J Ind Microbiol Biotechnol (2013) 40:601–611
DOI 10.1007/s10295-013-1257-7

ENVIRONMENTAL MICROBIOLOGY

Sporicidal efficacy of pH-adjusted bleach for control of bioburden on production facility surfaces

Anne Cornish Frazer · Josephine N. Smyth · Vishvesh K. Bhupathiraju

Received: 30 November 2012 / Accepted: 27 February 2013 / Published online: 27 March 2013

Abstract pH-adjusted bleach was one of the agents used to disinfect contaminated public buildings in the USA following the 2001 bioterrorist attack with *Bacillus anthracis* spores. A USEPA fact sheet describes the preparation of pH-adjusted bleach by combining diluted sodium hypochlorite (NaOCl) with a controlled amount of 5 % acetic acid. This paper reports a modification of this procedure to qualify the use of pH-adjusted bleach for routine disinfection of cleanroom surfaces in pharmaceutical manufacturing facilities whenever a short contact time is desirable or there is a need for enhanced germicidal or sporicidal activity. Adjustment of pH was obtained reproducibly with either acetic acid or HCl, confirming the feasibility of developing standard procedures for the controlled addition of acid to diluted NaOCl solutions without compromising operator safety and convenience. Efficacy testing using spores from an in-house isolate of *Bacillus pumilus* confirmed that NaOCl solutions in the pH 5–8 range have much greater sporicidal activity on surfaces than do unadjusted alkaline solutions (pH > 11). With a contact time of 0.5 min, the $\log_{10}$ reduction in spore viable counts was >5.4 for the five representative surfaces tested relative to untreated controls. Solutions of pH-adjusted NaOCl are known to be less stable than unadjusted alkaline solutions. Stability studies were performed by monitoring sporicidal efficacy, level of free available chlorine (FAC), and pH. Testing included several NaOCl concentrations and adjustment to different starting pHs. The efficacy of pH-adjusted solutions persisted in open containers for at least 12 h even though some FAC degradation occurred. In addition, solutions of 0.29 or 0.50 % NaOCl stored at room temperature protected from light retained efficacy for at least 4 weeks, indicating that short-term storage of solutions is possible following pH adjustment. The inorganic chemical degradation of pH-adjusted NaOCl solutions generates chlorate ion, an undesirable by-product. A comparison of chemical stability for 0.12, 0.25, and 0.50 % NaOCl solutions adjusted to different initial pHs indicated that the least chlorate formation occurred with 0.12 % NaOCl.

Keywords Sporicide · Cleanrooms · Hypochlorous acid · Bleach · pH adjustment · Disinfection

Electronic supplementary material The online version of this article (doi:10.1007/s10295-013-1257-7) contains supplementary material, which is available to authorized users.

A. C. Frazer (✉) · J. N. Smyth · V. K. Bhupathiraju
Bayer HealthCare LLC, Product Supply/Quality Control, 800 Dwight Way, Berkeley, CA 94710, USA
e-mail: ascfrazer@gmail.com

J. N. Smyth
e-mail: josephine.smyth@bayer.com

V. K. Bhupathiraju
e-mail: vishveshkb@drreddys.com

Present Address:
A. C. Frazer
Department of Plant and Microbial Biology, University of California, 271 Koshland Hall, Berkeley, CA 94720-3102, USA

Present Address:
V. K. Bhupathiraju
Quality Control, Biologics Development Center, Survey No. 47, Bachupally, Qutubullapur, R.R. District 500090, AP, India

Introduction

Commercial bleach solutions contain sodium hypochlorite (NaOCl) as the active ingredient and, since they are formulated with alkali for stability, the pH is high (pH > 11).

Disinfection with bleach is common in production facilities and laboratories but often without regard to solution pH. By contrast, pH control is an integral component in the chlorination of drinking water, in part to achieve necessary levels of germicidal activity [3, 18, 19]. In addition, pH adjustment of bleach or other hypochlorous acid solutions may be recommended for some disinfection and cleaning related to safe food handling practices [7] and in other settings [10, 11, 15, 19]. While there is ample evidence that alkaline NaOCl solutions are effective against vegetative bacteria, fungi, and yeast, as well as fungal conidia and viruses [6], the germicidal efficacy of NaOCl solutions is even greater if the pH is adjusted to the range of pH 5–8 [3, 6, 7]. Hypochlorite solutions that are pH adjusted are much more effective than alkaline solutions against bacterial endospores [4, 12, 19, 23]. Because NaOCl solutions are chemically reactive, conditions for their application should be designed to minimize the formation of disinfection by-products and chloramines [3, 6].

As summarized in Eq. (1), the hypochlorite anion (OCl^-) is a weak base in equilibrium with its corresponding acid, hypochlorous acid (HOCl). HOCl is the more germicidal form [2, 4, 12], and the pH of a solution governs the percent of undissociated HOCl that is present. The lethal activity of HOCl may be due to its oxidation of the amino acid side chains of proteins [8] leading to unfolding of tertiary structure and protein aggregation [24]. The ionization constant for HOCl dissociation to OCl^- and H^+ is 2.6×10^{-8} at 20 °C [13] so that little undissociated HOCl is present in alkaline solutions having a pH > 9.

$$HOCl \rightleftharpoons OCl^- + H^+ \quad pK_a = 7.6,\ 20°C \tag{1}$$

Control of pH is important in NaOCl solutions in order to avoid the presence of elemental chlorine gas (Cl_2) [3], which would be a hazard in the work place. The relationship between HOCl and Cl_2 is shown in Eq. (2). It is best to keep the pH greater than 4 to avoid formation of Cl_2. The term "free available chlorine" (FAC) refers to the mixture of oxidizing chlorine forms that have a chlorine atom in the 0 or +1 oxidation state and are not combined with ammonia or organic nitrogen [3]. In a hypochlorite solution, FAC includes OCl^-, HOCl, and Cl_2 [3, 6].

$$HOCl + H^+ + Cl^- \rightleftharpoons Cl_2 + H_2O \tag{2}$$

Bleach solutions at an alkaline pH are much more chemically stable than pH-adjusted ones, and chlorate anions (ClO_3^-) are formed in pH-adjusted bleach due to inorganic chemical degradation reactions. Degradation starts soon after pH adjustment and the extent and rate of degradation vary with temperature, concentration, and pH [1, 3]. As chemical degradation proceeds, pH-adjusted bleach solutions in the pH 5–8 range contain HOCl, OCl^-, and ClO_3^-. Solutions also become more acidic since H^+ ions as well as Cl^- are formed. The overall stoichiometry of degradation is shown in Eq. (3).

