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Research article

Decontamination of *Bacillus* spores adhered to iron and cement-mortar drinking water infrastructure in a model system using disinfectants

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ABSTRACT

Decontamination of *Bacillus* spores adhered to common drinking water infrastructure surfaces was evaluated using a variety of disinfectants. Corroded iron and cement-mortar lined iron represented the infrastructure surfaces, and were conditioned in a 23 m long, 15 cm diameter (75 ft long, 6 in diameter) pilot-scale drinking water distribution pipe system. Decontamination was evaluated using increased water velocity (flushing) alone at 0.5 m s^{-1} (1.7 ft s^{-1}), as well as free chlorine (5 and 25 mg L^{-1}), monochloramine (25 mg L^{-1}), chlorine dioxide (5 and 25 mg L^{-1}), ozone (2.0 mg L^{-1}), peracetic acid 25 mg L^{-1}) and acidified nitrite (0.1 mol L^{-1} at pH 2 and 3), all followed by flushing at 0.3 m s^{-1} (1 ft s^{-1}). Flushing alone reduced the adhered spores by 0.5 and 2.0 $\log_{10}$ from iron and cement-mortar, respectively. $\log_{10}$ reduction on corroded iron pipe wall coupons ranged from 1.0 to 2.9 at respective chlorine dioxide concentrations of 5 and 25 mg L^{-1}, although spores were undetectable on the iron surface during disinfection at 25 mg L^{-1}. Acidified nitrite (pH 2, 0.1 mol L^{-1}) yielded no detectable spores on the iron surface during the flushing phase after disinfection. Chlorine dioxide was the best performing disinfectant with >3.0 $\log_{10}$ removal from cement-mortar at 5 and 25 mg L^{-1}. The data show that free chlorine, monochloramine, ozone and chlorine dioxide followed by flushing can reduce adhered spores by > 3.0 $\log_{10}$ on cement-mortar.

1. Introduction

Bacillus spores are resistant to disinfection and other inactivation methods relative to vegetative bacteria (Young and Setlow, 2003; Rose et al., 2005; Raber and Burklund, 2010). Due to their resistance to disinfection, inactivation of spores can be used as a benchmark for decontamination of biological agents from drinking water infrastructure following intentional or unintentional contamination. *Bacillus* spores are persistent on common drinking water infrastructure surfaces like corroded iron (Szabo et al., 2007; Hosni et al., 2011) and cement-mortar (USEPA, 2008; Shane et al., 2011). Spore germination followed by chlorine disinfection has been studied as a decontamination method for *Bacillus* spores on home plumbing materials (PVC and copper) using L-alanine and inosine (Morrow et al., 2008) and in a pilot-scale system using nutrient media (Szabo et al., 2012). Though effective, germination may not be practical over a wide area of a distribution system due to the large amount of germinant that would need to be added. Therefore, data is needed on the decontamination of spores from drinking water infrastructure using common disinfectant solutions, or disinfectants that can be formulated using widely available equipment and reagents.

Free chlorine and monochloramine are common distribution system disinfectants that are ideal biological decontamination agents as they are well known and widely applied in the drinking water industry. Ozone and chlorine dioxide have some niche applications at water treatment plants focused on disinfection, oxidation and taste and odor control (AWWA, 1999). They are strong oxidants, but their reactivity may limit their application in a distribution system to small areas or lengths of pipe. Peracetic acid (PAA) is commonly used in the food and beverage industry for

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cleaning processing equipment and pipes, and has found use in the medical device industry (McDonnell and Russell, 1999; USEPA 2012). The acceptance of PAA in the food and medical industry may make it a more palatable decontaminating agent in drinking water. Acidified nitrite is not a widely used disinfectant, but it can be effective against spores in water, and it uses common reagents (Szabo et al., 2014). Flushing is a common practice in drinking water distribution systems and is easily implemented (although large volumes of contaminated water could be generated).

This study examines the effectiveness of decontaminating *Bacillus globigii* spores attached to corroded iron and cement-mortar coupons with free chlorine at two pH levels, monochloramine, chlorine dioxide, ozone, peracetic acid (PAA) and acidified nitrite, followed by flushing. Data on flushing alone is also presented to show the persistence of spores on water infrastructure without high levels of disinfectants present. The goal of this study is to present a summary of data on spore decontamination from water infrastructure, which comes from a pilot-scale experimental system that simulates real word drinking water infrastructure surfaces.

2. Materials and methods

2.1. Bacterial strains and cultures

Bacillus atrophaeus subsp. *globigii* (*B. globigii*) has been used as a surrogate for pathogenic *B. anthracis* in disinfection studies since it is more resistant to free chlorine (Sivaganesan et al., 2006). *B. globigii* spore preparation is presented in detail elsewhere (Nicholson and Setlow, 1990). Briefly, tryptic soy broth (TSB) was inoculated with *B. globigii* cells and incubated for 5 days at 35 °C with gentle shaking in a rotary shaker. Purified *B. globigii* endospores were produced using gradient separation. The presence of spores was confirmed using phase-contrast microscopy (<0.1% vegetative cells). Purified spores were stored in 40% ethanol at 4 °C. Before an experiment, purified spores were reintroduced into TSB and cultured in their vegetative form for 5 days at 35 ± 0.5° C and allowed to sporulate. The resulting spore suspension was used in experiments.

