

Inactivation of Biological Agents Using Neutral Oxone-Chloride Solutions

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Bleach solutions containing the active ingredient hypochlorite (OCl^-) serve as powerful biological disinfectants but are highly caustic and present a significant compatibility issue when applied to contaminated equipment or terrain. A neutral, bicarbonate-buffered aqueous solution of Oxone ($2\text{K}_2\text{HSO}_5 \cdot \text{KHSO}_4 \cdot \text{K}_2\text{SO}_4$) and sodium chloride that rapidly generates hypochlorite and hypochlorous acid (HOCl) in situ was evaluated as a new alternative to bleach for the inactivation of biological agents. The solution produced a free chlorine ($\text{HOCl} + \text{OCl}^-$) concentration of 3.3 g/L and achieved ≥ 5.8 -log inactivation of spores of *Bacillus atrophaeus*, *Bacillus thuringiensis*, *Aspergillus niger*, and *Escherichia coli* vegetative cells in 1 min at 22 °C. Seawater was an effective substitute for solid sodium chloride and inactivated 5 to 8 logs of each organism in 10 min over temperatures ranging from -5 °C to 55 °C. Sporocidal effectiveness increased as free chlorine concentrations shifted from OCl^- to HOCl . Neutrally buffered Oxone-chloride and Oxone-seawater solutions are mitigation alternatives for biologically contaminated equipment and environments that would otherwise be decontaminated using caustic bleach solutions.

Introduction

Bleach is a well-known biological disinfectant and serves as a convenient source of oxidative chlorine. The use of bleach solutions for neutralization of chemical and biological warfare agents is especially important due to terrorist activities affecting military and civilian populations (1–3). However, bleach possesses some undesirable chemical properties and can be problematic for the inactivation of certain biologicals. The sodium salt of hypochlorite found in commercial bleach is electrochemically generated in sodium hydroxide (pH 11) and is known to decompose upon storage (4–9). Bacterial spores are more resistant than vegetative bacteria to inactivation by free chlorine (10–12), and sporocidal effectiveness is dependent upon pH, temperature, and concentration of the oxidant (13). Live spores of *Bacillus anthracis* still active in the environment after an extensive bleach treatment on Vozrozhdeniye Island deposition site, Uzbekistan (14) indicate that caustic OCl^- solutions can be unreliable for decontamination efforts.

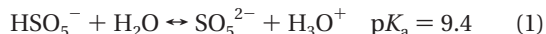
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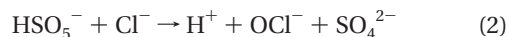
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The malicious routing of anthrax spores through the U.S. Postal Service in 2001 prompted the U.S. Environmental Protection Agency (EPA) to identify bleach as one of a few readily available decontaminants (15). As a follow-up treatment to chlorine dioxide for decontamination of the U.S. Hart Senate Office Building in Washington, D.C., solutions of HOCl were prepared by diluting bleach in water and acidifying with vinegar. Although this formulation is known to effectively kill spores (16), the additional weight burden in handling and transporting two liquids can be logistically cumbersome. This burden could be alleviated by an equally effective, alternative decontaminant that consisted of solid reagents.

An aqueous solution prepared by mixing solid reagents of Oxone, bicarbonate, and chloride salts is proposed as an alternative to bleach. Oxone is an acidic oxidant compound (pH 2) and used in swimming pools and spas to maintain high concentrations of chlorine (Cl_2) and bromine (Br_2) additives. The combination of Oxone with chloride at a low pH has also been used to generate Cl_2 for enhancing lignin degradation of kraft pulps (17, 18). The active species of Oxone, peroxymonosulfate (HSO_5^-), is stable up to a slightly alkaline pH (eq 1).



The oxidative effectiveness of an Oxone solution can be enhanced by the addition of other species under neutral pH conditions. In a previous study, acetone was added to a sodium bicarbonate-buffered Oxone solution to generate the cyclic peroxide dimethyldioxirane, which rapidly inactivated viruses, vegetative bacteria, fungi, protein, and bacterial spores (19, 20). Biological agent inactivation using neutrally-buffered Oxone-chloride solutions (eq 2) has not been described to date.