$$2HOCl + OCl^- \rightarrow ClO_3^- + 2Cl^- + 2H^+ \tag{3}$$

Following the bioterrorism attacks with *Bacillus anthracis* spores in the USA in 2001, pH-adjusted bleach was one of the agents used to assist in decontaminating buildings. Directions regarding how to prepare and use solutions for this purpose are described in a US Environmental Protection Agency (EPA) fact sheet [22]. For solutions prepared according to the EPA fact sheet, the final concentration of NaOCl is 0.5–0.6 % weight/volume and the pH is about 6.8 due to the addition of a specified proportion of 5 % acetic acid (i.e., distilled white vinegar). The pH-adjusted bleach solution can be used as a sterilant for non-porous surfaces, such as indoor office environments, by covering the surface with the solution for a contact time of 60 min. Repeated applications would be required to keep the surface wet throughout the 60-min contact time [22]. To qualify as an EPA sterilant, testing must show that three lots of the agent pass qualitative Method 966.04 of the Association of Official Analytical Chemists (AOAC), which uses bacterial spores of specified American Type Culture Collection (ATCC) strains inoculated onto surfaces of unglazed porcelain cylinders or surgical suture silk [14, 17, 20]. Tomasino and Hamilton [20] describe formulations for preparing solutions of 6,000 or 3,000 ppm NaOCl from concentrate, which use 5 % acetic acid to adjust the pH to 7.0 ± 0.5. The 6,000 ppm pH-adjusted NaOCl can be used as a positive control reference solution for testing sporicidal efficacy on carriers for Method 966.04 [20].

In contrast to the uncontrolled environments in which pH-adjusted bleach might be applied after a bioterrorist attack, cleanrooms used in pharmaceutical manufacturing are highly managed facilities with controlled access and low bioburden levels. Procedures for managing control of bioburden levels include cleaning, disinfection, and environmental monitoring. Desirable performance characteristics for cleanroom disinfectants include a short contact time as well as a level of efficacy that results in at least a two or three $\log_{10}$ reduction in the viable counts of representative microorganisms, i.e., sterilization of surfaces is not required [17, 21]. In addition, since bacterial endospores may be introduced into cleanrooms from time to time during routine operations, application of an agent that has sporicidal activity is often performed periodically [17, 21]. Regulatory agencies such as the US Food and Drug Administration (FDA) encourage the qualification of disinfectants for their intended use by testing their efficacy on surfaces similar to those present in a manufacturer's cleanrooms. In addition, it is desirable to include in-house environmental isolates in the qualification testing.

The goal of the work described in this paper was to evaluate procedures for preparing solutions of pH-adjusted bleach that would have an acceptable level of sporicidal activity with a short contact time and also be convenient to prepare and use. Spores were obtained from an in-house environmental isolate of *Bacillus pumilus*. The efficacy of pH-adjusted bleach was demonstrated on five different surface types and, in addition, it was shown that sporicidal efficacy depended on NaOCl concentration in the test system used. Since ready-to-use sterile alkaline solutions in the range of 0.25–0.50 % NaOCl are available from vendors, strategies were examined for adjusting the pH of such solutions, and efficacy and chemical degradation were monitored over time. In addition, a comparison was made of chemical degradation for solutions of 0.12, 0.25, and 0.50 % NaOCl adjusted to different starting pH levels.

Materials and methods

Reagents and media

Most testing was performed with commercially available ready-to-use sterile solutions that had nominal weight/volume concentrations of 0.25 or 0.52 % NaOCl. More concentrated commercial bleach, nominal 6.0 % NaOCl, was also used after appropriate dilution. The actual concentration of OCl^- in solutions was determined spectrophotometrically before use and is noted in the text, tables, and figure legends. Acetic acid and HCl used to adjust pH were reagent grade. The purified water was LAL Reagent Water, pH 6.8, (Lonza, Inc., Allendale, NJ, USA). This water was used to prepare and dilute the spore suspension that was applied to carriers as well as the solution used to soak untreated control carriers and to dilute the NaOCl solutions used for the testing listed in Table 1. Deionized water (18.2 MΩ cm^{-1}) was used to dilute 6.0 % commercial bleach for the 8-day chemical stability study. Cultures were grown on Trypticase Soy Agar (TSA) prepared media (BD BBL, Franklin Lakes, NJ, USA). The neutralizing solution, which was also used as the diluent for solutions recovered from carrier surfaces, was Dey/Engley (D/E) Neutralizing Broth (Hardy Diagnostics, Santa Maria, CA, USA). This is a rich broth containing numerous quenching agents effective for various disinfectants, including 0.6 % sodium thiosulfate for the inactivation of hypochlorite [21].

Spectrophotometric method for determining hypochlorite anion concentration

The UV absorption spectra are different for OCl^- and HOCl with OCl^- having an absorption maximum at 292 nm [13]. The FAC is present as the OCl^- form if the pH is greater than pH 10 [9, 24]. In the current study, NaOCl solutions were diluted with 0.01 N NaOH. If the pH was not greater than 10, it was adjusted with 1.0 N NaOH. Absorption of OCl^- was measured at 292 nm and a molar extinction coefficient of 360 M^{-1} cm^{-1} was used to calculate concentration [9]. In this paper NaOCl concentrations are given in either % weight/volume or mM units rather than following the practice in drinking water and wastewater treatment of expressing FAC in mg l^{-1} (i.e., ppm) of chlorine equivalents, usually expressed as "ppm available chlorine" [3]. For example, a solution having 0.17 % weight/volume NaOCl corresponds to 1,620 ppm available chlorine.

Estimating the concentration of HOCl

The percent of FAC present as HOCl was estimated from the pH and K_a according to reference 3, using Eq. (4), which assumes that the ionic strength of the solution is low enough to be ignored. A more accurate estimate of % HOCl would be obtained by taking ionic strength into consideration [3].

$$\%\,\mathrm{HOCl} = 100 \times \left(\frac{1}{1 + (K_a/[H^+])}\right) \quad (4)$$

Spore preparation and choice of strain for efficacy testing

Spores were prepared from an in-house isolate of *B. pumilus* designated as strain QC36715-1. TSA in 100-mm Petri plates was amended with $MnSO_4$ to a final concentration of about 0.15 mM. These were spread with an inoculum of *B. pumilus* QC36715-1 and incubated at 32.5 ± 2.5 °C for 5 days. Sterile purified water was added to the plates and the cultures were suspended with a spreader before transfer to a sterile 15-ml conical disposable centrifuge tube. The suspension was incubated in an 80 °C water bath for 30 min to inactivate vegetative cells. The heat-treated suspension was centrifuged at 2,000×*g* for 15 min at room temperature and the pellet was resuspended in sterile purified water. Three successive centrifugations at 2,000×*g* for 20 min at room temperature were performed to wash the pellet with sterile purified water. This spore suspension was stored 2–8 °C until use and had a viable count ± standard deviation ($n = 14$) of $3.3 \pm 0.5 \times 10^8$ colony-forming units (CFU) per milliliter.

