Efficiency of peracetic acid in inactivating bacteria, viruses, and spores in water determined with ATP bioluminescence, quantitative PCR, and culture-based methods

Eunyoung Park, Cheonghoon Lee, Michael Bisesi and Jiyoung Lee

ABSTRACT

The disinfection efficiency of peracetic acid (PAA) was investigated on three microbial types using three different methods (filtration-based ATP (adenosine-triphosphate) bioluminescence, quantitative polymerase chain reaction (qPCR), culture-based method). Fecal indicator bacteria (*Enterococcus faecium*), virus indicator (male-specific (F^+) coliphages (coliphages)), and protozoa disinfection surrogate (*Bacillus subtilis* spores (spores)) were tested. The mode of action for spore disinfection was visualized using scanning electron microscopy. The results indicated that PAA concentrations of 5 ppm (contact time: 5 min), 50 ppm (10 min), and 3,000 ppm (5 min) were needed to achieve 3-log reduction of *E. faecium*, coliphages, and spores, respectively. Scanning electron microscopy observation showed that PAA targets the external layers of spores. The lower reduction rates of tested microbes measured with qPCR suggest that qPCR may overestimate the surviving microbes. Collectively, PAA showed broad disinfection efficiency (susceptibility: *E. faecium* > coliphages > spores). For *E. faecium* and spores, ATP bioluminescence was substantially faster (~5 min) than culture-based method (>24 h) and qPCR (2–3 h). This study suggests PAA as an effective alternative to inactivate broad types of microbial contaminants in water. Together with the use of rapid detection methods, this approach can be useful for urgent situations when timely response is needed for ensuring water quality.

Key words | *Bacillus subtilis* spores, *Enterococcus*, filtration-based ATP bioluminescence, male-specific (F^+) coliphages, peracetic acid

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INTRODUCTION

Effective control of pathogenic microbes in water, including bacteria, viruses, and protozoa, has been an important issue for providing safe water to the public. In 2004, the World Health Organization (WHO) estimated that diarrhea associated with waterborne infectious microorganisms accounted for nearly 4.1% of the global burden of disease in disability-adjusted life years and 1.8 millions of deaths (WHO 2004). In emergency situations, such as floods and earthquakes, runoff waters often pick up large quantities of pathogenic microorganisms from farms, sewage, and waste waters and subsequently contaminate groundwater and surface water that are used for sources of drinking water. The tainted drinking water sources increase risk of disease transmission and likelihood of infection (Centers for Disease Control and Prevention (CDC) 2011). For example, after Hurricane Katrina in New Orleans in 2005, approximately 1,000 cases of gastrointestinal illness were reported, such as diarrhea and vomiting.

In order to reduce the risk to public health from exposure to pathogens in contaminated drinking water during emergencies, the United States Environmental Protection Agency (USEPA) and CDC have recommended boiling and chemical treatment for emergency water

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disinfection (USEPA 2006). The most commonly used chemicals are chlorine and its derivatives, which can inactivate many bacteria, including enteric bacteria (De Luca *et al.* 2008). However, chlorination shows relatively low effectiveness against viruses, protozoan cysts, and bacterial spores. These microbes are considerably associated with health risks and are known for low susceptibility to chlorination (Veschetti *et al.* 2003; Koivunen & Heinonen-Tanski 2005a; Zanetti *et al.* 2007). In addition, chlorination has the potential for forming carcinogenic disinfection by-products (DBPs) especially when the water contains high levels of organic matter (Kitis 2004; Crebelli *et al.* 2005). Therefore, alternative disinfectants have been proposed for effective inactivation of pathogens, assuring a practical application while minimizing the formation of DBPs.

Peracetic acid (PAA) may be a good alternative disinfectant since it is a strong oxidant and has been approved by the Food and Drug Administration (FDA) in the USA for various applications, including food, beverage, medical, and pharmaceutical industries (Kitis 2004; Koivunen & Heinonen-Tanski 2005b). Several studies have shown that it inactivates pathogenic bacteria, such as *Vibrio cholerae* (Kitis 2004) and indicator microorganisms in both the absence and presence of organic matter (Koivunen & Heinonen-Tanski 2005b; Zanetti *et al.* 2007). It has also been reported that PAA produces no or low amounts of DBPs and chemical residues compared to chlorination (Kitis 2004; Koivunen & Heinonen-Tanski 2005a; Crebelli *et al.* 2005) because PAA is easily decomposed into non-toxic compounds, such as acetic acid and oxygen (Kitis 2004; Crebelli *et al.* 2005). Owing to these advantages, PAA has been used for wastewater disinfection in England, Finland, Italy, Brazil and Canada (Kitis 2004). In the USA, the USEPA also included PAA as one of the recommended disinfectants for treating combined sewer overflows (USEPA 1999). However, there are limited studies that investigate its disinfection efficiency against various microbial types (bacteria, viruses, and protozoa) since they may have different susceptibility to PAA (Monarca *et al.* 2002; Bailey *et al.* 2011). Furthermore, no information is available about the use of PAA for drinking water disinfection as an alternative for the emergency situations requiring timely results of PAA effectiveness.