The hypochlorite ion and its conjugate, hypochlorous acid, possess strong oxidative potential, and the species distribution exists equally in a dilute aqueous solution of pH 7.5 at 25 °C. The species distribution can be manipulated by a change in pH with sodium bicarbonate and/or sodium carbonate. Benign byproducts of the decontaminating solution include sulfate and carbonate salts, CO_2 , and O_2 (an auto decomposition product of Oxone). Seawater serves as an alternative source of chloride in the decontaminating formulation.

This study presents data that characterizes (1) the generation and stability of free chlorine species ($\text{HOCl} + \text{OCl}^-$) in a buffered Oxone-chloride system, and (2) the effectiveness of buffered Oxone-chloride and buffered Oxone-seawater solutions, in comparison to bleach, for the inactivation of biological agents at various temperatures. The results will show the usefulness of these solutions as replacements to caustic bleach solutions for emergency response decontamination efforts.

Experimental Section

Materials. Oxone (triple salt, 42.8% as potassium peroxy-monosulfate), sodium chloride (ACS grade), sodium bicarbonate (99.7+%), ammonium sulfate (99.8%), ferrous sulfate (98%), monobasic potassium phosphate (99%), calcium chloride (96%), potassium chloride (99%), magnesium sulfate heptahydrate (>98%), manganese chloride hydrate (99.999%), and Triton X-100 were acquired from Sigma-Aldrich, Mil-

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waukee, WI. Potassium sulfate (ACS grade), sodium thiosulfate (>98%), sulfuric acid (ACS Plus), sodium hydroxide solution (50% w/w), sodium chlorate (Certified), magnesium chloride (99%), yeast extract (100%), L agar, nutrient broth (NB), and agar were obtained from Fisher Scientific, Pittsburgh, PA. Ethylenediamine (EDA, >99.5%), sodium carbonate (>99.5%), and sodium chlorite (technical grade, 85%) were obtained from Fluka, Buchs, Switzerland. White distilled vinegar (4% acidity) was obtained from Webbpak, Inc., Trussville, AL. Commercial bleach stock solutions (~6% by wt sodium hypochlorite) were obtained from the Clorox Company, Oakland, CA and KIK International, Houston, TX. Deionized water (DI) was collected at 18 MΩ from a Millipore Milli-Q system (Millipore Corporation, Molsheim, France). Seawater was obtained in May and June, 2003 and 2004, from the Gulf of Mexico, Panama City Beach, FL, and stored at 10 °C. The microorganisms, *Bacillus atrophaeus* (ATCC 9372), *Bacillus thuringiensis* (ATCC 35646), *Escherichia coli* (ATCC 15597), and *Aspergillus niger* (ATCC 1004), were purchased from American Type Culture Collection (ATCC), Manassas, VA. *B. anthracis* Sterne strain 34F2 (21) was derived from a veterinary vaccine culture supplied by Colorado Serum Company, Denver, CO.

Free Chlorine Measurements. Batch solutions were prepared in duplicate at 22 °C (±1 °C) using acid-washed glassware. Free chlorine was generated by combining NaCl (5% w/v) to a fresh solution of NaHCO₃ (6% w/v) and Oxone (10% w/v) in DI water. *Caution: Unbuffered solutions of Oxone and chloride will evolve toxic chlorine gas.* A 10⁴-fold dilution in DI water was performed at each assay time and analyzed for free and total chlorine using a Hach DR2000 spectrophotometer (λ=530 nm). *N,N*-Diethyl-*p*-phenylenediamine (DPD; Permachem reagent, Hach, Loveland, CO) was used to derivatize the target chlorine species. Bleach was diluted 10-fold to yield measurements within the range limit of the instrument. The individual concentrations reported for the HOCl and OCl⁻ species were based on standard water equilibrium calculations and log concentration-pH relationships (22).