Some efficacy testing was also performed with spores prepared from a second in-house isolate, *Bacillus megaterium* strain QC36589-1. Recovery of spores from carrier surfaces was similar for the two strains. Treatment with pH-adjusted NaOCl reduced spore survival to below the limit of detection for both strains. However, the *B. megaterium*

spores were more easily killed with alkaline NaOCl solutions. Hence, the efficacy testing reported in this work was performed only with the more resistant *B. pumilus* spores.

Efficacy testing procedure

A surface challenge test as outlined in the US Pharmacopeia [21] was used, in which a disinfectant is applied to an inoculated surface and the $\log_{10}$ reduction is determined after a specified contact time. The procedure for efficacy testing was modified from that described in European Standard EN 13697 [5]. Carriers of representative clean-room surfaces were 7.5 × 7.5 cm for polycarbonate, vinyl flooring, stainless steel, and polypropylene, and 7.5 × 5 cm for urethane epoxy. Carriers were placed in 150-mm-diameter Petri plates and each was inoculated with 25 µl of spore suspension that had been diluted with purified water to deliver about 1.1×10^6 CFU/carrier. The inoculum was dried by placing carriers uncovered in an incubator at 32.5 ± 2.5 °C for 30 min.

Testing was performed in a Biological Safety Cabinet within 2 h of inoculating the carriers. Duplicate carriers were used for each efficacy determination. A 0.25-ml volume of NaOCl solution was delivered as gently as possible over the inoculated area on a carrier at room temperature and allowed to stand for the desired contact time. This was 0.5 min for pH-adjusted solutions, and contact times of both 5 and 10 min were used for alkaline NaOCl solutions since they require a longer contact time to attain sporicidal efficacy. Treatment with the disinfectant was terminated by adding 1.0 ml of the neutralizing solution to quench disinfectant activity. The disinfectant and neutralizing solution were immediately mixed. Prior to transferring the solution mixture from the carrier to a sterile test tube, a sterile swab was rubbed vigorously over the inoculated area to help dislodge spores from the surface. For the untreated control carriers the inoculated area was covered with 0.25 ml of sterile water instead of NaOCl solution. The average ± standard deviation ($n = 27$) for the percent recovery of spores from the untreated control coupons was 100 ± 17 % of the inoculum applied.

Survivors of the NaOCl treatment of a carrier were quantitated by plating the quenched NaOCl mixture, or dilutions of the mixture. For suspensions with high CFUs, two sequential dilutions were plated to ensure that countable plates would be obtained. Duplicate TSA plates were inoculated for each dilution tested. TSA plates were incubated at 32.5 ± 2.5 °C to allow colonies to grow. Because the strain grew quickly and produced highly mucoid colonies, plates—especially for water-treated controls—were examined several times in order to count colonies while they were small and just becoming visible. As colonies continued to grow and produce mucus on a plate, the mucus over groups of older colonies would coalesce and obscure the count of individual older colonies. The first examination was made after about 14–15 h of incubation. Plates inoculated with spores from NaOCl-treated carriers were examined after 14–15 h and again within 20–24 h. These plates were then examined once or twice daily for at least 3 days so that colonies arising after a delay in growth initiation would be detected.

Efficacy calculations

The $\log_{10}$ of CFU per carrier for duplicate carriers was averaged. Efficacy was evaluated as the $\log_{10}$ reduction due to NaOCl treatment. This is the difference between the average $\log_{10}$ for duplicate untreated control carriers, which had been treated only with water, and the average $\log_{10}$ of carriers treated with NaOCl solution. The limit of detection was 3 CFUs/carrier. When carriers were treated with pH-adjusted solutions, the spore titer was generally below the limit of detection, i.e., no colonies were recovered. Thus, the number of survivors expressed as the average $\log_{10}$ ± (range for the duplicates)/2 is $< 0.48 \pm 0.00$ and the $\log_{10}$ reduction range is >5.4 to >5.7, varying somewhat with the CFU/carrier obtained for the untreated controls.

Effectiveness of the NaOCl neutralization procedure

The procedure for quenching NaOCl solutions with D/E neutralizing broth was developed using *B. megaterium* spores since they were more sensitive to NaOCl treatment. Test tubes were used to mix one part of NaOCl solution with different proportions of D/E neutralizing broth that had been previously inoculated with spores so that the mixture contained about 840 CFU/ml. After 10 min of contact, mixtures were plated to determine the effect of the treatment on viable counts. The disinfectant solutions contained 0.50 % NaOCl that was alkaline or adjusted to pH 7.2 with acetic acid. The estimated HOCl levels in the NaOCl solutions would have been about 0.005 or 48 mM, respectively. Both NaOCl solutions were quenched when mixed with four parts of D/E neutralizing broth.

The effectiveness of the quenching procedure when solutions were mixed on a carrier surface during efficacy testing was confirmed by placing 0.25 ml of 0.29 % NaOCl adjusted to pH 7.7 with acetic acid on duplicate polycarbonate carriers. Then, 1.0 ml of D/E neutralizing broth that had been inoculated with 1.1×10^6 CFU of *B. pumilus* spores was added to each carrier and immediately mixed. The mixtures were transferred to sterile tubes, diluted, and plated on TSA. The viable counts recovered from the neutralization confirmation carriers were 1.5×10^6 and 1.8×10^6 CFU per carrier, showing that the sporicidal activity of 0.25 ml of the NaOCl solution had been quenched by 1 ml of D/E neutralization broth. When the sporicidal

activity of 0.25 ml of the pH-adjusted NaOCl was tested with a 0.5 min contact time in the efficacy test, the observed $\log_{10}$ reduction was >5.7, confirming that the NaOCl solution used for neutralization testing had had high sporicidal activity.

pH adjustment

To obtain the data shown in Fig. 1, 30 ml of 0.34 % NaOCl solution was placed in each of two 50-ml conical centrifuge tubes and 1 N HCl was added to one tube and 5 % acetic acid to the other in 0.1-ml increments. After each addition of acid, the solution was mixed and the pH was measured. Acids were added until the solutions reached a pH of about 5. For efficacy or stability testing, HCl (1 or 6 N) or acetic acid (5 or 50 %) was added to adjust the pH of NaOCl solutions.

Stability testing

There were two approaches to preparing solutions for stability studies. One used ready-to-use NaOCl solutions and the other dilutions of concentrated commercial bleach.