The purpose of this study was two-fold. The first aim was to investigate the disinfection efficiency of PAA against three different microbial types: bacteria, virus, and protozoa. The reaction of PAA on *Enterococcus faecium* was tested to determine its effectiveness as a fecal indicator of microbial water quality (USEPA 1986). *Bacillus subtilis* spores were used as a surrogate for protozoa (Driedger *et al.* 2001; Radziminski *et al.* 2002; Cho *et al.* 2003) and coliphage MS2 as a representative surrogate of viral contamination (USEPA 2001). The second aim was to compare PAA disinfection efficiency using three detection methods for the evaluation of which method would be more accurate, faster and practical in emergency situations when timely results are required. A filtration-based ATP bioluminescence assay (Lee & Deininger 2004), quantitative polymerase chain reaction (qPCR), and conventional culture-based methods were conducted using *E. faecium* and *B. subtilis* spores. For male-specific (F^+) coliphage quantification, double agar layer method (DAL) (USEPA 2001) and reverse transcription (RT)-qPCR were conducted. Additionally, the effect of PAA on *B. subtilis* spores was examined using a scanning electron microscopy (SEM) to visualize the mode of disinfection.

METHODS

Microorganism preparation

E. faecium (ATCC 19434), *B. subtilis* (ATCC 6051), and male-specific (F^+) coliphage (MS2, ATCC 15597-B1) were purchased from American Type Culture Collection (ATTC, Manassas, VA, USA).

A 100 μL aliquot of *E. faecium* was added to 100 mL of tryptic soy broth supplemented with 0.6% yeast extract (TSBYE; Becton, Dickinson and Company, Sparks, MD, USA). This was cultured at 37 °C by shaking in an Incushaker (Benchmark, Ontario, Canada) for 16–24 h. The concentration of cultured *E. faecium* was determined by serial dilution with phosphate-buffered saline (PBS; Fisher Scientific, Fair Lawn, NJ, USA) and subsequent pour plates on tryptic soy agar (TSA), which were incubated for 16–24 h.

To prepare the *Bacillus* spores, *B. subtilis* was initially grown on TSA supplemented with 0.6% (w/v) yeast extract (TSAYE) at 30 °C for 24 h. A 100 μL aliquot of overnight culture was then cultured for 10–14 days on plates of TSAYE, which contained 10 mg L^{-1} of $MnSO_4$ (Baker Chemical

Co., Phillipsburg, NJ, USA) at 30 °C for sporulation (Ratphitagsanti *et al.* 2012). The spores were harvested after 95% of the population was sporulated, which was verified with the Schaeffer-Fulton stain method (Harley & Prescott 2002) and phase-contrast microscopy. The surfaces of the inoculated plates were flooded with 10 mL of cold, sterile deionized water and scraped with sterile disposable plastic spreaders. Spores were harvested and washed using sterile deionized water. The washing was performed by centrifugation at $9{,}700 \times g$ for 15 min at 4 °C. After repeated washing (at least four times), spore pellets were resuspended in sterile deionized water and sonicated for 10 min at room temperature to prevent spore clumping. Heat treatment was carried out at 80 °C for 15 min. Any remaining vegetative cells were removed via 0.1% lysozyme (MP Biomedicals, LLC., Solon, OH, USA) treatment, which was carried out by shaking at a ratio of 1:1 for 1 h at room temperature. The suspended spores were then centrifuged at $5{,}800 \times g$ for 10 min and washed with sterile deionized water. This step was repeated three times. Spore concentration was determined by the TSA plate count method and calculated as colony forming units (CFU) mL^{-1}.

Male-specific (F^+) coliphage was propagated from the ATCC stock according to the manufacturer's instructions. Its concentration was determined by USEPA 1601 DAL using *Escherichia coli* HS (pF_{amp}) as a host (USEPA 2001). Ten-fold dilutions of F^+ coliphage stock were prepared using TSB. Each tube of molten 0.7% TSA top agar (with host bacteria) was inoculated with 0.5 mL of each serial dilution of F^+ coliphage stock. Each tube was then poured into a 1.5% TSA bottom agar plate containing 1.5 mg L^{-1} of ampicillin sodium sulfate (Fisher BioReagents, Fair Lawn, NJ, USA) and 1.5 mg L^{-1} of streptomycin (Sigma, St Louis, MO, USA). After incubation at 36 °C for 16–24 h, visible plaques were counted and viral concentration was calculated as plaque forming units (PFU) mL^{-1} (USEPA 2001). All cultured *E. faecium* (1.0×10^9 CFU mL^{-1}), *B. subtilis* spores (2.3×10^{10} CFU mL^{-1}), and F^+ coliphages (1.0×10^8 PFU mL^{-1}) were stored at −80 °C until use.

