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Technical Note

Disinfection of *Bacillus* spores with acidified nitrite

Jeffrey G. Szabo [a,*], Noreen J. Adcock [b], Eugene W. Rice [a]

[a] U.S. Environmental Protection Agency, National Homeland Security Research Center, Water Infrastructure Protection Division, Cincinnati, OH 45268, USA
[b] U.S. Environmental Protection Agency, National Risk Management Research Laboratory, Water Supply and Water Resources Division, Cincinnati, OH 45268, USA

HIGHLIGHTS

- Acidified nitrite can be an effective disinfectant against spores.
- High ionic strength may inhibit the acidified nitrite disinfection.
- Disinfection is inhibited by lower temperature and higher pH.

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ABSTRACT

Disinfecting water generated from a bioterrorism contamination event will require large amounts of disinfectant since the volume of water flushed from a drinking water distribution system or wash water collected from a contaminated outdoor area can accumulate quickly. Commonly used disinfectants may be unavailable in the necessary amounts, so evaluation of alternative disinfectants is needed. This study focuses on disinfection of *Bacillus* spores in water using acidified nitrite. The effect of varying pH (2 or 3), temperature (5 °C or 24 °C), nitrite concentration (0.01 or 0.1 M), buffer (Butterfields or Phosphate Buffered Saline, PBS) and *Bacillus* species (*B. globigii* and *B. anthracis* Sterne) was evaluated. *B. globigii* was more resistant to disinfection under all water quality conditions. Disinfection was more effective for *B. globigii* and *B. anthracis* Sterne at 0.1 M nitrite, pH 2, and 24 °C. Disinfection of *B. anthracis* Sterne was enhanced in low ionic strength Butterfields buffer compared to PBS.

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1. Introduction

Inactivation of pathogenic *Bacillus* spores and surrogates with conventional drinking water disinfectants such as free chlorine and chloramines has been well studied (Brazis et al., 1958; Rice et al., 2005; Hilgren et al., 2007; Rose et al., 2007). This is also true for other water disinfectants such as ozone and chlorine dioxide (Foegeding, 1985; Larson and Mariñas, 2003; Hosni et al., 2009; Shams et al., 2011). This has prompted spore inactivation studies with novel disinfection methods such as plasma discharges, modified Fenton reagent, electrochemical chlorine generation, and a multitude of non-traditional water disinfectants (Russell, 1990; Abou-Ghazala et al., 2003; Cross et al., 2003; Lee et al., 2006; Raber and Burklund, 2010; Giovannozzi et al., 2011). This body of peer-reviewed literature shows that methods for *Bacillus* spore inactivation in water are plentiful, but not all are practical for various situations where water requires treatment. For example, chlorination or ozone can be implemented at a drinking water treatment plant, but Fenton's reagent or plasma discharges are not routinely used.

Should a water distribution system or indoor/outdoor area be contaminated with pathogenic *Bacillus anthracis* spores during a bioterrorist event, a large volume of water may need to be disinfected whether it was flushed from a distribution system or collected from washing down indoor or outdoor surfaces. Disinfection methods commonly used by the drinking water industry or procedures from the peer-reviewed literature would be the first place to look for an appropriate technique to inactivate *Bacillus* spores in contaminated tap water or wash water. However, disinfecting large volumes of water in the field can carry unique challenges not experienced at a drinking water treatment plant or in a laboratory. These include transport of large quantities of chemicals (e.g. for free chlorine or chloramines), placement and installation of large pieces of machinery (e.g. chlorine dioxide or ozone generators) and neutralization of high concentrations of residual disinfectant

* Corresponding author. Tel.: +1 513 487 2823.
E-mail address: szabo.jeff@epa.gov (J.G. Szabo).

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before final disposal (for chlorine, chloramines, or chlorine dioxide).

An alternative disinfectant that has not been widely studied for inactivation of spore forming bacteria and may avoid some of these logistical obstacles is acidified nitrite. Nitrite salts are cheap and available in large quantities, although transportation of these salts to a contaminated water source may pose some challenges. No mechanical generator is needed to produce nitrite, only adequate mixing in the water. Finally, disinfected water containing nitrite could be discharged to a municipal sewer without neutralization with a reducing agent, though pH adjustment may be required.

Acidification of nitrite salt acts as a disinfectant by forming nitrous acid, nitric oxide, peroxynitrite, dinitrogen trioxide, and nitrogen dioxide (Dykhuizen et al., 1998; Phillips et al., 2004). These compounds, particularly nitric oxide, can readily diffuse across cell membranes (Denicola et al., 1996) and can damage cells through direct oxidation (Fang, 1997) or formation of nitrosothiols (De Groote et al., 1995) and disruption of DNA replication (Nakaki et al., 1990). Acidified nitrite has been shown to be an effective disinfectant against microorganisms such as *Helicobacter pylori* (Dykhuizen et al., 1998), *Mycobacerium ulcerans* (Phillips et al., 2004), spores of *Clostridium difficile* (Wullt et al., 2003; Rao et al., 2006), and *Pseudomonas aeruginosa* (Yoon et al., 2006; Major et al., 2010). Beyond one isolated experiment, acidified nitrite inactivation of *Bacillus* spores has not been studied (Heaselgrave et al., 2010).