Separate assays for anion evaluation were diluted 10³-fold in DI water containing 50 mg/mL EDA to quench the free chlorine oxidant. Control solutions of 7.1% w/v K₂SO₄ with 5.4% w/v NaCl were adjusted to pH 7 with H₂SO₄ and treated with the same procedure. A buffered Oxone solution in the absence of chloride served as a positive control. Anion evaluation was conducted by ion chromatography (IC) using a Dionex DX500 IC system with a CD20 conductivity detector. A Dionex IonPac AS9-HC (4 mm) column equipped with an AG9-HC (4 mm) guard column was used for separation of target analytes. The mobile phase consisted of 9 mM Na₂CO₃ running isocratically at 1.2 mL/min. An ASRS-Ultra (4 mm) suppressor, used to lower the background conductivity of the mobile phase, was operated in chemical suppression mode with 25 mM H₂SO₄ at a flow rate of 6 mL/min. Anion standards of chloride, bromide, chlorate, chlorite, and sulfate were prepared in DI water.

Culture Preparations. Spores of *B. atrophaeus* and *B. thuringiensis* were prepared separately by streaking a lawn of each organism onto numerous plates of sporulation agar (18.0 g/L agar, 8.0 g/L nutrient broth, 1.0 g/L KCl, 0.25 g/L MgSO₄·7H₂O, 0.002 g/L MnCl₂·H₂O, 0.5 mM CaCl₂, 0.001 mM FeSO₄) and incubating at 37 °C for 2 weeks. Colonies were harvested after a 95% conversion of vegetative cells to spores (measured by standard spore stain and Gram stain procedures). Spore preparations were heated to 80 °C for 10 min to kill remaining vegetative cells. The viable spore count was then determined by standard dilution and plating methods on L agar (23). Spores were stored at 4 °C. Spore counts used in experiments for *B. thuringiensis* and *B. atrophaeus* were 10⁵–10⁶ CFU/mL and 10⁷–10⁸ CFU/mL, respectively. A

preparation of the *B. anthracis* Sterne strain was performed in a biological safety cabinet by the same procedure. The final spore count was 5.3 × 10⁸ CFU/mL.

Aspergillus niger spore cultures were prepared by streaking a lawn onto several potato dextrose agar (PDA) plates and incubating at room temperature for 10 days until black conidial spores covered all agar surfaces. A small amount (10 mL) of sterile saline solution (5 g/L NaCl, 0.5 g/L Triton X-100) was added to each plate, and the resulting slurry then filtered through sterile cheesecloth into a sterile container. Preparations contained ≥90% spores and were stored at 4 °C. Average cell counts of *A. niger* used for experiments were 10⁵–10⁶ CFU/mL.

Cultures of *E. coli* were prepared by inoculating sterile NBY (8 g/L nutrient broth and 3 g/L yeast extract) and incubating overnight at 37 °C while shaking at 175 rpm. Each culture was no older than 24 h. Average cell counts of *E. coli* cultures were 10⁷–10⁸ CFU/mL.

Bacteria and fungal spore preparations were washed with DI water to eliminate any residual chloride from reagents used in the cell and spore preparations.

Biological Inactivation. Buffered Oxone-chloride solutions were prepared in the same manner as described in the free chlorine experiments unless stated otherwise. Buffered Oxone-seawater formulations were prepared by adding bicarbonate to seawater followed by a slow addition of Oxone. 'Acidified' bleach solutions (EPA formulation) were prepared by a 1:10 dilution of bleach in DI water and adjusting to pH 5 with vinegar.

Experiments were initiated by placing a 1 mL aliquot of cells or spores into 9 mL of reactive oxidant or control solutions at temperatures of -5 °C (±2 °C), 10 °C (±1 °C), 22 °C (±1 °C), and 55 °C (±1 °C). A 1 mL aliquot of the resulting solution was removed at the selected exposure time and added to 1 mL of 1 N sodium thiosulfate to quench any residual oxidant. Treatment of biological simulants with thiosulfate alone showed no inactivation. Serial dilutions were performed in basal salts medium (BSM; 50 mM KH₂PO₄, 15 mM NH₄SO₄, 1 mM MgCl₂, and 0.02 mM FeSO₄) and samples plated onto L agar for quantitation. Experiments were run, at a minimum, in triplicate on separate days, and results were reported using standard deviation of average measurements.