Determination of PAA disinfection concentration

The working range of PAA (35%, FMC, Philadelphia, PA, USA) concentration and contact time for this study was determined according to previous studies and CDC's recommendation (Crebelli *et al.* 2005; Majcher *et al.* 2008; Rutala *et al.* 2008): 5 ppm for *E. faecium*; 500 ppm and 3,000 ppm for *B. subtilis* spores; and 50 ppm for male-specific (F^+) coliphage. Thirty-five percent of PAA was used to make 5, 50, 500, and 3,000 ppm (final concentration) by dilution for each contamination event. The responses of microorganisms against PAA disinfection treatments were monitored over a 1-h contact time.

PAA disinfection

E. faecium

To generate bacterial contamination in water, a 10 mL aliquot of cultured *E. faecium* suspension was spiked into 990 mL of 10% PBS and was then stirred gently with a magnetic bar. The initial level of *E. faecium* in this solution was designated as 0 min sample. 0.0143 g L^{-1} of PAA was added to the *E. faecium*-contaminated solution and the final concentration of PAA was 5 mg L^{-1} (5 ppm). The 10 mL PAA-treated samples were collected after each contact time of 5, 10, 15, 20, 25, 30, and 60 min, respectively. Sodium thiosulfate (10%; Fisher Scientific, Fair Lawn, NJ, USA) was added to stop PAA activity (Majcher *et al.* 2008). The levels of bacteria at 0 min and that of surviving bacteria after each contact time were enumerated with ATP bioluminescence, plate count method, and qPCR explained below, respectively.

B. subtilis spores

To generate water contamination events, a 10 mL aliquot of cultured *B. subtilis* spore suspension was spiked into 990 mL of distilled water and was then stirred gently with a magnetic bar. 1.43 g L^{-1} and 8.58 g L^{-1} of PAA (35%) were added into spore-contaminated water to make final concentrations of 0.5 g L^{-1} (500 ppm) and 3 g L^{-1} (3,000 ppm), respectively. The initial level of *B. subtilis* spores was designated as 0 min sample. The PAA-treated water samples were collected at the contact times of 5, 15, 30, and 60 min. Sodium thiosulfate (10%) was added to stop PAA activity. The levels of initial bacteria and surviving bacteria after each contact time were enumerated with ATP bioluminescence, plate count method, and qPCR, respectively.

Male-specific (F^+) coliphage

To generate water contamination events, a 10 mL aliquot of F^+ coliphage suspension was spiked into 990 mL of 10% PBS and was then stirred gently with a magnetic bar. 0.143 g L^{-1} of PAA (35%) was inoculated into coliphage-contaminated water. The final concentration became 0.05 g L^{-1} (50 ppm). The initial level of F^+ coliphages in this solution was measured using the DAL method and qPCR, respectively. This sample was designated as 0 min sample. The PAA-treated water samples were collected at the contact times of 5, 15, 30, and 60 min. Sodium thiosulfate (10%) was added to stop PAA activity. The levels of surviving bacteria after each contact time were enumerated with the DAL method and qPCR, respectively.

Determination of PAA disinfection efficiency using filtration-based ATP bioluminescence

E. faecium

The filtration-based ATP bioluminescence was performed using Profile®-1 ATP method (New Horizons Diagnostics, Columbia, MD, USA) (Lee & Deininger 2004). Fifty microliters of sample aliquots were concentrated by filtration on a Filtravette™ (New Horizons Diagnostics), a combination of a filter and a cuvette with a 0.45 μm pore size. After concentration, 50 μL of somatic cell-releasing agent was added to the Filtravette to remove non-bacterial cells and adjust pH for optimizing the downstream luminescence reaction. Next, 50 μL of bacterial cell-releasing agent (BRA) was applied for 1 min to lyse the concentrated bacterial cells. The extracted ATP was then mixed with 50 μL of luciferin/luciferase (LL). Light development was initiated at this point. The integrated light signal was measured using a Microluminometer (Model 3550i; New Horizons Diagnostics, Columbia, MD, USA). ATP luminescence was expressed in a relative luminescence unit (RLU), which is an indicator of the total ATP released from the bacteria present in the sample. The activity of LL was checked with serially diluted ATP standard solution (Sigma). Sterilized distilled water was used as a negative control.

B. subtilis spores

Heat shock treatment was carried out first to induce *Bacillus* spores in water to germinate (Lee & Deininger 2004). This step is essential because the heat shock transforms inert spores into vegetative cells and their ATP can be readily detected by luminescence assay. Lee & Deininger (2004) reported the fastest test conditions (germination time, temperature, and nutrient concentration) that showed a significantly high signal. This method was used in the current study because it detects spores sufficiently in a short time (within 5 min) even though there was variability in germination levels. First, 50 μL of preheated (37 °C) sterile TSBYE was added into the Filtravette as a nutrient source to trigger spore germination. The Filtravette contained concentrated spores from each sample. Each Filtravette was incubated at 37 °C for 15 min, which provided the optimal condition to generate the highest bioluminescence signal (Lee & Deininger 2004). After the germination step, gentle air pressure was used to filter out the liquid. At this step, the germinated vegetative cells remain on the surface of the Filtravette. Next, ATP bioluminescence assay was performed as described above. The differences in the RLU signal measured before and after the spore germination process indicated the amount of ATP that originated from the *Bacillus* spores present in the sample (Lee & Deininger 2004).

