CHAPTER

# 17

# Chlorine dioxide technologies for active food packaging and other microbial decontamination applications

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## 17.1 Introduction

Research scientists at the US Army-Natick Soldier RD&E Center (now named the US Army-CCDC Soldier Center) have invented a diverse ensemble of antimicrobial technologies based on the action of the biocide chlorine dioxide. These antimicrobial technologies have different attributes, since they were developed for different uses and were intended for different applications with different goals and different constraints, but all with the common purpose of inactivating target microorganisms using chlorine dioxide.

Two next-generation chlorine dioxide technologies were developed for use as concepts for active food packaging systems with antimicrobial activity with potential applications in the food industry to ensure microbial safety, extend shelf-life, and reduce waste and economic losses associated with perishable foods such as fresh fruits, vegetables, and berries. One system produces chlorine dioxide from precursors adsorbed onto films of the biopolymer polylactic acid (PLA) that can be used in food packaging. The second is a smart food packaging system designed with chemical precursors incorporated in an insertable sachet made of a superabsorbent hydrogel polymer called the Compartment of Defense (CoD). The CoD packaging system has the characteristic of being humidity-activated for the time-released, controlled production of $ClO_2$ inside the container. The CoD technology inactivates pathogens and prevents mold growth on perishable foods (fresh berries, fruits, and vegetables) that respire and create humidity in-packaging.

As an adaptable antimicrobial technology that works well on the species and strains of vegetative pathogens and bacterial spores that are causes of concern in the food industry for food safety and spoilage, these chlorine dioxide technologies work equally well in textile-related applications (parachutes and clothing) and in biodecontamination. For example, chlorine dioxide can be used to prevent mold growth that can occur in humid or warm wet environments and degrade military textiles during storage, such as parachute sleeves, clothing (uniforms, undergarments, socks, boots), tents

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and rigid-walled shelters, vehicle mats, and even metal surfaces coated with polyurethane containing antimicrobial compounds. Additionally, chlorine dioxide can inactivate microorganisms on clothing can cause cutaneous rashes or irritations, especially in instances when clothing is worn for prolonged periods without access to laundering and/or adequate hygiene facilities. Further, chlorine dioxide can inactivate bacterial spores of *Bacillus anthracis* (the causative agent of "Anthrax") that have been used in bioterrorism attacks.

In addition to the development of the active antimicrobial packaging made with films of PLA biopolymer and the humidity-activated CoD food packaging system, additional chlorine dioxide technologies were invented and developed for other applications. Presented herein is the ensemble consisting of next-generation chlorine dioxide decontamination technologies that can be adapted for use in myriad antimicrobial applications, either in the food industry, for the military, or to meet other needs. Specific examples of these novel technologies and their validation are demonstrated, which include technologies for sanitizing hard surfaces, disinfecting graywater, decontaminating porous materials, preventing mold growth on parachute sleeves, inactivating spores of *Clostridiodes difficile*, the pathogen notorious in hospitals and healthcare settings, and self-decontaminating textiles incorporated with a stimuli-responsive polymer for hospital gowns and other personal protective equipment. These demonstrations should encourage new uses of these antimicrobial technologies in the food industry, such as treating fresh produce before shipping to consumers, sanitizing hard surfaces in food processing facilities and food handling environments, and cleaning-in-place methods for production lines, and in other applications where controlling microorganisms is a concern.

## 17.2 Current uses of chlorine dioxide

The earliest set of chlorine dioxide decontamination technologies that were developed were designed to be portable, electricity-free, power-free, and environmentally friendly ("green"), and they were intended for use in austere or otherwise resource-constrained circumstances of military field deployments, crisis responses, natural disaster relief, or humanitarian aid (Doona et al., 2014, 2015; Setlow et al., 2009). These disinfectant technologies include a Novel Chemical Combination (NCC); a Portable Chemical Sterilizer (PCS) to sterilize surgical instruments (20 lbs, 2.1 ft3, 10 oz of water, 0 kW of electricity); a collapsible handheld trigger-sprayer (Disinfectant-sprayer For ENvironmentally-friendly Sanitization, D-FENS), and Field Decontamination Kits (FDKs).

The FDK technology uses gaseous chlorine dioxide and was invented at US Army CCDC SC (formerly Natick Soldier RD&E Center). This technology was deployed for use by WHO, Doctors Without Borders (MSF), NIH/NIAID, Public Health Canada, USAMRIID, and other global public health organizations during the Ebola crisis in West Africa in 2014–2015. Because of its suitability for use in remote or austere environments with constrained availability of power and its lightweight portability for rapid mobility, FDKs were used to sterilize Ebola-contaminated medical instruments and electronics and protect healthcare workers (Doona et al., 2015). Commercial industry has licensed these technologies and marketed salable products to civilian consumers for additional uses in laboratories, offices, and homes.

This early set of disinfectant/sterilization technologies were based primarily on the biocide chlorine dioxide, which inactivates a broad spectrum of microorganisms (e.g., viruses, bacterial cells and spores, yeasts, molds and mildews, and fungal spores) without acquiring increased resistance. Chlorine dioxide

($ClO_2$) is a well-known and versatile disinfectant, whose biocidal efficacy in either the gaseous state or in aqueous solution is well-established (Schaufler, 1933; Young and Setlow, 2003; Doona et al., 2014, 2015). Regulatory agencies have approved chlorine dioxide for different uses. The US Department of Agriculture (USDA) allows its uses in crop production and livestock production and for processed products (USDA, 2000); the US Food and Drug Administration (US FDA) allows its uses in poultry processing water and water used to wash fruits and vegetables (US FDA, 1998); and the US Environmental Protection Agency (US EPA) Emerging Pathogen Program lists chlorine dioxide as effective against coronavirus on hard surfaces (U.S. EPA, 2017).

Descriptions of the industrial-scale methods for generating $ClO_2$ can be found in chemical technology handbooks and encyclopedias (Vogt et al., 2003). Chlorine dioxide is employed safely in commercial uses across multiple industries and cross-cutting applications, with its largest consumers (ca. 4.5 million pounds used per day worldwide) being the pulp and paper industry and the approximately 700–900 municipal water systems in the United States that disinfect the water supply and render potability (Hoehn, 1992). Small-scale applications of chlorine dioxide include its use in personal hygiene consumer products such as mouthwashes and toothpastes (The $ClO_2$ Fact Sheet, 2013). Sterilizing medical electronics devices in austere environments in remote areas during the Ebola crisis in 2014–2015 (Doona et al., 2015) requires the use of dry chemical precursors that mix safely and controllably in water or aqueous solution to generate $ClO_2$ on-site, at-will, and at point-of-use for their intended application.