Results and Discussion

Chlorine Mass Balance. A stable concentration of 3.3 g/L (5% relative standard deviation [RSD]) free chlorine was generated from the buffered Oxone-chloride system (pH 7.2, 0.5% RSD) over 7 h (Figure 1). This concentration was 1 order of magnitude lower than a 38 g/L (4.2% RSD) free chlorine concentration measured for a bleach stock (pH 10.3, 0.9% RSD). The mass recovery of total chlorine species in the Oxone-chloride system began at 99.7% with a gradual decrease over 7 h to 92.5%. The loss is attributed to some volatilization of hypochlorous acid from solution. Free chlorine content continued to decrease over 2 days, suggesting the decontaminating solution would need to be prepared at the time of use. The solution half-life ('pot' life) was 34 h. As expected, chlorate (ClO₃⁻) was observed as the decomposition product of hypochlorite (5). The chlorite (ClO₂⁻) precursor was not observed because it is known to rapidly convert to chlorate (7).

Although the amount of chloride used in this experiment was in excess to HSO₅⁻, a separate experiment (not shown) resulted in a significantly lower free chlorine concentration (0.7 g/L, 17% RSD) when reagents were used in equivalent amounts. Additional kinetic experiments are needed to explain this result.

Rapid Inactivation Studies. Greater than 8-log inactivation of *E. coli* was obtained within 30 s upon exposure to

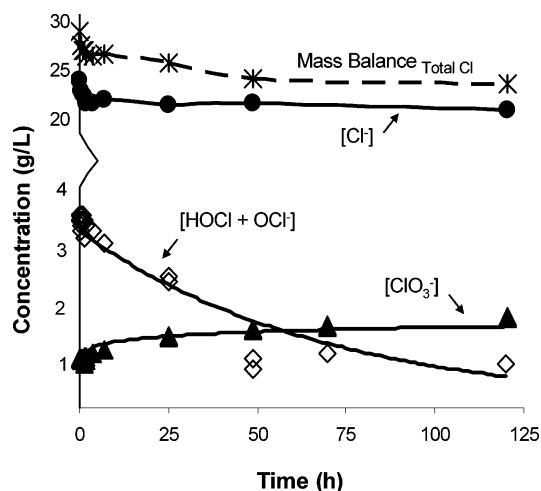
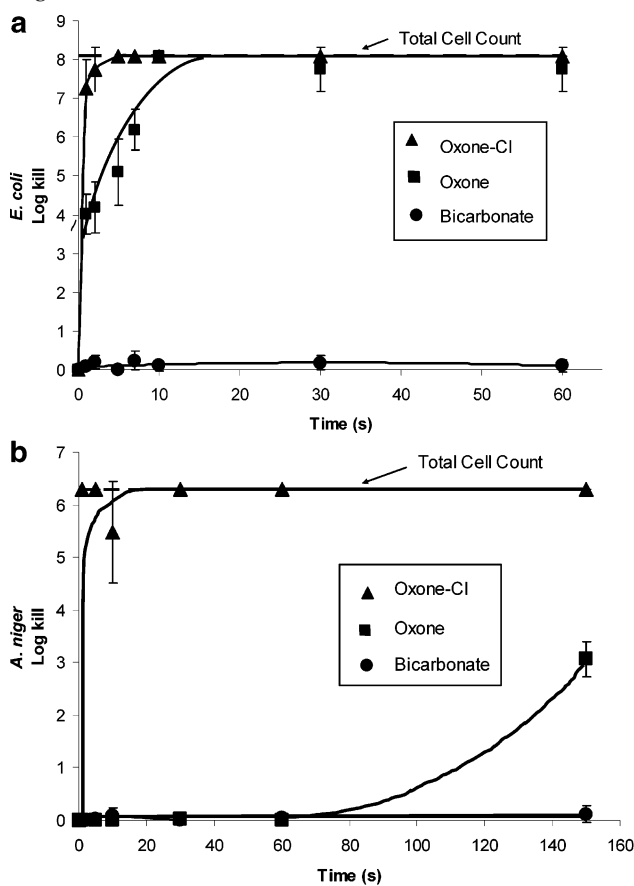