Determination of PAA disinfection efficiency using plate count method

E. faecium and *B. subtilis* spores

The numbers of *E. faecium* and *B. subtilis* in the samples were enumerated by counting colonies grown on R2A agar (Becton, Dickinson and Company, Sparks, MD, USA) (Lee & Deininger 2010) and TSA (Becton, Dickinson and Company), respectively. After incubation at 37 °C for 16–24 h, colonies were counted and calculated as CFU mL^{-1}.

F^+ coliphage

The numbers of coliphages were determined using a DAL method (USEPA 2001) as described above. TSB without antibiotics was used as a diluent and a negative control.

Determination of PAA disinfection efficiency using RT-qPCR

E. faecium and *B. subtilis* spores

DNA was extracted from 200 μL of each sample using a DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and then suspended in 50 μL of elution buffer. The eluates were used immediately or stored at −80 °C until further processing. Real-time quantitative PCR (qPCR) assay was carried out with a StepOne™ Real-Time System (Applied Biosystems, Foster City, CA, USA) in a 48-well format. For *E. faecium* real-time PCR, the reaction mix (20 μL) consisted of 5 μL of DNA template, 10 μL of TaqMan® PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 500 nM of each forward and reverse primer, and 250 nM of probe (Ludwig & Schleifer 2000). The sequences for primers and probes were as follows: 5′-AGAAATTCCAAACGAACTTG-3′ for forward primer, 5′-CAGTGC TCTACCTCCATCATT-3′ for reverse primer and 5′-6-carboxyfluorescein (FAM)-TGGTTCTCTCCGAAA-TAGCTTT AGGGCTA-minor groove binding (MGB)-3′ for probe (Ludwig & Schleifer 2000). Thermal cycling consisted of a holding stage (95 °C for 10 min) and a cycling stage (40 cycles at 95 °C for 15 s and 60 °C for 1 min) (Ludwig & Schleifer 2000). For spore real-time qPCR, the reaction mix (20 μL) consisted of 5 μL of DNA template, 10 μL of SYBR® Green PCR Master Mix (Applied Biosystems), and 500 nM of each forward and reverse primer (5′-CAGCCTTTGGGCAGGAAGA-3′ for forward primer and 5′-AGGACGCGCCTAAATCGA-3′ for reverse primer (Ratphitagsanti *et al.* 2012). Thermal cycling consisted of a holding stage (95 °C for 10 min) and a cycling stage (40 cycles at 95 °C for 15 s and 60 °C for 1 min). After amplification, melting curve analysis was performed by heating samples to 95 °C for 15 s, cooling to 60 °C for 1 min, and then heating the samples at 1.0 °C s^{-1} to 95 °C. Real-time qPCR was performed in triplicate for each sample. A negative control (distilled water) and a positive control (purified DNA of *E. faecium* or *B. subtilis*) were included in each run. The threshold cycle (C_T) values of the real-time qPCR results were determined using auto threshold. Ten-fold serial dilutions (10^1–10^8 CFU per reaction) of *E. faecium* or *B. subtilis* DNA were used to prepare their standard curves. Real-time qPCR assays were performed in triplicate to make standard curves by plotting C_T values versus log CFU. The concentrations of *E. faecium* and *B. subtilis* spores were calculated by using these standard curves.

F^+ coliphage

Coliphage RNA (50 μL) was extracted from each sample using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The eluates were used immediately or stored at −80 °C until further processing. RT was performed according to the manufacturer's instructions (Promega, Madison, WI, USA) with minor modifications. Briefly, 7.5 μL of extracted viral RNA was mixed with 100 nM of reverse primer in a reaction tube and RNase-free water was added up to 8.5 μL. The mixture was heated at 70 °C for 5 min and then immediately cooled on ice. The reaction mixture (11.5 μL) consisted of 4 μL of 5× reaction buffer (Promega), 1 μL of 10 mM dNTPs, 40 U of RNase Inhibitor (Invitrogen, Carlsbad, CA, USA), and 100 U of M-MLV reverse transcriptase (Promega). This RT mixture was incubated for 1 h at 42 °C, then for 5 min at 95 °C to produce cDNA. Real-time qPCR was performed as described by Ogorzaly & Gantzer (2006) with a StepOne Real-Time System (Applied Biosystems) in a 48-well format. Briefly, 5 μL of cDNA was added to 45 μL of reaction mixture containing 25 μL of TaqMan Universal PCR Master Mix (Applied Biosystems), 250 nM of each forward and reverse primer, and 750 nM of probe. Each primer pair and probe is as follows: 5′-TCAGTGGTCCATACCTTAGATGC-3′ for forward primer, 5′-ACCCCGTTAGCGAAGFFGCT-3′ for reverse primer and 5′-FAM-CTCGTCGACATGG-minor groove binding non-fluorescent quencher (MGBNFQ)-3′ for probe (Ogorzaly & Gantzer 2006). qPCR was performed in duplicate. Thermal cycling consisted of a holding stage (50 °C for 2 min and 95 °C for 10 min) and a cycling stage (50 cycles at 95 °C for 15 s and 60 °C for 1 min). The C_T values of the qPCR results were determined using auto threshold. Ten-fold serial dilutions (10^1–10^{10} PFU per reaction) of coliphage RNA were used for preparation of their standard curves. RT-qPCR was performed in triplicate to make standard curves by plotting the C_T values versus log PFU. The coliphage RNA concentration was calculated using these standard curves.