Three ready-to-use NaOCl lots were tested. Initial pH was adjusted to include low (pH 7.2), high (pH 8.1), and approximate midpoint (pH 7.7) values within the HOCl/OCl^- buffer range. Solutions were stored at room temperature in capped opaque HDPE 500-ml bottles, which protected the solutions from light. In addition, starting volumes for the stored solutions were chosen such that bottles were only partially filled to about a third or two-thirds of the bottle capacity, as might be encountered during routine use and subsequent short-term storage of pH-adjusted solutions. The time intervals studied were 4 weeks or more so that final data points were obtained at 29, 35, or 43 days of storage. Initial testing for chemical stability was done with 0.34 % NaOCl. Two bottles were adjusted to pH 7.2 by adding appropriate amounts of either 6 N HCl or 50 % acetic acid. No acid was added to the solution in a third bottle, which served as an unadjusted alkaline control. The volume of NaOCl solution in each bottle was adjusted to about 320 ml. It is expected that ready-to-use solutions will have some variability in the range of actual NaOCl concentration and that the nominal concentration, or a somewhat higher concentration, will be present at the expiration date of the solution. In order to determine whether such variability would make pH-adjusted solutions unreliable with respect to providing the desired high efficacy, the same volume of acid was added to two different lots of ready-to-use NaOCl solutions. The volume of 5 % acetic acid or 1 N HCl added was 1/50th of the volume of the NaOCl solution. NaOCl concentrations were 0.29 and 0.50 %, and the starting pH was 7.7 or about 8.1, respectively. For the lot with 0.29 % NaOCl, one bottle was used. The pH was adjusted with 5 % acetic acid and the initial volume adjusted to about 125 ml. For the 0.50 % NaOCl lot, the pH in two bottles was adjusted to about 8.1 by adding either 5 % acetic acid or 1 N HCl. No acid was added to a third bottle, which provided an unadjusted alkaline control. Immediately after acid was added, solutions were mixed, and 150-ml amounts were transferred to glass beakers. These solutions were then rapidly stirred on a magnetic mixer for 12 h to observe changes in efficacy that might occur when NaOCl solutions are used in open containers. For longer-term monitoring of these solutions, the starting volume in each bottle was adjusted to about 150 ml.

Concentrated commercial bleach was diluted to obtain 0.12, 0.25, and 0.50 % NaOCl solutions. In addition to unadjusted alkaline solutions, 5 % acetic acid was added to adjust the starting pH of solutions to about 8.1, 7.6, 7.0, and 6.0. The solutions were prepared in duplicate in test tubes of polypropylene and stored in the dark at room temperature. The starting volume was 40 ml and chemical stability was monitored over a period of 8 days.

Ion chromatography

Chlorate anion was analyzed by chromatography on an AS9-HC IonPac column (Thermo Scientific Dionex, Sunnyvale, CA, USA) eluted with 9 mM sodium carbonate. An ASRS 300 suppressor, 45 mA current, was used.

Results

Considerations for pH adjustment of NaOCl solutions

The effect of adding controlled amounts of either 1 N HCl or 5 % acetic acid to solutions of NaOCl is shown in Fig. 1. Consistent with the fact that OCl^- is a weak base in equilibrium with HOCl, its corresponding acid form, there is a buffer region from about pH 8.2–6.8 around the pK_a of 7.6. With continued acid addition outside of the buffer region there was a sharp decline in pH when HCl was used whereas the decline in pH was much more gradual for acetic acid addition and leveled off at about pH 5. This feature is of practical interest since Cl_2 is formed when the pH of hypochlorite solutions is lower than about pH 4 [3]. Thus if the desired pH for a NaOCl solution were below pH 7, the use of acetic acid is safer than HCl in preventing the formation of Cl_2 at low pH values. Similar titration curves were obtained using solutions of NaOCl from different vendors. Titration curves were reproducible, making it possible to use the curves to estimate how much acid should be added to obtain a specific pH for a given concentration and volume of NaOCl.

Table 1 Sporicidal efficacy of different concentrations of pH-adjusted NaOCl tested on carrier surfaces with a contact time of 0.5 min

NaOCl (%)[a]	pH	Survivors[b]	Log_{10} reduction[c]	Estimation of HOCl level		
				FAC (mM)	% of FAC that is HOCl[d]	HOCl (mM)[e]
0.17	7.8	<0.48 ± 0.00	>5.6	23	38	8.7
0.085	7.7	0.83 ± 0.35	5.2	11	43	4.7
0.042	7.7	4.71 ± 0.19	1.4	5.6	43	2.4
0.021	7.7	5.79 ± 0.05	0.3	2.8	43	1.2

[a] A 0.34 % NaOCl solution was adjusted to pH 7.8 with 1 N HCl and serial two-fold dilutions were tested for efficacy

[b] Viable counts given as the average log_{10} (CFU/carrier) ± (range/2). The average for untreated control carriers was 6.06 ± 0.02

[c] Calculated from the average log_{10} (CFU/carrier) for untreated control carriers−average log_{10} (CFU/carrier) for NaOCl-treated carriers

[d] Calculated from the pH and K_a at 20 °C using Eq. (4) in the text

[e] Calculated from the concentration of free available chlorine (FAC) and % HOCl

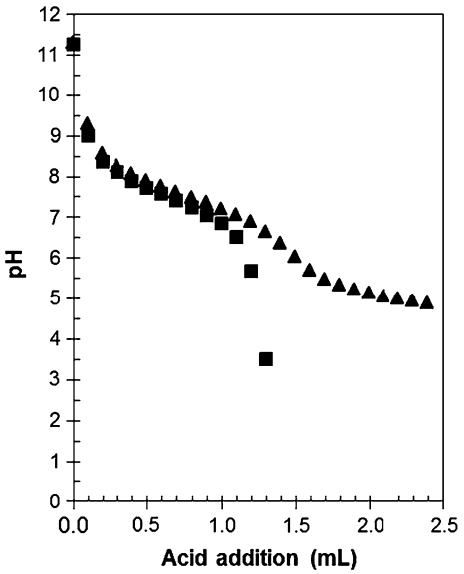

Fig. 1 Addition of acetic acid or HCl to 30 ml of 0.34 % NaOCl. Acids were added in increments of 0.1 ml

Dependence of sporicidal efficacy on the concentration of pH-adjusted NaOCl

HOCl is the chemical species responsible for sporicidal activity. An estimate of the HOCl concentration needed to obtain a high level of efficacy was obtained. Viable counts and log_{10} reduction results for pH-adjusted solutions of decreasing concentration are listed in Table 1, as well as the pH and FAC data used to estimate the HOCl concentrations. The solution with about 1.2 mM HOCl had little sporicidal efficacy whereas the log_{10} reduction was greater than 5 for solutions with 4.7 mM HOCl or higher. The solution with approximately 2.4 mM HOCl had an intermediate efficacy.

