Evaluation of *Bacillus subtilis* and coliphage MS2 as indicators of advanced water treatment efficiency

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Abstract The assessment of water treatment facilities for their efficiency using alternate indicators is of paramount importance. Current methods for assessing efficiency are limited by the specific characteristics of the microorganisms, such as their different sensitivities to disinfectants. A pilot study was carried out to compare different treatment scenarios for the future upgrade of the Sergio Cuevas Water Treatment plant (the largest in the Caribbean) in San Juan, Puerto Rico. The treatment units under investigation included a coagulation-flocculation-sedimentation unit, dual-media filters, micro-filtration units, intermediate ozone injection and contact columns as well as a biological filtration unit. The plant was challenged at different stages of treatment with *Bacillus subtilis* spores and MS2 coliphages in an attempt to test them as possible alternate indicators of treatment plant performance. These organisms were chosen because of their resistance to disinfection and desiccation, their low analysis costs and ease of detection. The removal of spores and coliphages by each treatment unit tested was calculated by seeding a known concentration (5–7 log$_{10}$) of spores and coliphages and following the removal or disinfection rates. The seeded indicators were detected using traditional culture techniques. Ballasted clarification was shown to be highly efficient at removing 99.1% (~3 log$_{10}$) of the spores and 85.1% (~0.86 log$_{10}$) of MS2. Ozone treatment inactivated 80.37% (~1.4 log$_{10}$) spores and 99.95% (~3.07 log$_{10}$) coliphages. The coliphage inactivation rate obtained confirmed data obtained by previous studies indicating that MS2 was less resistant to ozonation than *B subtilis* spores. The membrane technology had the best efficiency in terms of physical removal of spores achieving over 99.9% (>3 log$_{10}$) removal. Coliphage removal mechanisms remain to be determined and will be a future focus of the study. Preliminary results indicate that aerobic spores and coliphages may be useful as indicators to determine the efficiency of different drinking water treatment technologies.

Keywords Aerobic spore formers; *Bacillus subtilis*; ballasted clarification; coliphages; membrane technologies

Introduction

Treating water for consumption by conventional methods is sometimes inadequate for the effective control of certain pathogens such as *Cryptosporidium parvum* (Venczel et al., 1997). In order to obtain water of high microbiological quality and improve the treatment facilities, it is necessary to establish an appropriate microbiological surrogate that can serve as a tool for the monitoring of treatment efficiency and to optimise treatment plants. Nieminski et al. (2000) showed that aerobic spores were present in high numbers in raw waters, these high concentrations favouring their use as direct indicators of treatment plant performances. Additionally, some spores of the genus *Bacillus*, are known to be extremely resistant to heat, ultraviolet light, desiccation and exposure to disinfectants (Doih and McGloughlin, 1992). These characteristics make *Bacillus* spores an excellent alternative to currently used indicators which suffer from one or more shortcomings such as poor or no correlation with the removal of pathogens (Nieminski, et al., 2000). Microbial spores have been used previously by others when measuring the efficiency of removal of pathogens. Payment and Franco (1993) used anaerobic spores and found that they most closely followed the removal and inactivation of enteric viruses and protozoa. However, anaerobic
spores are not easily handled in laboratories and their use may present some problems because of the special incubating conditions required for their growth. Aerobic spores were proposed as indicators of virus disinfection because of their ease of detection and resistance characteristics (Toenniessen and Johnson, 1970), and Barbeau et al. (1997) tested several methods for their enumeration in drinking water. In the present study, B subtilis spores were used to measure the efficiency of microbial removal/inactivation in a pilot treatment plant in San Juan, Puerto Rico. Additionally, the coliphage MS2 was used in parallel to determine its possible use as a future indicator of treatment plant efficiency. The MS2 coliphage was proposed as an indicator because it represented viruses similar in size and shape to human enteric viruses, and was more stable and resistant to disinfection in environmental samples than human enteroviruses (Fujioka, 2002).

Materials and methods

Treatment technologies

The pilot plant used was located at the largest Caribbean water treatment plant, Sergio Cuevas, in San Juan, Puerto Rico. Treatment units were (i) a coagulation-flocculation-sedimentation unit, (ii) micro-filtration units and (iii) ozonation contact columns. The clarification unit could be operated under two different flocculation and settling modes: (a) typical conventional flocculation-lamellar settling and (b) micro-sand ballasted flocculation-lamellar settling.