Observation of PAA effect on *B. subtilis* spores using SEM

Round glass cover slips (5 mm; Electron Microscopy Sciences, Hatfield, PA, USA) were prepared by immersing in the order of 10% sodium dodecyl sulfate, 99% ethanol, and nano pure water (18.2 MΩ cm, Thermo scientific Barnstead Nanopure®, Dubuque, IA, USA). Each sample in the volume of 2.5 μL was loaded on each cover slip and then dried under pure nitrogen gas purging. The cover slip with a sample was mounted onto a metal stub with double-sided carbon tape, and then coated with a thin layer of gold (30 nm) using a gold sputter coater (Emitech K550X; Emitech, Ashford, Kent, UK). Images of the samples were taken using a SEM (Hitachi S-4300, Japan). Untreated samples (control) and those treated with PAA were selected for comparison.

RESULTS

PAA treatment of contaminated water with *E. faecium*

The reduction trend of *E. faecium* by the action of PAA over the 1-h contact time is summarized in Figure 1. Interestingly, the reduction rates differed depending on the detection methods. Based on the filtration-based ATP bioluminescence, *E. faecium* was significantly reduced with 5 ppm PAA treatment: 4-log reductions within 1 h. The greatest reduction (2 log) occurred within the first 5 min of contact time when measured with ATP bioluminescence assay. The plate count method showed that PAA treatment reduced the *E. faecium* level by 5 log within 5 min. The level of reduction remained constant over 1 h of contact time after the first 5-min treatment. The reduction rate of *E. faecium* determined with the plate count method was always higher than that determined with ATP bioluminescence assay. On the other hand, qPCR showed that PAA treatment resulted in only 1-log reduction over 1-h contact time (Figure 1). Thus, bacterial reduction rates measured with qPCR assay were always lower than those determined with ATP assay or plate count method.

PAA treatment of contaminated water with *B. subtilis* spores

A filtration-based ATP bioluminescence with heat shock method showed that *B. subtilis* spores were reduced by 2 log with 500 ppm of PAA within 5 min (Figure 2), whereas 3,000 ppm of PAA reduced the spores by 4 log within 5 min (Figure 3). The plate count method showed that the spores were reduced by 1.5 log within the first 5 min of contact time when 500 ppm of PAA was used (Figure 2). Filtration-based ATP bioluminescence with heat shock method showed 3-log reduction of *B. subtilis* spores under the same PAA treatment

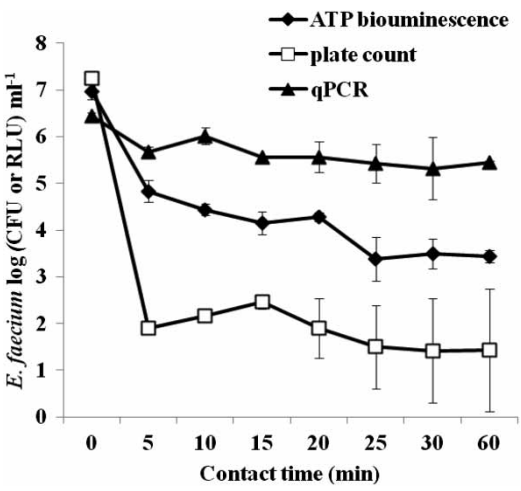

Figure 1 | Concentration of the surviving *E. faecium* measured with three methods when treated with PAA (5 ppm) during 1-h contact time: ATP bioluminescence (log [RLU] mL^{-1}), plate count (log [CFU] mL^{-1}), qPCR (log [CFU] mL^{-1}).

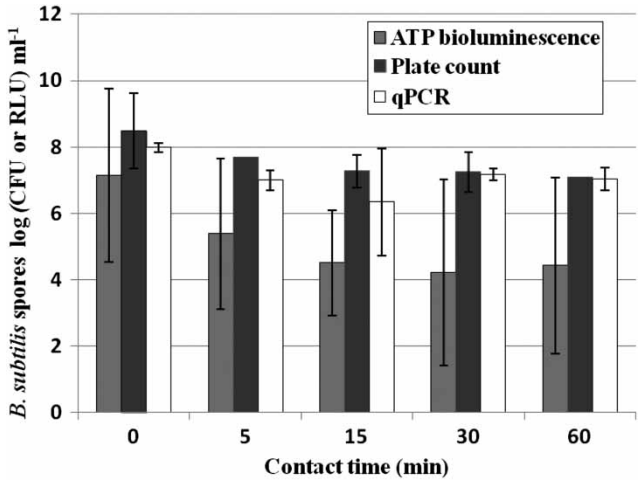

Figure 2 | Concentration of the surviving *B. subtilis* spores measured with three methods when treated with PAA (500 ppm) during 1-h contact time: ATP bioluminescence (log [RLU] mL^{-1}), plate count (log [CFU] mL^{-1}), qPCR (log [CFU] mL^{-1}).