Efficacy of pH-adjusted NaOCl on different types of cleanroom surfaces

Polycarbonate carriers were used for efficacy testing unless stated otherwise. Representative data for solutions with different starting conditions of NaOCl concentration and pH can be seen in Tables 1, 3, and 4. Several other types of surfaces were tested using 0.34 % NaOCl adjusted to pH 7.8 with acetic acid. The average log_{10} reduction for duplicate vinyl flooring, urethane epoxy flooring, and polypropylene carriers observed after a 0.5 min contact time was >5.6, >5.7, and >5.6, respectively (supplementary material Table S1). The percent recovery of inoculated spores from control carriers for these surfaces were similar to polycarbonate carriers. In addition, stainless steel carriers were used for efficacy testing of one of the stability time points listed in Table 3 (day 6), and the average log_{10} reduction of duplicate carriers after a 0.5 min contact time was >5.5. Thus, the efficacy of pH-adjusted NaOCl solutions was high for all the surface types tested.

Efficacy of NaOCl solutions after 12 h in open containers with and without addition of acetic acid or HCl

The sporicidal efficacy of pH-adjusted or alkaline NaOCl solutions before and after stirring for 12 h in open containers is presented in Table 2 and supplementary material Table S2. Sporicidal efficacy was maintained for the pH-adjusted solutions after 12 h of treatment. However, some FAC had degraded since the FAC as a percent of the initial level was 74 % (acetic acid adjusted) or 68 % (HCl adjusted) after the prolonged exposure to air and stirring. The pH for the stirred pH-adjusted solutions did not change, whereas the pH of the control alkaline solution dropped from pH 11.7 to pH 9.1 with the greatest change occurring during the first 2 h (Fig. 2).

Table 2 Sporicidal efficacy of pH-adjusted and unadjusted alkaline solutions of 0.50 % NaOCl tested on carrier surfaces before and after stirring the solutions for 12 h in glass beakers

Type of acid	5 % acetic acid	1 N HCl	None	
Starting pH	pH 8.2	pH 8.1	pH 11.7	
Contact time	0.5 min	0.5 min	5.0 min	10.0 min
Before stirring	>5.5	>5.5	0.5	>4.6
After stirring[a]	>5.6	>5.6	>2.8	>5.0

Values are the $\log_{10}$ reduction in viable counts due to NaOCl treatment and are calculated from the average $\log_{10}$ (CFU/carrier) for untreated control carriers−average $\log_{10}$ (CFU/carrier) of NaOCl-treated carriers

[a] After stirring for 12 h, the pH was found to be unchanged for pH-adjusted solutions. The control solution (i.e., no acetic acid or HCl added) was pH 9.1

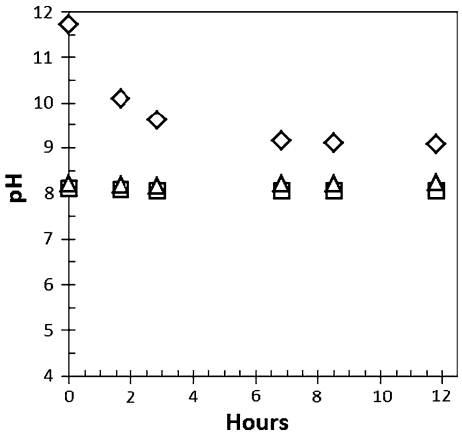

Fig. 2 The pH stability of ready-to-use solutions of 0.50 % NaOCl stirred for 12 h in glass beakers. Control for which no acid was added (*diamonds*), pH-adjusted with acetic acid (*triangles*), and pH-adjusted with HCl (*squares*)

The control solution, to which no acetic acid or HCl was added, showed higher sporicidal efficacy after 12 h of stirring in an open container than it had had when tested before this treatment since the $\log_{10}$ reduction for the 5-min contact time was only 0.5 before stirring and >2.8 afterwards. Vigorous stirring apparently facilitated uptake of CO_2 from the air, resulting in partial neutralization of the NaOH in the solution and a decrease in solution pH. The lower pH would increase the proportion of HOCl and result in higher sporicidal efficacy. The concentration of HOCl would have been about 0.005 mM for the solution at pH 11.7 and 2.0 mM at pH 9.1.

Efficacy and chemical stability during longer-term storage of pH-adjusted ready-to-use NaOCl solutions

Initial chemical stability testing with the 0.34 % ready-to-use NaOCl solutions adjusted to a starting pH of 7.2 showed that the overall concentration of FAC declined after pH adjustment, especially during the first 24 h (Fig. 3b). The pH of solutions after pH adjustment also changed, decreasing to 5.1 (acetic acid) or 4.5 (HCl) with most of the drop in pH occurring during the first 2 days and leveling off thereafter (Fig. 3a). The control solution, which had received no acid, did not change with respect to either FAC or pH. The estimated concentration of HOCl was high, starting at 32 mM and declining to 19 mM (acetic acid) or 24 mM (HCl) by day 29 (Fig. 3c). Efficacy testing was not performed with these solutions but it is expected that efficacy would have been high since the concentration of HOCl was well above the 8.7 mM level shown to provide high efficacy (Table 1).

For the ready-to-use 0.29 % NaOCl solution adjusted to starting pH 7.7, sporicidal efficacy remained high throughout the 35 days of testing (Table 3), even though the decrease in FAC and increase in acidity showed that chemical degradation occurred over time (Table 3; Fig. 3). The pH range at which high efficacy was demonstrated at various times was 5.4–7.7. For the ready-to-use 0.50 % NaOCl solution adjusted to a starting pH of about 8.1, sporicidal efficacy of pH-adjusted solutions remained high throughout the first 20 days of testing, for which the $\log_{10}$ reduction was >5.5 to >5.7 (Table 4; supplementary material Table S3). On day 35, the solution that had been pH adjusted with HCl had a $\log_{10}$ reduction of >5.4 whereas the solution that had been pH adjusted with acetic acid had a $\log_{10}$ reduction of 4.8. The control solution, which was not pH adjusted, had lower sporicidal efficacy throughout the testing period and results for duplicate carriers appeared more variable than with pH-adjusted solutions (supplementary material Table S3). Thus, a high level of sporicidal efficacy persisted for pH-adjusted solutions in spite of the decline in overall FAC. The pH range at which high efficacy was demonstrated at various times was 5.5–8.2. The testing with the 0.29 and 0.50 % solutions showed that it is not necessary to have a precise NaOCl concentration or starting pH in order to have high sporicidal activity. However, the degradation observed in stored pH-adjusted solutions is of concern since it results in the formation of chlorate, an undesirable degradation product [1, 3]. The least degradation during storage occurred when the initial pH was the lowest.