### 17.2.1 Chemical methods of generating $ClO_2$

The uses of $ClO_2$ and the methods for its generation rely on the unique chemical attributes of the element chlorine (chemical symbol Cl). As a member of Group VIIA (halogens) of the periodic table, Cl is found in diverse chemical species with different reactivities and the Cl atom in different oxidation states and (Table 17.1). Many of the chemical methods that generate $ClO_2$ mentioned above are categorized as occurring by processes involving the: (i) reduction of chlorate, (ii) acidification of chlorite, (iii) oxidation of chlorite ion, and (iv) reduction of chlorite ion.

Chemical systems in categories (i), (ii), and (iii) all have substantial practical drawbacks that make them unsuited for small-scale, electricity-free, point-of-use applications. In the method of category (i) reduction of chlorate ($ClO_3^-$ with Cl in the +5 oxidation state), the chemical reaction involves the reaction of the small molecule chlorate in strong acid solution with a reductant such as sulfur dioxide, methanol, hydrogen peroxide, or hydrochloric acid. The reduction of chlorate accounts for more than 95% of the $ClO_2$ used in the world today, primarily in the pulp and paper industry, through the large-scale production of $ClO_2$ for the manufacture of high-quality white paper products. The chemical

**Table 17.1 Chemical species and names, symbols, and oxidation states of chlorine-containing molecules.**

| Name | Chemical symbol | Oxidation state |
|---|---|---|
| Chloride | $Cl^-$ | −1 |
| Chlorine gas (dichlorine) | $Cl_2$ | 0 |
| Hypochlorite (bleach) hypochlorous acid | $OCl^-$ $HOCl$ | +1 |
| Chlorite chlorous acid | $ClO_2^-$ $HClO_2$ | +3 |
| Chlorine dioxide | $ClO_2$ | +4 |
| Chlorate | $ClO_3^-$ | +5 |
| Perchlorate | $ClO_4^-$ | +7 |

reaction takes place in large-scale equipment and with the use of strong acid solutions. In the method of category (ii) acidification of chlorite ($ClO_2^-$, Cl in the +3 oxidation state), the addition of acid to chlorite solution forms unstable chlorous acid ($HClO_2$) that subsequently disproportionates to produce $ClO_2$. In the method of category (iii) oxidation of chlorite, chlorite ($ClO_2^-$) is oxidized by chemical reaction with dichlorine ($Cl_2$) gas. In all three of these cases, the uses of hazardous materials (acids in categories i and ii and dichlorine gas in category iii) require special considerations for transportation, storage, and disposal. Acids are also incompatible with many surface materials, and transporting large-scale equipment or cylinders of gas for the on-site, small-scale production of $ClO_2$ in high-intensity, rapid-mobility environments is impractical.

In contradistinction, chemical systems that produce $ClO_2$ by the method of category (iv) reduction of chlorite ion ($ClO_2^-$) do so handily by adding safe, dry reagents (chlorite and reductants) to water to effectuate the controlled generation of chlorine dioxide on-site with distinct advantages in terms of their ease-of-use, convenience, and safety to users and the environment. In the method of category (iv) reduction of chlorite ion ($ClO_2^-$), chlorite reacts with a reductant to produce chlorine dioxide ($ClO_2$, with the Cl in the +4 oxidation state).

## 17.2.2 Chemical mechanism proposed for the reduction of chlorite

The astute chemist might notice the paradox that the process of category (iii) is the *oxidation* of chlorite ($ClO_2^-$, Cl in the +3 oxidation state) to chlorine dioxide ($ClO_2$, Cl in the +4 oxidation state), and that the process of category (iv) is the *reduction* of $ClO_2^-$ to produce $ClO_2$. While the former situation for the loss of an electron seems fairly straightforward ($ClO_2^- \rightarrow ClO_2 + e^-$), the latter situation might seem counterintuitive, unless one understands the role(s) of transient intermediates formed in the chemical reaction system.

Consider the reaction of $ClO_2^-$ with the reductant sulfite ($SO_3^{2-}$). This chemical reaction is thermodynamically favorable (molar enthalpy of reaction $\Delta H = -648.3$ kJ/mol) but kinetically inert—it does not proceed to produce $ClO_2$ and/or exothermic heat on any type of practical timescale. However, adding substoichiometric amounts of an electron-transfer effector that reacts readily with $ClO_2^-$ is postulated to establish new reaction pathways through the formation of oxidizing transient intermediates ($ClO^\bullet$), which even react with the reductant sulfite ($SO_3^{2-}$). Table 17.2 depicts a reaction scheme for this chemical reaction with the $ClO^\bullet$ intermediate.

In Table 17.2, proposed reaction step i denotes *formation* of the transient oxidizing intermediate chlorine monoxide radical (designated as $ClO^\bullet$, Cl in the +2 oxidation state) by the $ClO_2^-$-effector reaction; steps ii–iii denote the *propagation* of $ClO^\bullet$ by reactions involving the reductant; and step iv

**Table 17.2 Mechanism with transient intermediates.**

| **Overall reaction** |
|---|
| $ClO_2^- + R^{2-} \rightarrow$ no reaction |
| Overall effector-driven reaction |
| $ClO_2^- + R^{2-} + Eff^- \rightarrow ClO_2, SO_4^{2-}, C_2O_4^{2-}, CO_2$ (fast reaction, exothermic) |
| Effector-driven reaction mechanism |
| i $ClO_2^- + Eff^- \rightarrow ClO^\bullet + Eff^{\bullet-}$ |
| ii $ClO^\bullet + R^{2-} \rightarrow ClO^- + R^{\bullet-}$ |
| iii $ClO_2^- + R^{\bullet-} \rightarrow ClO^\bullet +$ salt |
| iv $ClO_2^- + ClO^\bullet \rightarrow ClO^- + ClO_2$ |

denotes the *termination* of $ClO^{\bullet}$ and concomitant formation of $ClO_2$ by the reaction with $ClO_2^-$. Other mechanisms and intermediates (e.g., $Cl_2O_2$) could plausibly account for this reaction, but the overall result of an electron-transfer chemical effector accelerating an otherwise inert chemical reaction by initiating additional chemical reaction pathways involving transient intermediates capable of oxidizing $ClO_2^-$ would be the same.