Under the micro-sand ballasted mode, the three mixing tanks had a total contact time of 10 min at a nominal flow rate of 132 gpm (594 L/min) that could be increased to 264 gpm (1,188 L/min). The surface hydraulic loading rate ranged from 16–32 gpm/ft² (800–1,600 L/min/m²). The three mixing tanks were coagulation, injection, and maturation. The coagulant was introduced allowing for a 2 min contact time followed by the injection tank where micro-sand and a polymer were introduced for a 2 min mixing time. In the maturation basin 6 min of mixing were allowed at lower mixing intensity to help mature the sand-ballasted flocs before entering the up-flow lamellar clarifier. Two micro-sand recirculating pumps were installed (one in backup) to pump the sludge through hydrocyclones to separate the micro-sand from the sludge for reuse. In addition, the unit included a settled water tank that was used to pump settled water to the micro-filtration units. Another settled water-mixing tank was used in the unit for different testing purposes such as adding powdered activating carbon before the membranes and pH adjustment.

The micro-filtration units consisted of two different membrane technologies: pressure vessels (CMF) and submerged membranes (CMF-S). Both were skid mounted and each had its own compressor for air backwashing. Pressure vessels were composed of three membrane modules each having a capacity to treat 7–10 gpm (31.5–45.0 L/min). Water was pumped from a settled water break tank through the membranes. The unit was completely automated with automatic self-cleaning. The submerged membrane unit (also completely automated) was composed of four modules each having a capacity of 6–8 gpm (27.0–36.0 L/min). The modules are directly submerged in the settled water break tank where suction pressure was applied.

The ozonation unit consisted of one ozone generator, two contact columns connected in series for ozone transfer and all equipment required to measure ozone residual in air or water. Each contact column was 10 inches (25 cm) in diameter and 22 ft (6.7 m) high. At the nominal flow of 20 gpm (90 L/min), each contact column allows for 4.5 min contact time. The generator produced O3 from pure oxygen for better production efficiency and had a capacity to apply 20 mg/L at nominal flow. The off-gas was recovered from each contact column and sent to an ozone furnace for destruction.
Bacteria and coliphage

*B. subtilis* spores and MS2 (5–6 log_{10}) were injected in each of the treatments to be tested and the percent removal/inactivation determined.

Sporulation and harvesting

A late-log inoculum of *B. subtilis* (ATCC 6633) vegetative cells was inoculated into sterile Tryptic Soy Broth and incubated at 35°C for 24 h. A dilution of the resulting stock was prepared using phosphate buffer (42.5 mg/L KH_{2}PO_{4}, 40.6 mg/L MgCl_{2}.6H_{2}O, 20 mg/L MnCl_{2}, pH 7.0) and inoculated onto R2A agar (35°C, 124 h) until 99% of the cells had sporulated as observed by phase-contrast microscopy. The R2A media was flooded with phosphate buffer, the spore layer scraped with a sterilised bent glass rod and spores collected by centrifugation at 3,000 rpm (5 min, 4°C). The resulting pellet was washed ×4 with phosphate buffer/0.1% Tween-80 (to preclude clumping).

Coliphages

The coliphage MS2 was used in the study using *E. coli* C3000 (ATCC 15597) as the host bacterium. The stock solution was prepared by infecting an overnight culture of the bacterium and then allowing the phage to replicate using the double-layer method. The progeny phages were collected by scraping the top layer, vortexing in a phosphate buffer, centrifuging as above with the supernatant being collected and titred.

Injection and sampling

For each treatment, *B. subtilis* spores and MS2 phages were injected in the system in order to have approximately 6 log_{10} of each indicator. The stock suspensions of spores and coliphage were diluted in settled water, the volume used being dependent on the residence time in each treatment. A peristaltic pump was used to dose a constant flow (50 mL/min) of the solution. Samples were collected every 15 min into sterile 1 L plastic bottles containing sodium thiosulphate and analysed (in duplicate) within 6 h.

Clarification unit – ballasted mode

Under the ballasted mode, spores and phages were seeded over a period of 2 h. No samples were taken during the first 45 min to allow system stabilisation after which samples were collected every 15 min.

Ozone

To evaluate the ozonation system, spores and phages were injected for 2.5 h with the first 90 min for system stabilisation after which samples were taken as above. Samples were – settled water background, settled water seeded and ozonated water.

Pressure vessels and submerged membranes

The micro-filtration system was seeded over a period of 75 min. The first 10 min allowed system stabilisation before sampling. Samples were settled water background, break tank water (seeded water) and filtered water (in the effluent of each membrane unit).