Chemical stability comparison of 0.12, 0.25, and 0.50 % pH-adjusted NaOCl solutions

Testing to compare the chemical stability of pH-adjusted NaOCl solutions over an 8-day period showed that the least inorganic degradation occurred with the lowest concentration (Fig. 4a, b, supplementary material Table S4 and Figure S1), since the percent of initial FAC for each of the

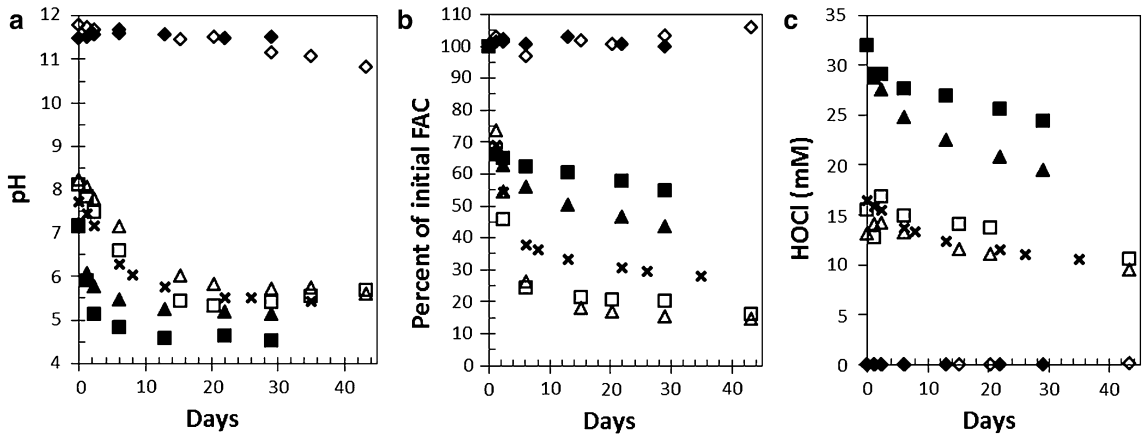

Fig. 3 Chemical stability of ready-to-use solutions of different NaOCl concentrations with and without pH adjustment stored at room temperature, protected from light in HDPE bottles. **a** pH stability. **b** Free available chlorine (FAC) stability. **c** Estimated HOCl concentration. *Open symbols* used for 0.50 % solutions and *closed symbols* for 0.34 % solutions. Adjustment of pH by addition of acetic acid (*triangles*), pH-adjusted with HCl (*squares*), controls for which neither acid was added (*diamonds*). 0.29 % solution with acetic acid used for pH adjustment (*times symbol*). The FAC initially present was 67.3 mM (0.50 % solutions), 45 mM (0.34 % solutions), and 38.3 mM (0.29 % solution)

Table 3 Sporicidal efficacy of 0.29 % NaOCl solution adjusted to pH 7.7 with acetic acid and tested on carrier surfaces at various times during storage

Day tested	Survivors[a]	Untreated controls[a]	Log_{10} reduction[b]	pH	FAC (mM)	HOCl[d] (mM)
0	<0.69 ± 0.21	6.06 ± 0.02	>5.4	7.7	38.3	16.5
1	<0.48 ± 0.00	5.95 ± 0.03	>5.5	7.4	26.3	15.8
2	<0.48 ± 0.00	6.02 ± 0.03	>5.5	7.1	20.7	15.5
6[c]	<0.48 ± 0.00	5.96 ± 0.05	>5.5	6.3	14.4	13.7
8	<0.48 ± 0.00	6.01 ± 0.03	>5.5	6.0	13.8	13.4
13	<0.48 ± 0.00	6.04 ± 0.00	>5.6	5.7	12.6	12.4
22	<0.69 ± 0.21	6.04 ± 0.00	>5.4	5.5	11.6	11.5
26	<0.48 ± 0.00	6.04 ± 0.04	>5.6	5.5	11.2	11.1
35	<0.48 ± 0.00	6.04 ± 0.00	>5.6	5.4	10.6	10.5

[a] Values are viable counts given as the average log_{10} (CFU/carrier) ± (range/2) for untreated controls or survivors after NaOCl treatment. The contact time was 0.5 min

[b] Values are the log_{10} reduction in viable counts due to NaOCl treatment and are calculated from the average log_{10} (CFU/carrier) for untreated control carriers−average log_{10} (CFU/carrier) of NaOCl-treated carriers

[c] Carriers used on day 6 were stainless steel, all other carriers were polycarbonate

[d] Calculated from the concentration of FAC and the % HOCl

initial pH groups was always highest for 0.12 % and lowest for the 0.50 % solutions. The chlorate anion concentration in the solutions on day 8 reflected the greater extent of degradation in the higher concentration solutions (Fig. 4d). The estimated level of HOCl in 0.12 % NaOCl solutions adjusted to an initial pH of 6.0, 7.0, 7.6, and 8.1 compared to the alkaline controls that were not pH-adjusted (pH > 10) is shown in Fig. 4c. It is expected that sporicidal efficacy would be high for the solutions showing a decrease in HOCl from about 15–13 mM (initial pH 6.0), and 12–10 mM (initial pH 7.0), since they are above the 8.7 mM HOCl level that showed high sporicidal efficacy (Table 1). The chemical stability testing indicates that to minimize the formation of the chlorate anion, while providing sufficient HOCl for high sporicidal efficacy, pH-adjusted 0.12 % NaOCl solutions would be useful in facilities disinfection.

Discussion

The results reported in this paper help to extend concepts regarding disinfection with pH-adjusted NaOCl to the disinfection of manufacturing facilities. Testing of pH-adjusted ready-to-use NaOCl solutions confirmed that the sporicidal efficacy for solutions tested in the pH 5–8 range

Table 4 Sporicidal efficacy of pH-adjusted and unadjusted alkaline solutions of 0.50 % NaOCl tested on carrier surfaces at various times during storage

Type of acid	Acetic acid		HCl		None		
Contact time	0.5 min		0.5 min		5 min	10 min	
Day tested	LR	pH	LR	pH	LR	LR	pH
0	>5.5	8.2	>5.5	8.1	0.5	>4.6	11.8
1	>5.6	8.0	>5.6	7.8	0.4	4.6	11.7
2	>5.6	7.8	>5.6	7.5	0.6	3.8	11.7
6	>5.6	7.1	>5.6	6.6	0.7	>5.6	11.6
15	>5.6	6.0	>5.6	5.4	0.7	5.1	11.4
20	>5.7	5.8	>5.7	5.3	0.8	>5.3	11.5
35	4.8	5.7	>5.4	5.5	1.4	>5.2	11.0