### 17.2.3 Microbiological validation of the PCS and D-FENS

The PCS and D-FENS systems use a three-component reduction of $ClO_2^-$ to rapidly produce concentrated $ClO_2$ solutions with the evolution of copious exothermic heat. As a suitcase sterilizer, the PCS is a modern field autoclave designed to off-gas $ClO_2$ that acts in concert with heat and steam to inactivate bacterial spores and sterilize surgical instruments. The D-FENS, a collapsible handheld sprayer for surface decontamination applications in laboratories, offices, and homes, uses the same reaction chemistry as the PCS, but D-FENS invokes the principles of kinetics control and postreaction dilution to produce relatively dilute $ClO_2$ solutions without concomitant heat production.

Achieving sterility with the PCS was demonstrated using live cultures of *Bacillus stearothermophilus* spores and indicators of *B. stearothermophilus* or *Bacillus atrophaeus*. The efficacy of the D-FENS system was validated by spraying the solution onto a representative porous surface consisting of Petri dishes containing agar and Baird-Parker nutritive media supplemented with egg yolk tellurite and inoculated with a three-strain cocktail of *Staphylococcus aureus*. Plates sprayed with the D-FENS solution showed no subsequent colony growth, whereas untreated plates showed the growth of dark colonies characteristic of *S. aureus*. In-house cross-laboratory testing of *S. aureus* dried on metal coupons showed that the D-FENS solution (100 ppm $ClO_2$) inactivated > 7-logs of *S. aureus*, whereas hydrogen peroxide or ozone inactivated <3.4-logs and <4.8-logs, respectively (Doona et al., 2014, 2015).

## 17.3 Next-generation $ClO_2$ technologies

While the PCS and D-FENS accomplished their goals of portable power-free sterilization and surface decontamination, certain attributes of these systems (e.g., producing concentrated $ClO_2$ and copious heat for the PCS; requiring three-components and two-step mixing for D-FENS) might not be desirable for other microbial decontamination applications, and so other custom-made systems and configurations were needed for those instances. Accordingly, three new chemical reductions of chlorite were invented and developed to produce $ClO_2$ for antimicrobial food packaging concepts, disinfecting graywater, sanitizing hard surfaces, decontaminating porous materials (i.e., textiles), self-decontaminating protective garments, and other innovative applications.

All of these next-generation chemical systems that produce chlorine dioxide could be used in the food industry in applications such as treating fresh produce before shipping to consumers, sanitizing surfaces in food processing facilities, and cleaning-in-place methods for production lines. An active food packaging system with antimicrobial properties was developed using one of the next generation chemical systems in conjunction with a film made of PLA biopolymer as a biodegradable food packaging material, because of the interest in recent years of reducing food packaging waste and increasing environmental sustainability (Ray et al., 2013). Additionally, an active food packaging system was also developed that inserted a sachet consisting of superabsorbent hydrogel polymer incorporated with chemical precursors into a clamshell plastic container and called the CoD. The CoD packaging system

is activated by high humidity for the time-released, controlled, sustained production $ClO_2$ in-container. Both of these antimicrobial food packaging concepts could be used by the food industry to inactivate pathogens, prevent mold growth, and extend the safe shelf-life of perishable foods that respire in-packaging (fresh berries, fruits, and vegetables) and thereby reduce economic losses.

These next-generation technologies can also be adapted for other applications. For example, the active food packaging system (called CoD) can be used in other military applications in which mold growth is also a concern, such as clothing (uniforms, undergarments, socks, boots), tents and rigid-walled shelters, and vehicle mats, especially when stored in warm and humid environments. Additionally, the next-generation chlorine dioxide technologies could be used in conventional cleaning and disinfectant applications, such as disinfecting surgical or laboratory instruments, sanitizing surfaces in hospitals, nursing care facilities, kitchens or galleys, or other janitorial-type applications (showers, laundries, latrines, public restrooms, and corridors), or cleaning surfaces in vehicles. Descriptions of these next-generation chlorine dioxide technologies, their chemical bases for generating chlorine dioxide, demonstrations of their operation, and tests validating their microbiological efficacy in: (*i*) preventing mold growth on parachute sleeves, (*ii*) inactivating the notorious pathogen in hospitals and healthcare settings *Clostridiodes difficile*, and (*iii*) creating self-decontaminating textiles for protective garments that inactivate bacterial spores are presented below.

### 17.3.1 Disinfectant for environmentally friendly decontamination, all-purpose (D-FEND ALL)

D-FEND ALL was developed to be especially convenient for field use in applications on a slightly larger scale than a typical handheld spray-bottle (approx. 1 L) could accommodate. For example, the dry precursors of the D-FEND ALL system were added to a beaker of water ($6 < pH < 8$) and stirred, to produce the pale yellow color of $ClO_2$ almost immediately. With variations in the stating conditions, $ClO_2$ concentrations in the range of 5–5900 ppm were produced quickly in the 3–15 min timeframe and showed no noticeable generation of exothermic heat. The reactivity of the reductant is such that it produces dilute concentrations of $ClO_2$ rapidly and without generating substantial exothermic heat. This chemical system was an integral and essential component of an environmentally friendly graywater recycling system to render potable water in expeditionary base camps.