FIGURE 1. Stability of a buffered Oxone-chloride solution at pH 7.2 and 22 °C.

buffered Oxone-chloride and buffered Oxone alone (Figure 2a). Rapid inactivation was also observed of *A. niger* spores when exposed to Oxone-chloride (Figure 2b), but the effect was delayed when exposed to Oxone alone. *E. coli* and *A. niger* were used in this study to simulate the biological warfare agents of *Yersinia pestis* and *Francisella tularensis*. Only a 2-log inactivation of these agents was achieved in 10 min when exposed to a potable water free chlorine concentration of 0.8–1.6 mg/L (24). Oxone-chloride or Oxone-seawater would be expected to inactivate these pathogens faster based on the higher free chlorine concentration that is generated.



Differences between inactivation by Oxone-chloride and Oxone were more pronounced with bacterial spores. Greater than 5.8 log of *B. thuringiensis* (Figure 2c) and *B. atrophaeus* (Figure 2d) spores were inactivated within seconds by Oxone-chloride solutions. Oxone had no appreciable effect over the course of the experiment.

Effect of Temperature. Greater than 7-log inactivation of *E. coli* was obtained in 10 min at temperatures of –5, 10, 22, and 55 °C with all formulations containing Oxone (Figure 3a). Bleach was equally effective at all temperatures. These results were expected because *E. coli* is known to be readily susceptible to household disinfectants and strong oxidants such as bleach and hydrogen peroxide. The complete inactivation by the bicarbonate-chloride control (pH 7.2), and the lack of inactivation by seawater (pH 8.1) at 55 °C was unexpected. These results were reproduced in separate experiments and may be attributed to physiological pH and temperature effects.

Oxone-chloride, Oxone-seawater, and bleach solutions all achieved complete inactivation (approximately 6.5 logs) of *A. niger* fungal spores over a 10 min exposure at 22 and 55 °C (Figure 3b). Effectiveness of these solutions at 10 °C was nearly comparable to bleach, considering a positive standard deviation in the measurements. Oxone-chloride appeared to be slightly less effective at –5 °C. The Oxone data showed variability, and no kill was observed at –5 °C. Overall results suggest that Oxone-chloride and Oxone-seawater formulations can be used in cleanup efforts after natural disasters, such as treatment for mold growth in flooded homes in the aftermath of Hurricane Katrina. Further research is needed to determine if these solutions can be effective against viruses and parasites.