Values are the $\log_{10}$ reduction (i.e., LR) in viable counts due to NaOCl treatment and are calculated from the average $\log_{10}$ (CFU/carrier) for untreated control carriers−average $\log_{10}$ (CFU/carrier) of NaOCl-treated carriers on the day of testing

was greatly enhanced compared to alkaline NaOCl, providing both a greater $\log_{10}$ reduction and a shorter contact time. The efficacy obtained for surfaces representative of those in manufacturing areas was high and consistently exceeded the two $\log_{10}$ reduction criterion for spores, which may be considered adequate in qualification studies [17, 21]. It was also demonstrated that there can be considerable latitude with respect to both starting NaOCl concentration and pH as evidenced by the high efficacy demonstrated for 0.17–0.50 % NaOCl solutions with pH-adjusted initially to pH 7.7–8.2 and for solutions tested as low as pH 5.4 after storage during stability studies. In addition, pH-adjusted solutions had high efficacy when stored for 35 days or longer even though estimated HOCl concentrations declined gradually. After 1 day of storage, the FAC in pH-adjusted solutions was 66–73 % of the starting value. Chemical degradation of pH-adjusted hypochlorite solutions during storage results in the formation of chlorate, an undesirable by product, as well as chloride, and hydrogen ions [1, 3]. In addition to ready-to-use, NaOCl solutions prepared from diluting more concentrated commercial bleach are common in facilities disinfection. Concentrations often used include 0.12, 0.25, or 0.50 % NaOCl. When solutions at these concentrations were prepared from concentrated bleach and compared with respect to chemical stability after pH adjustment, it was shown that the least degradation occurred with the 0.12 % solutions. This suggests that from the point of view of minimizing the formation of inorganic disinfection by product, while providing concentrations of HOCl that should exhibit high sporicidal efficacy, pH-adjusted solutions of about 0.12 % NaOCl offer advantages in the disinfection of manufacturing facilities.

The use of filter-sterilized disinfecting solutions for pharmaceutical manufacturing facilities is often recommended;

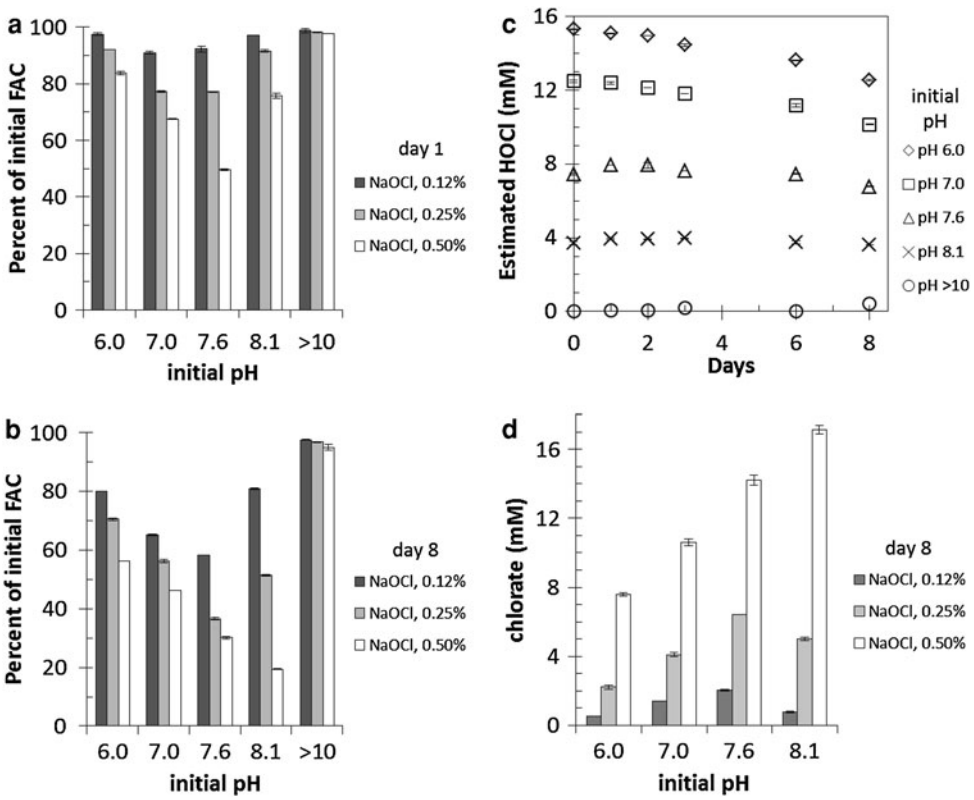

Fig. 4 Chemical stability of 0.12, 0.25, or 0.50 % NaOCl solutions with initial pH adjustment to 6.0, 7.0, 7.6, or 8.1 by adding acetic acid, as well as solutions without acid added (initial pH > 10). **a** Free available chlorine (FAC) stability on day 1 of storage. **b** Free available chlorine (FAC) stability on day 8 of storage. **c** Estimated HOCl concentration of the 0.12 % solutions. **d** Chlorate anion concentrations in solutions on day 8. Duplicate solutions were tested and the *error bars* represent the duplicates range/2

however, the efficacy of pH-adjusted NaOCl solutions is so high that physically sterilizing them may be redundant. Nevertheless, if it is deemed advisable to prepare a sterile acid solution to use in the pH adjustment, 5 % acetic acid or 1 N HCl could be filter sterilized [20]. Once qualification testing demonstrates that an appropriate starting percent NaOCl and acid addition procedure provides adequate sporicidal efficacy in an appropriate pH range, measuring or manipulating the pH during routine standard application operations is not needed. Increased acidity due to chemical degradation reactions in stored solutions could increase the risk of corrosion on surfaces like stainless steel.

The high efficacy observed for pH-adjusted 0.085 and 0.17 % NaOCl solutions (Table 1) is consistent with other studies. For instance, Kaatz et al. [10] showed that disinfecting with phosphate buffered hypochlorite (1,600 ppm available chlorine, pH 7.6) reduced the isolation of *Clostridium difficile* by 98 % from the surfaces of a patient's hospital room. Levine [12] reported that a two $\log_{10}$ reduction in viable *Bacillus metiens* (i.e., *Bacillus cereus*) spores was obtained in less than 20 s with a 1,000 ppm available chlorine solution, pH 7.3, compared to 64 min of contact required for a similar reduction in counts with a pH 11.3 solution. Sagripanti and Benifacino [19] demonstrated that NaOCl solutions having the equivalent of 500 ppm available chlorine, pH 7.0, had high-level sporicidal activity when tested on *Bacillus subtilis* subsp. *globigii* (i.e., *Bacillus atrophaeus*) spores. By comparison, solutions at pH 11 had an intermediate level of sporicidal activity. The pH of the solutions decreased from 7.0 to about 5.5 over approximately 12 days, and sporicidal efficacy also declined during the same period of storage [19]. A 0.12 % NaOCl solution, pH-adjusted with HCl to 6 ± 1, has been validated for the disinfection/cleaning of a pharmaceutical manufacturing facility and used routinely to control bioburden (Kathleen Scully Bupp, pers. comm.). In addition, a previous study qualifying the use of bleach and other disinfectants in a pharmaceutical manufacturing facility demonstrated that a 0.12 % NaOCl solution adjusted to a starting pH of 6.8 with acetic acid had a high level of efficacy after aging the solution for 6 h (Frazer and Matsubara, unpublished).