The D-FEND ALL system was used to generate $ClO_2$ solutions in the concentration range of 20–200 ppm, as determined using a $ClO_2$ indicator strip. For microbiological validation, individual strips (1" × 2") of military fabric samples using the Advanced Combat Uniform (ACU, made of 50%:50% nylon/cotton) or an experimental weatherproof fabric printed with a camouflage pattern were spot-inoculated with spores of *Bacillus amyloliquefaciens* and *Bacillus anthracis* Sterne. Inoculated fabric strips were immersed in the 20–200 ppm $ClO_2$ solutions in pouches and paddle-mixed with a Stomacher for 2–4 min. After 10 min, excess $ClO_2$ was quenched with reductant, and the solution was serially diluted and spread-plated on agar with ST-1 and nutrient agar, respectively. The $ClO_2$ solutions at concentrations of 20, 40, 80, and 100 ppm inactivated 0, 1.3, 2.0, and 7.3-logs of *B. amyloliquefaciens* spores, respectively. All of the $ClO_2$ concentrations (20–100 ppm) inactivated 7.5-logs of *B. anthracis* Sterne spores. Further, the ACU samples were unaffected visually by exposure to $ClO_2$ at all of the concentrations tested, whereas the experimental weatherproof fabric samples were unaffected at concentrations at or below 80 ppm, but became bleached at the 200 ppm concentration.

### 17.3.2 Active food packaging concept using PLA

The potential for PLA to be used as antimicrobial active packaging systems has been reported previously (Ray, 2013). PLA can be synthesized from renewable bioderived monomers and is an alternative to conventional petroleum-based polymers for commercial use in food packaging applications with a focus on films and coatings that are suitable for short shelf life and ready-to-eat food products. The use of PLA as a chlorine dioxide-releasing film for the microbial decontamination of fresh produce used acidification chemistry with the chemical precursors incorporated into the PLA films. Moisture in the PLA package activated the release of gaseous $ClO_2$.

The chemical precursors used in the D-FEND ALL (see Section 17.3.1) system and in the CoD system (see Section 17.3.3) were tested with PLA as a candidate active food packaging material. Samples of PLA film (16 in. wide, 0.2 mm thickness) were cut into 2-in. square coupons and spotted with drops of concentrated solutions of either the oxidant and reductant on two separate PLA squares, or of both reagents onto spatially discrete regions of a single PLA coupon. After drying over night at $T = 25$ °C in a covered glass dish, the spotted coupons were placed in a beaker of water at the same time and the yellow color of $ClO_2$ appeared over the next 3–15 min. These results indicate that these chemical systems could be used with the biopolymer PLA as active food packaging that produced the antimicrobial chlorine dioxide. The oxidation–reduction chemical precursors could also be incorporated into a plastic spray bottle or container that, when filled with water, would generate a sanitizing solution for spraying onto or receiving microbially contaminated objects, respectively.

### 17.3.3 The Compartment of Defense active food packaging concept

A third chemical system was invented that used another alternative reductant. Tests were carried out using a 100 ppm $ClO_2$ solution made by mixing chlorite and this reductant in water to inactivate spores of the foodborne pathogen *Bacillus cereus*. The 100 ppm $ClO_2$ solution inactivated $5.5 \times 10^4$ CFU/mL of the *B. cereus* spores directly in solution or by immersing fabric samples spot-inoculated with the *B. cereus* spores into the disinfectant solution, paddle-mixing the sample, quenching the remaining $ClO_2$ with reductant, and plating on nutrient agar medium.

The third chemical system was also used in conjunction with a polymeric material (i.e., superabsorbent hydrogel) to absorb humidity and controllably generate gaseous $ClO_2$ in-container as an active food packaging system called the CoD. To demonstrate the operation and microbial validation with the CoD system, a superabsorbent hydrogel pad was impregnated with dry $ClO_2$-producing precursors and placed inside a plastic clamshell container. The container was made of the biopolymer PLA or polyethylene terephthalate (PET) and had vent holes to allow the free-flow exchange of moisture with its surroundings. Additionally, bioindicator strips of *G. stearothermophilus* or *B. atrophaeus* spores and a $ClO_2$ chemical indicator strip were placed inside the plastic container. The entire container was placed inside a desiccator containing a saturated solution of KCl to created a humid (87–90 %RH) environment. The entire desiccator with all its contents were placed in a warm ($T = 25$–$35$ °C) incubator. After 24–48 h of incubation, the $ClO_2$ indicator showed approx. 25 ppm level of $ClO_2$ had been reached and the spore bioindicators were inactivated. This example demonstrated the proof-of-concept for the CoD active packaging concept. Specifically, the test confirmed that the oxidation–reduction reaction had taken place through the absorption of moisture from the humid environment water into the absorbent polymeric pad substrate to produce $ClO_2$. The low-level concentration of $ClO_2$ was produced over time

in a time-released manner. This packaging concept can be used for fresh produce, vegetables, berries, plants, and other tissues that respire in-packaging to create a humid environment and trigger the controlled production of $ClO_2$.

### 17.3.4 The Biospray technology and the inactivation of *Clostridiodes difficile* spores

The fourth next-generation system, called Biospray, uses a biological reagent to generate $ClO_2$ solution in a sprayer to disinfect equipment and facilities in microbiology and molecular biology laboratories, particularly those that routinely handle pathogens or infected biological tissues. These reagents could be incorporated into PLA films used as food packaging or in collapsible plastic spray bottles to sanitize food processing and handling equipment, food contact surfaces, or surfaces in kitchens, galleys, hospitals, or nursing care settings.

Biospray was validated against spores of *Clostridiodes difficile*, a notorious opportunistic pathogen in hospitals and nursing care settings. Infections with *C. difficile* constitute a public health threat, with over half a million cases and 29,000 deaths estimated to have occurred in the United States in 2011 (Lessa et al., 2015). *C. difficile* spores germinate, grow out, and produce toxins that cause severe digestive infections and enteric lesions. Eliminating this environmental contaminant from surfaces, objects, foods, etc. would likely reduce the incidence and expenses of illnesses from *C. difficile* infection.