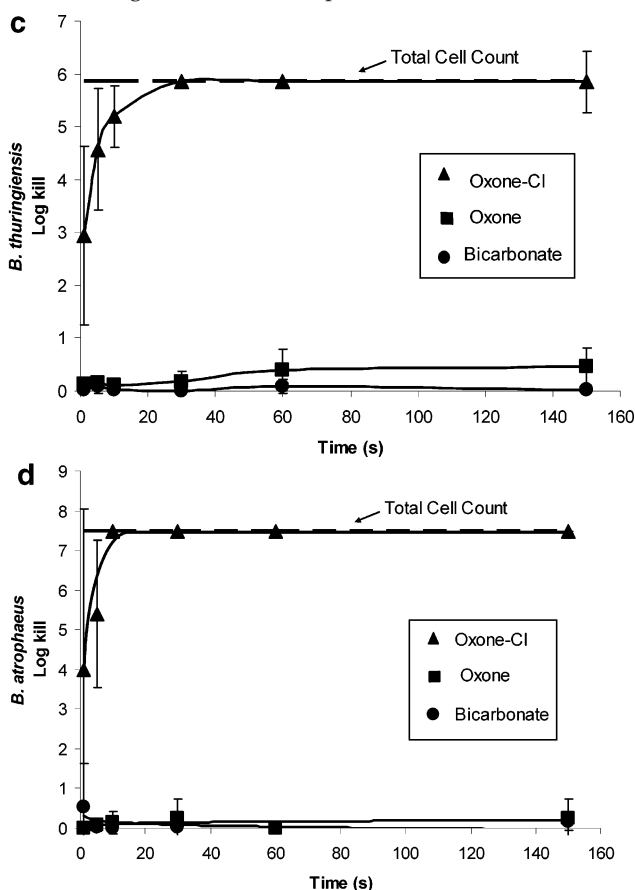


FIGURE 2. Inactivation comparison of (a) *E. coli* (8.08 log), (b) *A. niger* (6.3 log), (c) *B. thuringiensis* (5.85 log), and (d) *B. atrophaeus* (7.47 log) by buffered Oxone and buffered Oxone-chloride solutions at 22 °C.