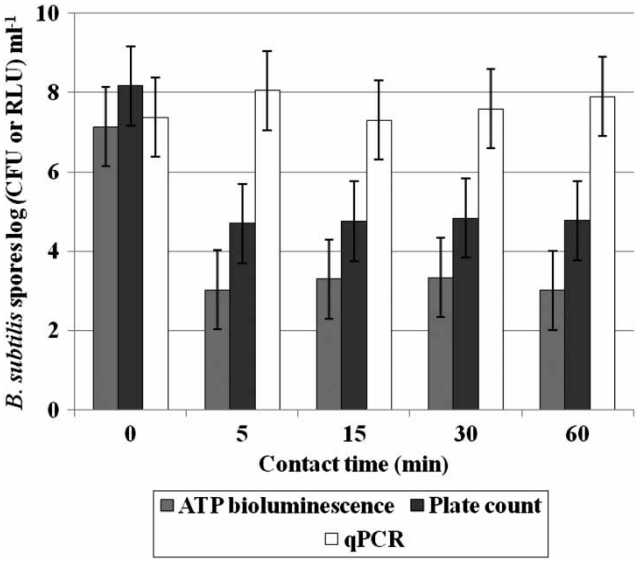

Figure 3 | Concentration of the surviving *B. subtilis* spores measured with three methods when treated with PAA (3,000 ppm) during 1-h contact time: ATP bioluminescence (log [RLU] mL^{-1}), plate count (log [CFU] mL^{-1}), qPCR (log [CFU] mL^{-1}).

conditions within 1 h of contact time (Figure 2). When spores were disinfected with 3,000 ppm of PAA, both ATP bioluminescence with heat shock and plate count methods showed similar spore reduction rates (~4 log) (Figure 3). As shown in the *E. faecium* results, however, both 500 and 3,000 ppm of PPA resulted in only 1-log reduction over 1-h contact time when measured with qPCR (Figures 2 and 3).

PAA treatment of contaminated water with F^+ coliphage

For measurement of coliphages, two methods (DAL and qPCR) were used because coliphages do not contain ATP, therefore ATP bioluminescence cannot be used. The DAL method shows that the 50 ppm PAA treatment inactivated F^+ coliphages by ~2.5 log within 5 min and reduced up to 4 log over 1 h of contact time (Figure 4). Their RNA seemed to remain intact during the 30-min contact time, although both culturable coliphages and their genetic materials were reduced similarly (~4-log reduction) over the 1-h treatment (Figure 4).

B. subtilis spores observed by SEM

B. subtilis spores affected by PAA were observed by SEM to determine the mechanism of PAA on spores. Figure 5 shows

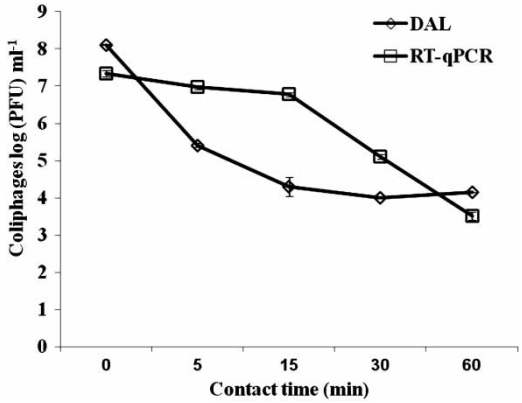

Figure 4 | Concentration of the surviving male-specific (F^+) coliphages measured with two methods when treated with PAA (50 ppm) during 1-h contact time: DAL (log [PFU] mL^{-1}), RT-qPCR (log [PFU] mL^{-1}).

the difference in morphology between untreated spores and those treated with PAA (500 and 3,000 ppm) and the external layers of *B. subtilis* spores were damaged (Figures 5(b) and 5(c)). More severe damage on the layers was observed via SEM when the spores were treated with higher concentrations of PAA (3,000 ppm; Figure 5(c)).

DISCUSSION

Disinfectants are usually regarded as effective when they can reduce the targeted organism by at least 3 log (99.9%) in their numbers. PAA has been demonstrated to inactivate the indicator organism *E. coli* with such high disinfection efficiency (Koivunen & Heinonen-Tanski 2005a). However, the variability of microbial resistance against disinfectants and the lack of significant correlation between the indicator organism, *E. coli* and other pathogenic microorganisms have suggested the importance of assessing disinfectant efficiency by using more than one indicator organism (Gehr *et al.* 2003; Koivunen & Heinonen-Tanski 2005a, b; Zanetti *et al.* 2007). In this study, we evaluated PAA disinfection efficiency using three different microbial types (*E. faecium*, *B. subtilis* spores, and male-specific (F^+) coliphage).