To demonstrate that $ClO_2$ inactivates spores of *C. difficile* irrespective of the chemical precursors or methods used, the Biospray and CoD (see Section 17.3.3) chemistries were tested as aqueous $ClO_2$ solutions on spores of *C. difficile*. Specifically, spores of *C. difficile* wild-type 630Δ*erm* strain were treated with 0–25 ppm aqueous solutions of $ClO_2$ produced using CoD or Biospray. At 15 min, excess $ClO_2$ was quenched, and the spore suspension was plated on brain heart infusion-supplemented agar with 0.1% taurocholic acid (TCA). The plates were incubated at $T = 35$ °C for ~24 h, and germinated spores were counted as CFU (Fig. 17.1). Samples were done in two biological replicates with three technical replicates of each. While each concentration of $ClO_2$ inactivated *C. difficile* spores to some

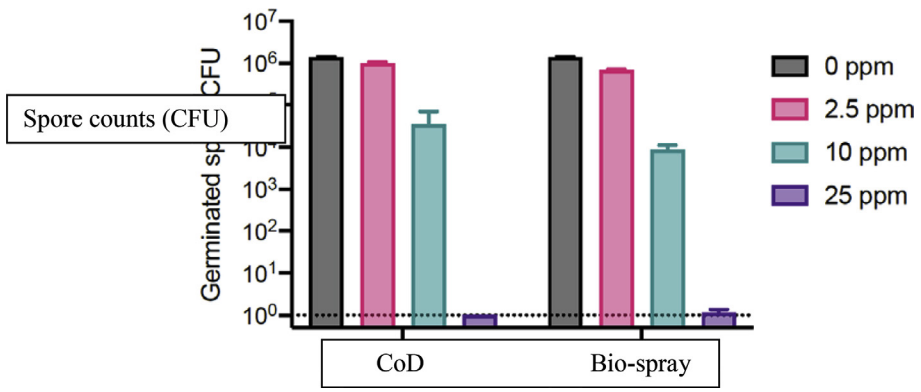


**FIG. 17.1**

Inactivation of *C. difficile* spores by $ClO_2$ from two different sources.

extent (1–2 logs at 10 ppm $ClO_2$), the 25 ppm concentration of $ClO_2$ from both solutions sterilized (inactivated 6-logs) of the *C. difficile* spores, thereby demonstrating that it is the action of $ClO_2$ rather than its method of production that is responsible for decontaminating the microorganisms.

Phase contrast microscopy provided further interesting observations of the $ClO_2$-inactivated *C. difficile* spores. Specifically, phase contrast images of untreated control *C. difficile* wild-type 630Δ*erm* spores showed typical phase-bright character (Fig. 17.2A). However, phase contrast images taken after treatments of the *C. difficile* spores with 25 ppm aqueous $ClO_2$ produced using the CoD or Biospray systems also showed phase-bright character (Fig. 17.2B, C). Similar results were found in the inactivation of *Bacillus subtilis* spores by $ClO_2$ (Young and Setlow, 2003), in which the $ClO_2$-inactivated *B. subtilis* retained their phase-bright character. And although the precise mechanism of $ClO_2$ killing *B. subtilis* spores was not identified, $ClO_2$ was thought to target a location on the spores' inner membrane (IM).

The interactions of *Bacillus* and *Clostridia* spores with high hydrostatic pressures offer some insight into future research needs for inactivating bacterial spores with chlorine dioxide. First, *Bacillus* and *Clostridia* spores have some key morphological and physiological differences that account for very different responses of *B. subtilis*, *C. difficile*, and *C. perfringens* spore to germination by high hydrostatic pressures (Doona et al., 2016). High hydrostatic pressure treatments of *Bacillus* spores at conditions of $P = 150$ MPa and $T = 37$ °C stimulate GRs on the IM and conditions of $P = 550$ MPa and $T = 50$ °C stimulate IM SpoVA channels, so that in both instances, DPA subsequently releases from the spore core, the cortex lytic enzymes (CLEs) become activated, and hydrolysis of the spore cortex peptidoglycan effectuates the conversion to phase-dark spores.

In contradistinction, *C. difficile* spores did *not* respond to high hydrostatic pressure conditions of $P = 150$ MP and $T = 37$ °C, because *C. difficile* does not have IM GRs (GRs are located in the outer layers (OL) in a few *C. difficile* spores). HPP at $P = 550$ MPa and $T = 50$ °C causes DPA release with cortex hydrolysis in *Bacillus* spores, but DPA release *without* cortex hydrolysis for *C. difficile* spores. *Bacillus* spores have two CLEs that are located in spores' OLs and that degrade peptidoglycan spore cortex, whereas *C. difficile* has one CLE and *C. difficile* spores have a specific Csp protease(s) located in the OLs that activates a CLE zymogen (pro-SleC) leading to subsequent cortex hydrolysis, whereas *Bacillus* spores do not have this Csp protease. Accordingly, with conditions of $P = 550$ MPa and $T = 50$ °C, *C. difficile* spores rapidly release approx. 90% of the DPA from the spore core *without*

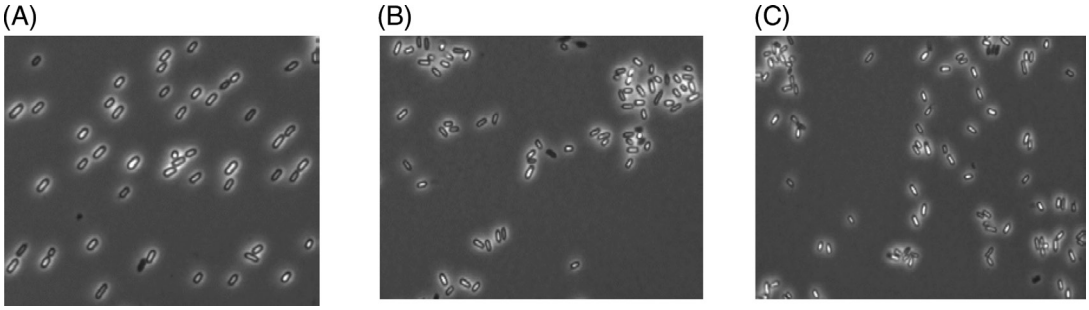


**FIG. 17.2**

(A–C) Phase contrast images of *C. difficile* spores as phase-bright untreated controls (A) and retaining phase-bright character after treatment with 25 ppm $ClO_2$ from the CoD (B) or Biospray (C) systems.

conversion to phase-dark spore (<2%), apparently because the Csp protease and SleC (the CLE) were not activated and the cortex was not hydrolyzed. Apparently, $ClO_2$ inactivates both *Bacillus* and *C. difficile* spores without casing cortex hydrolysis in either. These results suggest that there might be similar loci for spore inactivation by $ClO_2$ for both *Bacillus* and *Clostridial* spores. This finding warrants further research into the mechanism of bacterial spore inactivation by $ClO_2$, and including the effects of $ClO_2$ on *C. perfringens* spores.