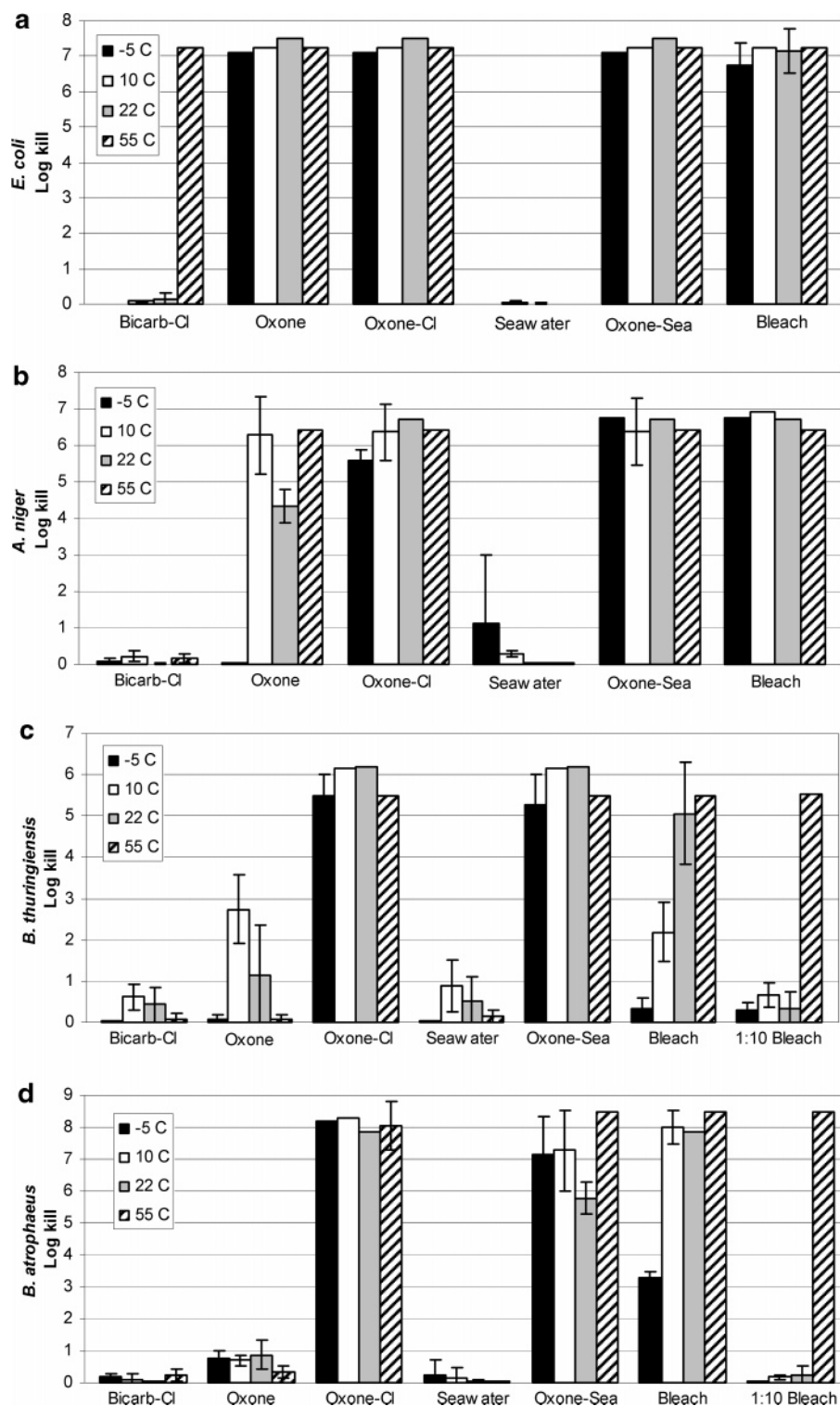


FIGURE 3. Efficacy of oxidant solutions at various temperatures for the inactivation of (a) *E.coli*, (b) *A. niger*, (c) *B. thuringiensis*, and (d) *B. atrophaeus* within 10 min. Total cell counts for experiments at -5 , 10 , 22 , and 55 °C, respectively: *E. coli*, 7.00, 7.39, 7.59, and 7.40 log; *A. niger*, 6.76, 6.90, 6.69, and 6.40 log; *B. thuringiensis*, 5.70, 6.16, 6.18, and 5.51 log; *B. atrophaeus*, 8.20, 8.27, 7.85, and 8.49 log. 'Bleach' was undiluted. '1:10 Bleach' was prepared in DI water and not acidified. No data was obtained for 1:10 bleach with *E. coli* and *A. niger*.

Oxone-chloride solutions inactivated spores of *B. atrophaeus* and *B. thuringiensis* within 10 min at all temperatures studied (Figure 3c,d). Oxone-seawater appeared equally effective at each temperature, although with slight variability in the data. This was unexpected because the additional trace elements of bromide and iodide in seawater were expected to produce hypohalous oxidants, HOBr and I_2/HOI , respectively, in the presence of Oxone (22). These species would also possess sporicidal properties. IC analysis confirmed that

the trace level of bromide present in the buffered Oxone-seawater formulations had been consumed.

Bleach effectively inactivated *B. atrophaeus* at 10 °C, 22 °C, and 55 °C, but poor sporicidal effects were observed for *B. thuringiensis* at -5 and 10 °C and for *B. atrophaeus* at -5 °C. This temperature effect is believed to be caused by an increase in bleach viscosity and the tendency for spores to clump together at lower temperatures. Poor sporicidal effectiveness was observed over the temperatures studied,

TABLE 1. Effect of HOCl Species on Kill of *B. atrophaeus* Spores at a 10 s Exposure Time^a

formulation	pH	[HOCl] (mM)	[OCl ⁻] (mM)	initial log count	log kill	SD
Oxone-Cl	5.0	50	0.16	7.93	7.93	<0.01
	6.5	50	4.0	7.93	7.93	<0.01
	7.2	45	20	7.47	7.47	<0.01
	8.5	4.0	50	8.08	0.95	0.07
acidified bleach	5.0	25	0.08	8.08	8.08	<0.01

^a Buffer concentration was 2.8% w/v NaHCO₃ at pH 5; 3.5% w/v NaHCO₃ at pH 6.5; 6.2% w/v NaHCO₃ at pH 7.2; and 9.2% w/v NaHCO₃ with 5.1% w/v Na₂CO₃ for pH 8.5; all with 10% w/v Oxone and 5.4% NaCl.