E. faecium has been used as one of the recommended indicators for bacterial water quality in fresh and marine water by the USEPA for almost three decades because of its association with gastrointestinal disease (USEPA 1986). As shown in Figure 1, its reduction rates against PAA over

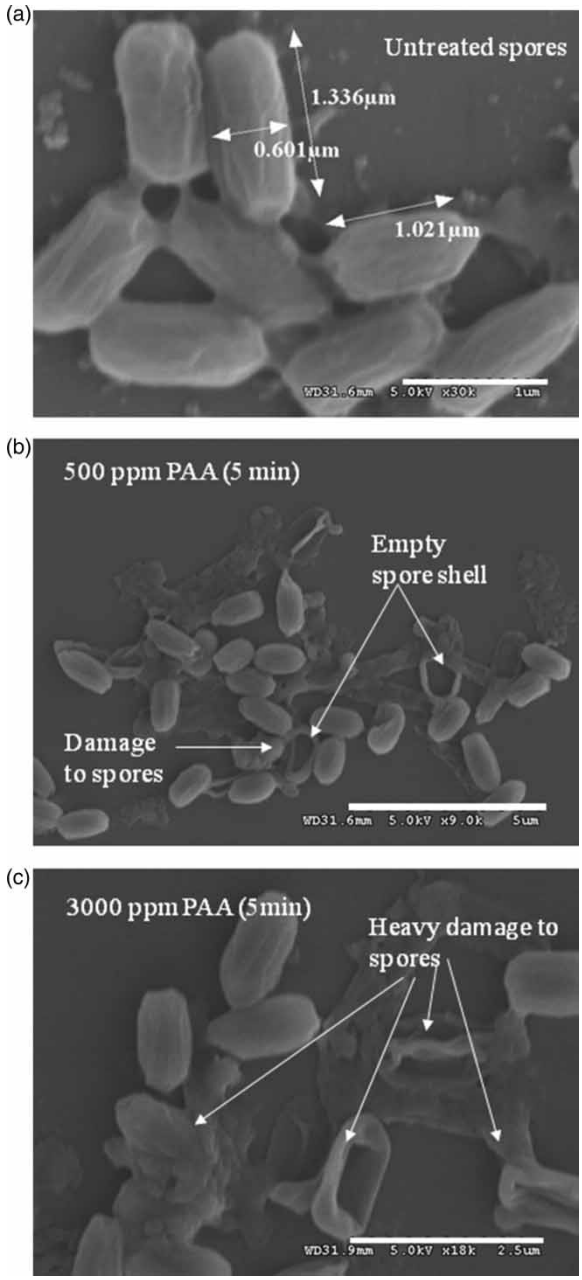

Figure 5 | SEM images of *B. subtilis* spores: (a) without PAA treatment (1-μm scale bar); (b) after the treatment with PAA at 500 ppm (5-μm scale bar); (c) after the treatment with PAA at 3,000 ppm (2.5-μm scale bar).

time could be calculated differently depending on the detection methods. The concentrations of *E. faecium* did not decrease any further after 25 min. It was possible that most of the PAA had been consumed during the bacterial cell oxidations and reached its reaction capacity after 25 min. To confirm this, it would have been ideal if the residual PAA after 25 min had been measured. However, it was speculated that it was not necessary because more than 3-log reduction of *E. faecium* was already achieved, which was confirmed with the plate count method. A filtration-based ATP bioluminescence has shown the potential to detect only viable cells in water samples (Lee & Deininger 2010). This can possibly explain why the reduction rate measured by a filtration-based ATP bioluminescence was lower than that of the plate count method. Bacterial cells may be injured by PAA, but still maintain viability during 1-h contact time even though they were unable to develop colonies on culture media. It has been reported that disinfectants can cause reversible damage on bacteria, and injured bacteria can survive in stressful environments by entering viable but non-culturable (VBNC) state (Colwell 2000; Oliver 2005). In VBNC state, bacteria are no longer culturable on conventional culture media, but the cells have been shown to display active metabolism, such as respiration, gene transcription, and membrane integrity (Lleò *et al*. 2001). Furthermore, it has been reported that pathogenic bacteria in VBNC status may retain pathogenic factors/genes, which can make them a reservoir of infectious disease transmission (Colwell 2000; Lleò *et al*. 2001; Camper 2004; Oliver 2005; Keep *et al.* 2006). Therefore, it would be essential to find an appropriate method to detect not only those culturable, but also those which have entered VBNC state. However, it was not clear whether these VBNC bacteria were accurately detected with the filtration-based ATP bioluminescence in our experiments. To confirm this, additional tests, such as Live/Dead kit (Lleò *et al*. 2001) and flow cytometry (Oliver 2005), are recommended. Our results indicated that filtration-based ATP bioluminescence may detect the VBNC population which could not be detected by plate count method. This method has limitations because it does not provide information on the identification of bacteria present in water samples. However, it can be used as a rapid and practical evaluation tool by providing more accurate numbers of viable bacteria on site, especially for emergency situations (Deininger & Lee 2001). The plate count method has been widely used because of its low cost, simplicity of methodology, and positive correlation with health risks (Camper 2004). However, as shown in Figure 1, the underestimated rate of surviving microbes measured by plate count method may raise a question