### 17.3.5 Chlorine dioxide to control mold (fungal spores)

As discussed above, $ClO_2$ is a potent biocide that functions at low concentrations to inactivate a broad spectrum of microorganisms on surfaces, even the sensitive surfaces of whole tomatoes, fresh berries, sliced apples, and other fresh produce commodities, without damaging the tissue during treatment (Setlow et al., 2009). The CoD system (Section 17.3.3) was developed to prevent mold growth that causes spoilage of fresh produce and berries. Mold growth causes a similar problem with cotton parachute sleeves. Cotton, if stored wet or improperly dried, can support the growth of mold that degrades tensile strength. Therefore, tests were carried out to determine the ability of $ClO_2$ produced with the CoD system to prevent mold growth on cotton parachute sleeves. The versatile biocide $ClO_2$ from the CoD system will be shown to prevent mold growth on the cotton without compromising the mechanical strength of the cotton fabric.

The purpose of the cotton deployment sleeve is to control the opening of the parachute during deployment for increased safety and reliability of the system. The jumper's entire parachute is packed into this narrow, almost 20-foot long sleeve, which is then packed into a bag and container that is worn on the jumper's back. After the jumper exits the aircraft, the parachute deployment sequence initiates, the container and bag open to release the sleeve, then the parachute inflates as it exits from the sleeve and creates intense heat due to friction. The rapid inflation process occurs in less than 1 s and simultaneously pushes the sleeve off the parachute.

With the paramount goal of protecting the safety of the jumpers, maintaining the tensile strength of the textiles used in parachutes and sleeves for multiple uses over a large number of deployments is critical in ensuring the reliability and durability of the parachute systems over many years of parachute service life. Cotton fabric is used as the construction material of the sleeve, because cotton's thermo-resistance allows it to withstand the intense frictional heat associated with deployment without compromising the tensile strength of either the sleeve or the parachute. In contrast, high-tech, synthetic fibers have been investigated for use as the construction material for deployment sleeves and found not suitable for these purposes.

A cotton parachute sleeve previously deployed in a wet landing environment and exhibiting spots of a black mold was tested with three (3) different disinfectants. Mold from the contaminated cotton sleeve was viewed with phase contrast microscopy, to confirm the presence of refractile (phase-bright) spores. Examining the moldy sleeve with light microscopy showed black spores (presumably *Aspergillus niger* spp.) with hyphae on the cotton. Samples (1-$in^2$) were cut from the cotton sleeve, placed in individual Petri dishes containing sterile potato dextrose agar (PDA), wetted with a few drops of sterile water to ensure contact with the agar, and incubated at 25 °C or 30 °C. At both temperatures, the PDA plates exhibited luxuriant mold growth within 3–4 days. One of the moldy cotton samples was transferred to a fresh, sterile Petri dish of PDA with sterile water. A second moldy cotton sample was placed in a plastic pouch containing 10 mL of 100 ppm $ClO_2$ solution, mixed in a paddle-Stomacher

**Table 17.3 Tensile strength and elongation of cotton cloth treated with aqueous decontaminants.**

| Treatment | Breaking strength (lbs) |
|---|---|
| Requirement | 80–96 |
| $ClO_2$ | 92.5 |
| $ClO_2$ | 94.5 |
| $ClO_2$ | 83.5 |
| Avg | 90.2 ± 5.9 |
| | |
| $H_2O_2$ | 37 |
| $H_2O_2$ | 29 |
| $H_2O_2$ | 38 |
| Avg | 34.7 ± 4.9 |
| | |
| $OCl^-$ | N/A |

*Note: All tests in warp direction.*

for 2-min intervals over a 20-min period, then transferred to a fresh Petri dish with PDA. Both samples were stored at 30 °C for 5 days. After incubation, the untreated moldy sample exhibited luxuriant mold growth. The $ClO_2$-treated sample showed no discernible indications of mold growth or damage to the cotton, thereby demonstrating the efficacy of $ClO_2$ in inactivating fungal spores (*Aspergillus niger* spp.).

To test the effects of $ClO_2$ treatment on the tensile strength of the textile substrate, the cotton fabric samples were immersed for 30 min in disinfectant solutions of either $ClO_2$, hydrogen peroxide ($H_2O_2$), or household bleach ($OCl^-$), then rinsed in deionized water and dried. Samples were tested for tensile strength, also called ultimate breaking strength, as measured by applying a controlled force to strips of the 4 oz cotton fabric used in the deployment sleeve according to standard method ASTM D5035-11 (ASTM, 2019).

The break strength requirement for the cotton fabric is 80 lbs. Results in Table 17.3 show that $ClO_2$ is the only disinfectant solution that does not degrade the break strength of cotton (average = 90.15 lbs) below minimum requirements. Conversely, results showed that hydrogen peroxide solutions weakened the break strength of cotton by more than 50% (average = 34.53 lbs), and treatment with typical household chlorine bleach ($OCl^-$) degraded the integrity of cotton so severely that the sample could not be prepared and tested with the standard procedure (Table 17.3). Future R&D of mold prevention technologies for parachute textiles will focus on gaseous $ClO_2$ applications using the CoD system to simultaneously prevent mold growth and retain cotton's mechanical properties.

## 17.4 Nonthermal processing for inactivating *B. anthracis* spores

High pressure processing (HPP) is perhaps the most well-recognized nonthermal processing technology that is growing worldwide in its use for food pasteurization. The inactivation of bacterial spores for food sterilization with HPP is possible, but the food processing industry has not implemented this application commercially. Hydrostatic pressure has also been used to decontaminate *B. anthracis* spores in buffer solutions (Cléry-Barraud et al., 2004). $ClO_2$ is a recognized nonthermal technology as a chemical sanitizer for rinsing fresh produce, and $ClO_2$ inactivates bacterial spores (Doona et al., 2015).

Accordingly, $ClO_2$ in solution (D-FEND ALL), gaseous $ClO_2$ (PCS), and HPP were used to inactivate *B. anthracis* spores on novel fabrics intended for use in self-decontaminating protective garments.