Detection and enumeration of indicators

Aerobic spore-forming bacteria were enumerated by membrane filtration (47 mm, 0.45 µm; HAWG047s3, Millipore; APHA, 1992; Barbeau *et al.*, 1997). Spore-forming bacteria were enumerated by placing membranes onto the medium, which were then pasteurised for 15 min at 75°C (Barbeau *et al.*, 1997) and incubated at 35°C for 24 h before counting colonies. MS2 phage was enumerated by the single-layer method by adding 100 mL sample
to 100 mL of 2× Tryptic Soy broth containing 15 g agar. A fresh culture of *Escherichia coli* C3000 (5 mL) was then added. All plates were enumerated after 6 h and 12 h to avoid possible background bacterial overgrowth that may mask the viral plaques.

### Results and discussion

Results showed that all the seeded treatment units performed well in terms of spore and coliphage removal. However, some tendencies were observed by comparing results obtained by the different treatment units. Ballasted clarification gave a 99.1% removal which could be explained by the more efficient turbidity and particle removal achieved by the ballasted mode observed throughout the testing (Table 1).

For coliphage removal, the ballasted mode achieved 85.1% (<1 log₁₀) removal proving its effectiveness in physically removing more particles and hence turbidity (Table 2). These results were consistent with previous studies on sand ballasted clarifiers (Rastogi *et al.*, 1999; Alvarez *et al.*, 1999).

Membrane technologies showed a high efficiency achieving a removal of 99.99% (4 log₁₀). With ozone treatment 99.95% MS2 was inactivated but only 80.37% of the spores. The results also indicated that both aerobic spores as well as coliphages, seemed to work well as surrogate indicators of water treatment efficiency. As expected there was a higher removal/inactivation of MS2 than for spores confirming the greater ozone resistance of spores. The results showed that aerobic spore formers can be found in all steps of the treatment plant (data not shown) but, upon analyses, the different morphotypes indicated the presence of different microorganisms. This may possibly preclude the analyses of these naturally-occurring spore formers as indices of water treatment efficiency. Similarly, coliphages were also found in the influent water. However, the low concentration of these coliphages may also preclude their use as an index of treatment efficiency. We are currently using molecular fingerprinting to trace the seeded spores. This will allow us to determine if colonisation of the system by these bacteria under natural conditions is possible, and to trace only the seeded spores, eliminating the background noise as a result of the naturally occurring spore formers.

### Conclusions

Aerobic spores and coliphages can be used to evaluate or improve treatment plant performance as our results consistently showed throughout the study. Naturally occurring coliphages may not be used as indices of water treatment performance. More studies need to be done to determine if the viability or removal of seeded spores correlated to those of

**Table 1** Aerobic spore removal/inactivation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean influent CFU/L (± SD)</th>
<th>Mean effluent CFU/L (± SD)</th>
<th>Mean removal/inactivation (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ballasted clarification (n = 2)</td>
<td>2.08 × 10⁵ (5.80 × 10⁴)</td>
<td>1.63 × 10³ (9.30 × 10²)</td>
<td>99.1 ± 0.5</td>
</tr>
<tr>
<td>CMF (n = 3)</td>
<td>8.55 × 10⁵ (8.0 × 10⁵)</td>
<td>4.54 (6.64)</td>
<td>99.99 ± 0.001</td>
</tr>
<tr>
<td>CMF-s membranes (n = 2)</td>
<td>8.83 × 10⁵ (7.5 × 10⁵)</td>
<td>1.11 (1.75)</td>
<td>99.99 ± 0.00001</td>
</tr>
<tr>
<td>Ozone (av dose 74.17 g/m²)</td>
<td>1.45 × 10⁶ (7.81 × 10⁵)</td>
<td>3.21 × 10⁵ (3.93 × 10⁵)</td>
<td>99.37 ± 18.9</td>
</tr>
</tbody>
</table>

**Table 2** Removal/inactivation of MS2 coliphage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean influent PFU/L (± SD)</th>
<th>Mean effluent PFU/L (± SD)</th>
<th>Mean % removal (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ballasted clarification</td>
<td>1.35 × 10⁶ (3.0 × 10⁵)</td>
<td>2.0 × 10⁴ (8.7 × 10⁵)</td>
<td>85.11 ± 0.77</td>
</tr>
<tr>
<td>Ozone (av dose 74.17 g/m²)</td>
<td>1.0 × 10⁵ (1.0 × 10⁵)</td>
<td>5.32 × 10¹ (8.3 × 10¹)</td>
<td>99.95 ± 0.027</td>
</tr>
</tbody>
</table>
protozoan parasites. The latter will allow us to better protect public health from waterborne pathogens.

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References