It was demonstrated that either HCl or acetic acid is suitable for adjusting pH. The sporicidal efficacy of 0.34 % ready-to-use NaOCl solutions adjusted with either acid was the same. Sporicidal efficacy for 0.50 % ready-to-use solutions adjusted with either acid was also the same initially in open containers or capped bottles. The decline in pH during storage was less for acetic acid pH-adjusted solutions whereas the decrease in FAC was more than seen for HCl-adjusted solutions. Addition of acid to diluted NaOCl solutions should be safer with acetic acid than for HCl providing a lower risk of releasing Cl_2. These observations are consistent with the findings of Kuroiwa et al. [11] who tested seven acids for effects on sporicidal efficacy of NaOCl solutions when pH was adjusted to 5.0. The authors found that sporicidal efficacy was highest and very similar for the acetic acid and HCl adjusted solutions. By contrast, solutions with pH adjusted by addition of formic, phosphoric, or sulphuric acids had lower efficacy while addition of lactic or citric acids resulted in solutions with no sporicidal efficacy.

Bleach has applications in addition to disinfection in manufacturing and laboratory operations. For instance, it is well established that alkaline NaOCl is effective for nucleic acid contamination control [16]. Although pH-adjusted NaOCl is much more effective than an alkaline solution with respect to germicidal activity, it is less effective in cleaning equipment or surfaces that are contaminated with protein material [3, 7]. When designing procedures for the application of bleach in manufacturing facilities, the goal of the operation should guide the selection of which pH range to utilize, depending on the chemical reactions of interest.

Acknowledgments The authors are indebted to John Matsubara and Sol Nho for excellent technical assistance, Cristina Vigano for recommending the methodology of European Standard EN 13697, Wayne Yamamoto for helpful discussions, and John D. Coates for his sustained interest.

References

1. Adam LC, Fabian I, Suzuki K, Gordon G (1992) Hypochlorous acid decomposition in the pH 5–8 region. Inorg Chem 31:3534–3541
2. Albrich JM, McCarthy CA, Hurst JK (1981) Biological reactivity of hypochlorous acid: implications for microbicidal mechanisms of leukocyte myeloperoxidase. Proc Natl Acad Sci USA 78:210–214
3. Black and Veatch Corporation (2010) White's handbook of chlorination and alternative disinfectants, 5th edn. Wiley, Hoboken
4. Brazis AR, Leslie JE, Kabler PW, Woodward RL (1958) The inactivation of spores of *Bacillus globigii* and *Bacillus anthracis* by free available chlorine. Appl Microbiol 6:338–342
5. CEN (European Committee for Standardization) (2001) EN 13697 chemical disinfectants and antiseptics—quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas—test method and requirements without mechanical action (phase 2/step 2). CEN, Brussels
6. Dychdala GR (2001) Chlorine and chlorine compounds. In: Block SS (ed) Disinfection, sterilization, and preservation, 5th edn. Lippincott, Williams and Wilkins, New York, pp 135–157
7. Fukuzaki S (2006) Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. Biocontrol Sci 11:147–157
8. Hawkins CL, Pattison DI, Davies MJ (2003) Hypochlorite-induced oxidation of amino acids, peptides and proteins. Amino Acids 25:259–274

9. Hussain A, Trudell P, Repta AJ (1970) Quantitative spectrophotometric methods for determination of sodium hypochlorite in aqueous solutions. J Pharm Sci 59:1168–1170
10. Kaatz GW, Gitlin SG, Schaberg DR, Wilson KH, Kauffman CA, Seo SM, Fekety R (1988) Acquisition of *Clostridium difficile* from the hospital environment. Am J Epidemiol 127:1289–1294
11. Kuroiwa K, Nakayama H, Kuwahara T, Tamagawa K, Hattori K, Murakami K, Korai H, Ohnishi Y (2003) Augmenting effect of acetic acid for acidification on bactericidal activity of hypochlorite solution. Lett Appl Microbiol 36:46–49
12. Levine M (1952) Spores as reagents for studies on chemical disinfection. Bacteriol Rev 16:117–125
13. Morris JC (1966) The acid ionization constant of HOCl from 5 to 35°C. J Phys Chem 70:3798–3805
14. Official Methods of Analysis (1990) AOAC method 966.04: sporicidal activity of disinfectants, 15th edn. AOAC International, Gaithersburg
15. Park GW, Boston DM, Kase JA, Sampson MN, Sobsey MD (2007) Evaluation of liquid- and fog-based application of Sterilox hypochlorous acid solution for surface inactivation of human norovirus. Appl Environ Microbiol 73:4463–4468
16. Prince AM, Andrus L (1992) PCR: how to kill unwanted DNA. Biotechniques 12:358–360
17. Priscott PK, Dai Y (2008) Disinfectants program. In: Prince R (ed) Microbiology in pharmaceutical manufacturing, 2nd edn. PDA/DHI, Bethesda, pp 93–114
18. Rose LJ, Rice EW, Jensen B, Murga R, Peterson A, Donlan RM, Arduino MJ (2005) Chlorine inactivation of bacterial bioterrorism agents. Appl Environ Microbiol 71:566–568
19. Sagripanti J-L, Bonifacino A (1996) Comparative sporicidal effects of liquid chemical agents. Appl Environ Microbiol 62:545–551
20. Tomasino SF, Hamilton MA (2007) Comparative evaluation of two quantitative test methods for determining the efficacy of liquid sporicides and sterilants on a hard surface: a precollaborative study. J AOAC Int 90:456–464
21. US Pharmacopeia 32 rev. and the National Formulary 27th edn (2008) General chapter 1072: disinfectants and antiseptics. US Pharmacopoeial Convention, Rockville
22. USEPA (2012) Anthrax spore decontamination using bleach (sodium hypochlorite). http://www.epa.gov/pesticides/factsheets/chemicals/bleachfactsheet.htm. Accessed 27 Nov 2012
23. Whitney EAS, Beatty ME, Taylor TH Jr, Weyant R, Sobel J, Arduino MJ, Ashford DA (2003) Inactivation of *Bacillus anthracis* spores. Emerg Infect Dis 9:623–627
24. Winter J, Ilbert M, Graf PCF, Ozcelik D, Jakob U (2008) Bleach activates a redox-regulated chaperone by oxidative protein unfolding. Cell 135:691–701