### 17.4.1 Decontaminating bacterial spores on protective garment fabrics

A series of novel fabrics were tested for their abilities to self-decontaminate when challenged with bacterial spore surrogates of *B. anthracis*. In general, the fabrics consisted of a repellant omniphobic coating on the outer surface of a fabric; a cloth layer adsorbed with the antimicrobial compounds (8-hydroxyquinoline, abbreviated HQ, and 1,2-benzisothiazol-3(2H)-one, abbreviated BIT); and an interior liner impregnated with activated carbon. The premised function of these test fabrics was that when challenged with dry aerosols of bacterial spores on their outer surface, humidity would trigger the release of the antimicrobial compounds from the cloth layer to inactivate the bacterial spores. Spores of *B. anthracis* Delta Sterne, a surrogate of the bioweapon *B. anthracis*, were inoculated on the exterior surfaces of fabric samples as dry aerosols using a unique biodispersal chamber (Fig. 17.3) in accordance with the standard method ASTM E2894-12 (Harnish et al., 2014).

### 17.4.2 Dry aerosol inoculation of fabrics

Spore crops were prepared using standard media concentrations to yield smaller crop densities and prevent agglomeration of the spores. Mild heating inactivated vegetative cells prior to cleaning by centrifugation and scraping off debris. The clean spores were lyophilized using a Labconco Freeze Dry System and stored frozen. For testing, preweighed amounts of lyophilized spores were deposited into the loading region (a) of the chamber located in the square black section in the upper, right-side region of the chamber (Fig. 17.3), and the system was turned "on." With air flowing, spores transferred out of the loading area as a dry aerosol, conveyed through the stainless steel cylinder and tubing on top of the chamber (b), released into glass-enclosed biodispersal chamber (c, the entire left-hand side of

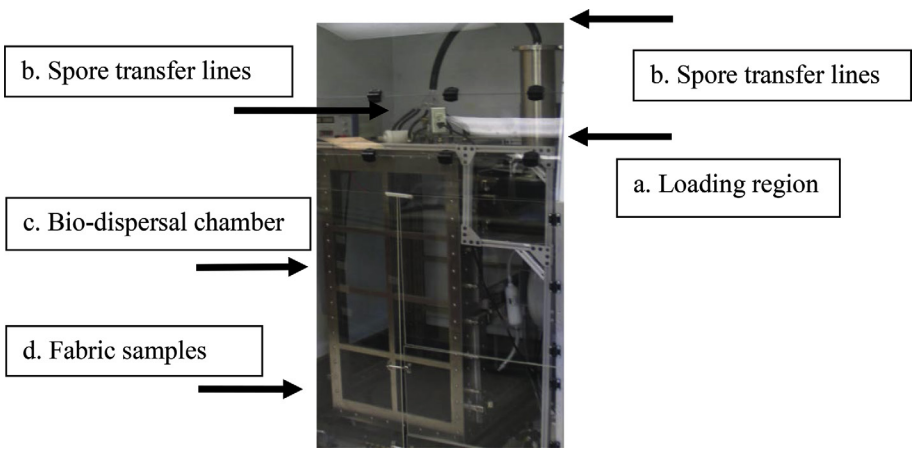


**FIG. 17.3**

Biodispersal chamber for inoculating samples with dry aerosols of spores.

**Table 17.4 Results of challenging fabrics with spores and prolonged high-humidity storage.**

| | Dry aerosol deposition time | Humidity incubation (%RH, $T$, $t$) | Recovered (CFU/cm$^2$) | % Kill |
|---|---|---|---|---|
| Control fabric | 24 h | 0 | $2.39 \times 10^6$ | 0 |
| Control fabric | 24 h | 18%, 37 °C, >24 h | $\sim 10^6$ | 0 |
| Fabric-1 | 24 h | 0 | $1.14 \times 10^6$ | 0 |
| Fabric-1 | 24 h | 89%, 37 °C, 15 d | $2.21 \times 10^6$ | 0 |
| Fabric-1 | 24 h | 89%, 37 °C, 28 d | $2.76 \times 10^6$ | 0 |
| Fabric-2 | 24 h | 0 | $1.80 \times 10^6$ | 0 |
| Fabric-2 | 24 h | 89%, 37 °C, 15 d | $1.67 \times 10^6$ | 0 |
| Fabric-2 | 24 h | 89%, 37 °C, 28 d | $1.71 \times 10^6$ | 0 |

Fig. 17.3), and deposited onto the multiple 1-in$^2$ fabric samples located at the bottom of the biodispersal chamber on a rotating surface (d).

After inoculation, the test samples were placed in humidity-controlled glass containers (covered desiccators containing saturated KCl solutions producing conditions of 89%RH and $T = 37$ °C) and the entire desiccator was stored isothermally in incubators (Feeherry et al., 2003). Inoculated control samples were covered and stored at $T = 35$ °C and 18 %RH. The inoculated fabric test samples were withdrawn at intervals over a 28-day period (over a 60-day period for control samples) for enumeration.

To recover and enumerate the spores from the inoculated fabrics, samples were placed in sterile pouches with sterile water and mixed with a paddle stomacher (Feeherry et al., 2003), and the water was subsequently serially diluted and plated on Nutrient Agar. In all cases, 100% of the spore load was recovered (Table 17.4). The fabrics did not inactivate contaminating bacterial spores and the fabrics are not inherently self-decontaminating.

### 17.4.3 Alternative methods of decontamination

To decontaminate the bacterial spores on the inoculated fabrics, alternative methods of applying external agents were tested. Initial experiments used aqueous solutions of $ClO_2$ or bleach ($OCl^-$) to treat the fabrics, according to the following methods to determine the quantity of spores removed from the fabrics by the mechanical agitation of the rinsing process versus the quantity of spores inactivated by the chemical decontaminating agent.

In the first method, the inoculated fabric samples were immersed in 20 mL of sterile water in a Stomacher bag labeled RINSED and agitated mildly by gently moving the fabric sample through the water. Next, the fabric sample was moved to a second Stomacher bag containing 20 mL of sterile water and labeled RECOVERED. The count of spores removed by the rinsing process were determined by serially diluting the solution in the RINSED stomacher bag, spread-plating on NA plates, incubating, then enumerating the plates (Table 17.5).