2.2. Pilot scale drinking water distribution system

The drinking water distribution system simulator (DSS) is shown in Fig. 1 and has been described previously (USEPA, 2008; Szabo et al., 2012). Briefly, the DSS consists of 23 m (75 ft) of 15 cm (6 inch) diameter PVC pipe connected in a rectangular shape to an in-line recirculation tank. Total DSS volume is 832 L (220 gal). A 3780 L (1000 gal) feed tank supplied Cincinnati tap water to the DSS. This setup allows tap water to enter and exit the DSS while still having flow circulating around the rectangular pipe to impose shear on the inner pipe surfaces. Cincinnati tap water flowing into and out of the DSS was set at 3.78 L min^{-1}, which maintained stable water quality. Free chlorine and pH ranged from 0.9 to 1.1 mg L^{-1} and 8.4 to 8.6, respectively, pressure ranged from 68,950 to 82,740 Pa (10–12 psi), and temperature fluctuated between 25 °C and 30 °C. These flow and water quality values were the baseline operating conditions unless otherwise noted. Flow circulating around the loop that affects shear forces on the coupons and pipe inner surface was set at 314 L min^{-1} (83 gal min^{-1}), which resulted in a flow velocity of 0.3 m s^{-1} (1 ft s^{-1}). When flushing was used as a decontamination method, flow was increased to achieve a flow velocity of 0.5 m s^{-1} (1.7 ft s^{-1}).

Thirty 6.5 cm^2 coupons were inserted flush with the pipe inner surface. Half were unlined iron cut from a drinking water main and half were cement-mortar made using AWWA C104/A21.4–08 (AWWA, 2008). Coupons were allowed to condition and form biofilm in the DSS for 1 month prior to contamination.

2.3. Experimental procedure

Coupon surfaces were sampled before spores were injected to ensure that no detectable spores were present. Spore injection achieved 1×10^6 cfu/ml in the DSS bulk phase, which was allowed to circulate with flow present for 2 h. Spores were flushed out of the DSS after the 2 h contact period. Thereafter, a disinfectant was introduced into the DSS, mixed in the bulk phase and held for 22 h (target concentrations of 5 and/or 25 mg L^{-1}, or 0.1 M for acidified nitrite). During the decontamination period, no flow was present and the water was stagnant. The only exception to this protocol was ozone (2 mg/L), where decontamination occurred for 12 h in the presence of flow at 314 L min^{-1} before flushing. After the decontamination period, any remaining disinfectant was flushed out of the DSS and water was subsequently recirculated around the DSS at a flow velocity of 0.3 m s^{-1} (1 ft s^{-1}) for 22 h to flush any remaining spores off of the coupon surfaces. Coupon and bulk water sampling times are listed in Tables 1 and 2.

Infrastructure coupons were removed by unscrewing them from the pipe. Biofilm and corrosion particles were removed from the coupon using a sterile scalpel and suspended in 100 ml of 0.05 mol L^{-1} sterile KH_2PO_4 buffer (pH 7.2). The buffer was supplemented with 0.1 ml of sterile 10% (wt/vol) sodium thiosulfate when disinfectant was present. Suspended biofilm and corrosion particles were dispersed for 30 s using a tissue homogenizer. The homogenized suspension was vortexed and then serially diluted. *B. globigii* was enumerated by spread plating on tryptic soy agar (TSA) plates and incubating for 24 ± 2 h at 35° C. All samples were plated in duplicate.

2.4. Decontaminating agents

Free chlorine came from 10% Champion (Woodridge, IL) bleach/pool shock and was injected directly into the pipe system to achieve the desired free chlorine concentration. The pH was adjusted using concentrated (12.1 N) hydrochloric acid. Monochloramine was prepared by adding reagent grade ammonium hydroxide and free chlorine (10% bleach/pool shock) to the pipe system at a 5:1 Cl_2:N ratio by weight to obtain a 25 mg L^{-1} monochloramine concentration in the loop water. Chlorine dioxide came from TwinOxide® (Best, Netherlands) pouches, the contents of which were diluted in the DSS to achieve the desired concentration. Ozone was made using a Clearwater Tech, Inc. (San Luis Obispo, CA) CD 2000 ozone generator with an air flow of 9 standard cubic feet per hour (SCFH) and vacuum of 5.4 in Hg and injected into the DSS through the suction side of a pump. PAA was added directly to the pilot scale system using dilutions of Solvay Chemicals, LLC (Brussells, Belgium) Proxitane® WW-12 microbiocide (18.5% hydrogen peroxide, 12% peroxyacetic acid). Acidified nitrite was prepared by first injecting concentrated (12.1 N) hydrochloric acid into the pipe system and lowering the pH to 2 or 3. Reagent grade sodium nitrite dissolved in deionized water was then added to the pipe system and allowed to mix. If needed, disinfectants were diluted in deionized water before introduction into the pilot scale pipe system.

2.5. Measurement of disinfectant concentrations

Free chlorine and monochloramine residuals were measured using a Hach® (Loveland, CO) DR/2400 spectrophotometer using Hach methods 8021 (DPD) and 10,171 (indophenol), respectively. AccuVac® Ampules containing N,N-Diethyl-p-phenylenediamine (DPD) were used for free chlorine analysis and powder pillows