The current study examines inactivation of spores of *B. anthracis* Sterne and *Bacillus globigii* in water with acidified nitrite. Batch experiments were conducted at room temperature (~24 °C) and 5 °C and pH 2 and 3. Test conditions were replicated in phosphate buffered saline (PBS) and Butterfield's buffer to replicate waters with different ionic strengths. Results are presented on how water quality affects inactivation with acidified nitrite and how inactivation under these experimental conditions compares to traditional disinfectants.

2. Materials and methods

2.1. Bacillus spores generation and storage

B. globigii was obtained from Dugway Proving Ground (Dugway, UT) and *B. anthracis* Sterne was obtained from Centers for Disease Control and Prevention (Atlanta, GA). Briefly, generic spore medium was inoculated with vegetative *Bacillus* cells and incubated for 5 d at 35 °C with gentle shaking in a rotary shaker. Purified *Bacillus* endospores were produced using gradient separation as previously described (Nicholson and Setlow, 1990). The presence of spores was confirmed using phase-contrast microscopy (<0.1% vegetative cells). Spores were stored in 40% ethanol at 4 °C until use.

2.2. Acidified nitrite inactivation test protocol

10 mL of *Bacillus* spores were centrifuged (5900× g) for 15 min and resuspended three times in 10 mL of sterile PBS (ThermoFisher Scientific, Waltham, MA) or Butterfield's buffer (Hardy Diagnostics, Santa Maria, CA). A fourth centrifuged sample was resuspended in 10 mL of sterile PBS or Butterfields buffer at the test pH. PBS contained 0.14 M NaCl, 0.01 M KCl and 0.01 M KH_2PO_4, and Butterfields contained 3.10×10^{-4} M KH_2PO_4. A sterile 500 mL glass beaker with a sterile stir bar containing 150 mL of sterile PBS or Butterfields's buffer adjusted to pH 2–3 was used for these experiments. An aliquot of the washed spore suspension was added to the test beaker to achieve an initial spore density of 6.0 ± 0.5 $\log_{10}$ CFU mL^{-1}. The test began when an aliquot of a stock sodium nitrite (ThermoFisher Scientific, Waltham, MA) solution was added to the test beaker to achieve an initial nitrite concentration of 0.01 M or 0.10 M. The test beaker and sodium nitrite solution were allowed to equilibrate to room temperature (24 °C ± 1 °C) or at low temperature (5 °C ± 1 °C) before the experiment began. pH was measured before nitrite introduction and at the end of the test. The stir bar was activated during spore and nitrite introduction, was allowed to stir for 2 min and was then turned off. In addition to the test beaker, control experiments identical to the test experiments were undertaken at pH 2 and 3 with no nitrite present to determine any spore inactivation due to pH effects alone. All test and control experiments were performed in duplicate.

Samples were removed at regular intervals of 0 min (spores stirred in the beaker for 2 min with nitrite not added), 60 min, 300 min, 1440 min (1 d) and 2880 min (2 d). Additional sample points were added if preliminary tests indicated that inactivation would proceed at a rate such that an adequate inactivation profile could not be captured within the standard sampling times. Sample points were timed with a NIST traceable timer. The stir bar was started 2 min before each sampling point to ensure adequate mixing in the beaker, and was turned off after the sample was removed. 1 mL was removed from the reaction vessel at each sample point and added to 9 mL of nutrient broth to quench the reaction. Further serial dilutions up to 10^{-5} occurred in PBS.

2.3. Bacillus enumeration

Initial densities and the number of survivors were determined using the spread plate method which is described in Standard Method 9215C (APHA, 2012). Samples were usually plated immediately, but were occasionally stored at 4 °C for no longer than 2 h. 0.1 mL was plated on tryptic soy agar (TSA) (ThermoFisher Scientific, Waltham, MA), except when densities were low enough that 1.0 mL was needed. For these samples, 0.2 mL aliquots were spread onto each of five plates and the number of colony forming units CFU mL^{-1} was recorded as the sum of the CFU from the five plates. This yielded a detection limit of 10 CFU mL^{-1} in the test beaker. Plates were incubated at 37 °C for 24 h. All samples were plated in duplicate.

2.4. Data analysis

Data shown in Table 1 are the $\log_{10}$ transformed value of the mean of two duplicate experiments for each experimental condition. The time to achieve a 5-log inactivation was determined by linear interpolation between two time points on either side of the 5-log inactivation threshold. Linear extrapolation was used to determine the time to a 5-log inactivation when this level of inactivation was not reached. Ionic strength was calculated as follows: $I = \frac{1}{2}\sum_{i-1}^{n} C_i Z_i^2$, where I is ionic strength (M), c is concentration (M) and z is charge.

3. Results and discussion

Table 1 presents the $\log_{10}$ number of *Bacillus* spores surviving after exposure to acidified nitrite. Note that control studies were performed at each pH with no nitrite present. The largest spore decrease in the control experiments was 0.3 log after 2 d. This indicates that the nitrogen compounds formed during acidification of nitrite were responsible for spore inactivation and not the acidification alone.

The data in Table 1 can be used as a tool to help guide decisions about disinfecting water containing *Bacillus* spores before disposal. The number of $\log_{10}$ survivors at each time point is presented, along with the time required to achieve a 5-log level of

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