The counts of bacterial spores remaining on the fabric samples *after* the water rinse were determined by vigorously agitating the sample in the RECOVERD bag with a Stomacher, then serially diluting the RECOVERED water, spread-plating it on NA plates, incubating the plates, and enumerating survivors (Table 17.5). Carrying out the same process with immersing the fabric samples in 20 mL of water (RINSE), then moving the fabric samples to 20 mL of household bleach (5–6% hypochlorite, $OCl^-$) and adding a small quantity of solid reductant at 10 min of exposure to quench the $OCl^-$ yielded

**Table 17.5 Results of physical decontamination by aqueous rinsing.**

| | Inoculum (CFU/cm$^2$) | RINSED (CFU/mL) | RECOVERED (CFU/mL) | Log-kill (removed) |
|---|---|---|---|---|
| Fabric-1 | **$1.61 \times 10^6$** | | | |
| Fabric-1 | | $1.4 \times 10^4$ | $2.7 \times 10^4$ | 0.46 |
| Fabric-1 | | $8.6 \times 10^3$ | $9.0 \times 10^3$ | 0.31 |
| Fabric-1 | | $3.2 \times 10^3$ | $5.9 \times 10^3$ | 0.45 |
| | | **$8.6 \pm 5.4 \times 10^3$** | **$1.4 \pm 1.1 \times 10^4$** | **0.41 ± 0.08** |
| Fabric-2 | **$2.51 \times 10^6$** | | | |
| Fabric-2 | | $9.6 \times 10^2$ | $1.01 \times 10^3$ | 0.31 |
| Fabric-2 | | $3.2 \times 10^3$ | $1.81 \times 10^3$ | 0.19 |
| Fabric-2 | | $7.2 \times 10^3$ | $5.9 \times 10^3$ | 0.26 |
| | | **$3.8 \pm 3.2 \times 10^3$** | **$2.9 \pm 2.6 \times 10^3$** | **0.25 ± 0.06** |
| Fabric-1 Bleach rinse—10 min | **$1.61 \times 10^6$** | $1.1 \times 10^2$ | 0 | >4 |
| Fabric-2 Bleach rinse—10 min | **$2.51 \times 10^6$** | $2.0 \times 10^0$ | 0 | >6 |

different results from the water-only rinse. Table 17.5 shows that rinsing the inoculated fabrics with copious water effectuated physical decontamination to only a minor extent (less than 0.5-log reduction), and that exposure to $OCl^-$ for both types of fabrics completely inactivated all of the spores remaining after the rinsing process.

In the second approach, the inoculated 1-in$^2$ fabric samples were cut in half with sterile scissors. The control half was enumerated without further treatment, while the second half of the sample was subjected to a lethal treatment (aqueous bleach $OCl^-$, aqueous or gaseous $ClO_2$, or HPP) before enumerating survivors. Treatment with a bleach rinse (5–6% hypochlorite, $OCl^-$) for 30 min inactivated all contaminating spores (>5-logs, see Table 17.6). $ClO_2$ sterilized contaminating bacterial spores on all of the inoculated fabric samples, irrespective of whether the inoculated fabric samples were immersed in aqueous solutions of $ClO_2$ made using the D-FEND ALL technology (data not shown) or whether the inoculated fabric samples were treated with gaseous $ClO_2$ in the PCS for 30 min (>4.60-log inactivation, see Table 17.6). Treating the inoculated fabric samples with HPP at conditions of $P = 550$ MPa, $T = 65$ °C, time $t = 100$ min effectuated >6-log inactivation of all contaminating *B. anthracis* Sterne spores (Table 17.6). To prevent cross-contamination of the pressure-transmitting fluid, the inoculated fabric samples were sealed in sterile pouches for the HPP experiments. It is important to note that 1 mL of sterile water was added to the pouch with the inoculated fabric sample to achieve the >6-log inactivation. HPP treatments of the same inoculated fabrics at the same HPP conditions *without* adding water to the pouch did *not* inactivate the bacterial spore dry powder.

**Table 17.6 Alternative treatments to decontaminate spores on fabrics.**

| Fabric sample | Control (CFU/mL) | Bleach—30 min (CFU/mL) | Log (kill) | Control (CFU/mL) | Gaseous ($ClO_2$)—30 min | Log (kill) | Control (CFU/mL) | HPP | Log (kill) |
|---|---|---|---|---|---|---|---|---|---|
| Fabric-2 | $1.10 \times 10^5$ | 0 | 5.04 | $4.00 \times 10^4$ | 0 | 4.60 | $1.34 \times 10^6$ | 0 | 6.13 |
| Fabric-1 | $3.90 \times 10^6$ | 0 | 6.59 | $5.40 \times 10^5$ | 0 | 5.73 | $1.28 \times 10^6$ | 0 | 6.11 |

Fabric samples inoculated with the surrogate *B. anthracis* Delta Sterne spores ($10^6$ CFU/cm$^2$) using the ASTM E2894-12 dry aerosol method and incubated for 28 days at elevated humidity-temperature conditions (89 %RH and 37 °C) showed no discernible inactivation of spores—the spores were not even activated by this prolonged humidity incubation. The presence of the antimicrobial compounds HQ and BIT in the fabrics did not interact with and inactivate the bacterial spores, presumably because the repellant coating acts as a physical barrier preventing any such interaction. The HQ and BIT were not even leached from the fabrics to the surface to effectuate inactivation of the contaminating spores. The repellant coating, however, does not protect spores from externally applied sterilants, whether aqueous or gaseous $ClO_2$ or chemical-free high hydrostatic pressures, and all three methods sterilized the fabrics.

## 17.5 Conclusions

In 2014–2015, a gaseous chlorine dioxide technology invented at US Army CCDC SC (Natick Soldier RD&E Center) was adapted for use in the Ebola-stricken areas of West Africa by USAMRIID, NIH (NIAID), Doctors without Borders, Public Health Canada, and other global public health organizations for use in remote environments with limited infrastructure for power and clean water. This technology was also used to decontaminate the biodispersal chamber prior to its use in inoculating protective fabrics with *B. anthracis* Sterne. As the inventory of CCDC SC's innovative chlorine dioxide technologies continues to grow with D-FEND ALL, the CoD, and Bio-spray, the uses of these technologies for food packaging, sanitizing hard surfaces, decontaminating porous materials, preventing mold growth, or disinfecting pathogens such as *B. cereus, C. difficile*, Ebola virus, and perhaps Coronavirus to prevent the spread of COVID-19 and meet the needs in broad-based or niched dual-use applications for military and civilian consumers will likely continue to increase.

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