whether this method can sufficiently safeguard public health (Colwell 2000; Lleò *et al.* 2001; Keep *et al.* 2006). In addition, conventional culture-based methods usually take 1–2 days or sometimes 7 days depending on species or media types (Camper 2004). qPCR analysis showed that PAA could only achieve 1-log reduction (Figure 1). PAA is thought to act by releasing active oxygen or producing oxidized sulfhydryl bonds, which attack bacterial cells, disrupt cell walls and membranes, and target enzymes and the base of the bacterial DNA (Kitis 2004; Koivunen & Heinonen-Tanski 2005a). Therefore, since qPCR assay detects the target DNA sequence, regardless of their intact cell structure or viability, it is obvious that qPCR may overestimate the true survival rates of *E. faecium*, indicating that qPCR may not be an accurate assessment tool in this disinfection case.

B. subtilis spores have been used as a surrogate to assess the disinfection efficiency of *Cryptosporidium* oocysts or *Giardia* cysts during water treatment processes (Facile *et al.* 2000; Bitton 2005). There are three main advantages of using *B. subtilis* spores as a surrogate: their lack of pathogenicity on humans, easy handling of culture, and straightforward quantification (Dow *et al.* 2006). It was found that *B. subtilis* spores were very resistant to PAA disinfection, especially compared to *E. faecium* (Figures 2 and 3). Three-log reductions were achieved with the concentration (C) × time (t) value of 15,000 mg × min L^{-1} for the spores whereas only 50 mg × min L^{-1} was needed for the 3-log reduction of *E. faecium* (Figures 1 and 3). It is not surprising that spores are very much resistant to chemicals (Kitis 2004; Setlow 2006). The main factors of disinfection resistance are: (1) spore coat proteins that likely react with and detoxify chemical agents; (2) low impermeability of the spore's inner membrane; (3) the protection of spore DNA by its saturation with α/β-type small acid-soluble spore proteins; and (4) DNA repair for agents that eliminate spores by damaging their DNA (Setlow 2006). SEM images clearly showed empty spore shells when treated with PAA while untreated spores remained intact (Figure 5(a)). The damage to the external layers of spores was more severe as the concentration of PAA increased, indicating its increased sporicidal activity (Figures 5(b) and 5(c)). This observation implied that PAA appears to cause damage to the external layers of spores, which is consistent with previous studies (Kitis 2004; Setlow 2006; Rutala *et al.* 2008).

The reduction rates of spores measured by the three methods varied depending on PAA concentration. Plate count method and filtration-based ATP bioluminescence showed different spore reduction rates when 500 ppm of PAA was used, whereas both methods showed similar spore reduction rates against 3,000 ppm of PAA (Figures 2 and 3). Lower reduction rates of spores measured with the plate count method compared to the ATP assay (500 ppm PAA) indicated that PAA could harm spores to some extent at the concentration of 500 ppm. However, it did not completely inactivate the spores. In contrast, similar reduction rates were observed with both methods when 3,000 ppm of PAA was used. These results indicated that this high amount of PAA had totally inactivated the spores. CDC recommends that concentrations ranging from 500 ppm to 10,000 ppm PAA are enough to decontaminate bacterial spores in health settings (Rutala *et al.* 2008). Based on our analysis, we could suggest that 500 ppm PAA may not be sufficient and that a higher dose is needed to ensure a safe level of disinfection.

Male-specific (F^+) coliphage is a surrogate for human viral contamination in ground water proposed by the USEPA (USEPA 2001). Because viral infection may be the causative agent of approximately 50% of all acute gastrointestinal illnesses, it is useful to assess the disinfection efficiency of PAA against F^+ coliphage (USEPA 2001; Bitton 2005; Jiang *et al.* 2007). Our study showed that coliphages were more resistant than enterococci (Figures 1 and 4). Approximately 3- or 4-log reduction of coliphages was achieved with 50 ppm PAA, which was 10 times higher than the amount of PAA required to achieve the same degree of *E. faecium* reductions. This observation agrees well with previous findings (Kitis 2004; Koivunen & Heinonen-Tanski 2005b; Zanetti *et al.* 2007). It seems that PAA had a greater effect on the viability of coliphages than on their RNA at the beginning of contact time (15 min), but as the time went by (60 min), it also affected the RNA of coliphages because the reduction of coliphages was measured similarly (~4-log reduction) over the 1-h contact time with both methods (Figure 4).

CONCLUSIONS

The results from this study show that PAA has a good potential to inactivate microbial contaminants in urgent situations

within a reasonably short contact time. Its disinfection efficiency differed based on the type of targeted microbes. Bacteria are the most susceptible to the action of PAA, followed by viruses and spores. The results also support that filtration-based ATP bioluminescence is capable of detecting bacterial cells rapidly (~5 min) and is a more reliable method than the other two methods. SEM observation showed that PAA targeted the external layers of spores. Our study also suggests that PAA may be applied as a disinfectant in ground water contaminated with sewage, and treatment of municipal wastewater prior to discharge into receiving water bodies. Further studies with different types of water (e.g., wastewater, surface water) and mixed microbial groups are warranted to expand the applicability of PAA.

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