluent were significantly lower than those in culturable cells and fecal coliforms by two to four orders of magnitude. As a result, a great number of E. coli remained in a VBNC state in the thermophilic digester effluent. For mesophilic digestion, the differences in the reductions in E. coli and fecal coliforms counts estimated by the four methods were less than two orders of magnitude, indicating that the mesophilic digester effluent had a higher proportion of culturable E. coli cells. Reactivation induced by centrifugal dewatering, however, was not verified in this study, because there was no increase in the numbers of two indicators based on a culture assay after laboratory centrifugation. The comparison of the measurements from this research with the USEPA and EU regulations indicated that pasteurization after anaerobic digestion and preservation in the closed tank were necessary to maintain the microbial quality of digester effluents. For thermophilic digestion, the reductions in total and viable but nonculturable bacteria. Further research on the PMA-qPCR method will help to enhance our understanding of the behaviors of viable pathogens in the microbial community.

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