TABLE 2. Comparison of 'Aged' Solutions of Acidified Bleach and Oxone-Chloride on Spores at 22 °C and 10 Min Exposure

organism	formulation	pH	age	initial log count	log kill	SD
<i>B. atrophaeus</i>	acidified bleach	4.94	10 m	7.30	7.30	<0.01
		4.94	24 h	8.08	6.75	2.37
	Oxone-chloride	8.42	24 h	7.47	7.47	<0.01
<i>B. thuringiensis</i>	acidified bleach	4.94	10 m	5.48	5.48	<0.01
		4.94	24 h	4.46	4.46	<0.01
	Oxone-chloride	8.42	24 h	5.60	5.60	<0.01

with the exception of 55 °C, using a 10-fold dilution of bleach (0.4% OCl⁻). These results are similar to another study in which a 10-fold dilution of bleach was ineffective for inactivation of *B. subtilis* over a 30 min exposure time (25). Hypochlorite has been shown to damage spore coat components rather than the DNA of this organism (26). Further studies are needed to determine the mechanism by which bleach solutions differ in spore kill in comparison to buffered Oxone-chloride solutions.

An additional experiment was performed at 22 °C (single measurement—results not shown) with 8.7 logs of avirulent *B. anthracis* (Sterne strain). Oxone-chloride achieved complete inactivation in 10 min, while bleach and Oxone-seawater were slightly less effective. It was reported that a 4-log inactivation of the Sterne strain was obtained in a 2-h period when exposed to 0.8 g free chlorine/L at 25 °C (24). The virulent Ames strain of *B. anthracis* was inactivated by only 2 logs under the same conditions. The higher free chlorine concentration generated by the Oxone-chloride system suggests that a higher level of inactivation is possible. The exposure time required to inactivate high concentrations of dry spores would require further study. Wet spore preparations were used in our experiments, but testing with dry spores would more closely mimic a real world threat.

Effects of Hypochlorous Acid. The HOCl species is believed to contribute to inactivation of bacteria rather than the OCl⁻ species (13). The temperature data (Figure 3) shows that Oxone-chloride containing a 0.3% concentration of HOCl + OCl⁻ has a disinfecting capacity at least equal to that of a 4% bleach solution containing strictly OCl⁻.

The increased inactivation of *B. atrophaeus* based on higher HOCl:OCl⁻ ratios in the Oxone-chloride solution is shown in Table 1. Lower HOCl concentrations at pH 8.5 resulted in less effective spore kill at a 10 s exposure time; however, complete kill was achieved within 5 min (data not shown).

A dilution of bleach followed by pH neutralization with acid has been shown to effectively kill microorganisms by shifting the equilibrium from OCl⁻ to HOCl. Freshly prepared solutions of acidified bleach (EPA formulation) were as equally effective as 24 h aged Oxone-chloride solutions achieving 7 log and 5 log inactivation of *B. atrophaeus* and *B. thuringiensis*, respectively (Table 2). Although the pH of the Oxone-chloride solution increased over 24 h from pH 7.2 to 8.8, the efficacy of the formulation was unchanged. The

24 h aged acidified bleach remained effective, although inactivation of *B. atrophaeus* was diminished by 2 logs at a 10 min exposure time.

The dissemination of biological weapons has detrimental effects toward human health and the surrounding environments. Based on the results of this study, buffered Oxone-chloride and buffered Oxone-seawater solutions perform as well as, if not better than, bleach for the rapid inactivation of simulated biological agents over a wide range of operational temperatures. The active oxygen available from peroxy-monosulfate for chloride oxidation is limited to conditions below pH 9 thereby supporting conditions that minimize surface corrosion. The reactive chlorine species in these solutions are stable, but the volatility of HOCl suggests that the formulation should be prepared at the time of demand. Seawater can serve as a substitute for solid sodium chloride in the formulation, assuming that no extreme factors such as heavy pollution or elevated nitrogen levels are attributed to the source water which would otherwise exert an oxygen or chlorine demand on the mixture. All reagents are commercially available in large quantities, and the transport of these solids would be logistically less burdensome for operations in the field. The buffered Oxone-chloride and buffered Oxone-seawater formulations are therefore excellent alternatives to bleach.

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