Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

REVIEW ARTICLE

Terminal sterilization of medical devices using vaporized hydrogen peroxide: a review of current methods and emerging opportunities

B. McEvoy[1] and N.J. Rowan[2]

1 STERIS Applied Sterilization Technologies, IDA Business and Technology Park, Tullamore, Ireland
2 Bioscience Research Institute, Athlone Institute of Technology, Athlone, Ireland

Keywords
Bacillus, bacterial endospores, medical devices, sterilization, terminal gaseous sterilization, vaporized hydrogen peroxide.

Correspondence
Brian McEvoy, STERIS Applied Sterilization Technologies, IDA Business and Technology Park, Tullamore, Ireland.
E-mail: brian_j_mcevoy@steris.com

2019/0278: received 15 February 2019, revised 3 July 2019 and accepted 25 July 2019

doi:10.1111/jam.14412

Summary

Medical devices are an important and growing aspect of healthcare provision and are increasing in complexity to meet established and emerging patient needs. Terminal sterilization plays a vital role in the provision of safe medical devices. While terminal sterilization technologies for medical devices include multiple radiation options, ethylene oxide remains the predominant nonthermal gaseous option, sterilizing *c.* 50% of all manufactured devices. Vaporized hydrogen peroxide (abbreviated VH2O2 by the International Organization for Standardization) is currently deployed for clinical sterilization applications, where its performance characteristics appear aligned to requirements, constituting a viable alternative low-temperature process for terminal processing of medical devices. However, VH2O2 has operational limitations that create technical challenges for industrial-scale adoption. This timely review provides a succinct overview of VH2O2 in gaseous sterilization and addresses its applicability for terminal sterilization of medical devices. It also describes underappreciated factors such as the occurrence of nonlinear microbial inactivation kinetic plots that may dictate a need to develop a new standard approach to validate VH2O2 for terminal sterilization of medical devices.

Introduction

Medical devices play an important role in the provision of healthcare, where the global market for which is predicted to surpass $400bn by 2020 (International Trade Administration 2016). According to the International Organization for Standardization (ISO 2016b), a medical device may be defined as an 'instrument, apparatus, implement, machine, appliance, implant, reagent for *in vitro* use, software, material or other similar or related article, intended by the manufacturer to be used, alone or in combination, for human beings, for one or more of the specific medical purpose(s)', with such purposes further described in ISO13485:2016 (ISO 2016b). As indicated in the definition, medical devices provide critical care and diagnostic applications through patient contact. An important consideration is safe use, whereby patient infection does not arise as a consequence of such contact. Such hospital-acquired infections (HAIs) are defined by the World Health Organization as infections developing after 48 h of hospitalization that were not present or incubating at the time of admission (Rowan and Anderson, 1998; RAISIN Working Group 2009). HAIs are estimated to affect some 1.7m patients in the United States, with an estimated annual cost to healthcare of $9.8bn (Hensley and Monson 2015). HAIs also impact negatively upon patient health, well-being and quality of life. Infections acquired in hospital may lead to sepsis, a 'life threatening organ dysfunction caused by a dysregulated host response to infection' (Dugani *et al.* 2017; Masterson *et al.* 2019). There are some 750 000 cases of sepsis annually in the United States, with a cost to healthcare of *c.* $20bn (Guirgis *et al.* 2017). While there are many known sources of HAIs (Bauld 2016; Dasenbrock *et al.* 2016), terminally sterilized medical devices are

unlikely to be a point source of infection due to associated rigorous sterilization and validation processes that deliver a sterility assurance level (SAL) far above the minimum requirements to achieve sterilization for patient safety.

Sterility assurance and terminal sterilization processes

Sterility assurance level may be defined as the 'probability of a single viable micro-organism occurring on an item after sterilization' (ISO 2018), and a sterilization process may be defined as a 'series of actions or operations needed to achieve the specified requirements for sterility' (ISO 2018). In order to achieve a required SAL, a sterilization process is applied to medical devices prior to patient use; see Fig. 1. Traditional technologies used in such terminal sterilization processes are described in Table 1 and are Category A sterilization methods according to the US Food and Drug Administration (FDA), as they are well established, they have a long history of use and consensus standards (administered by the ISO) are available. Standards such as ISO14937:2009 help inform manufacturers and users of the key aspects to be evaluated in defining the sterilization process and subsequent qualification and validation. Key considerations such as equipment and process definition, sterilization agent characterization and process validation are described in Table 2.

Medical device sterilization processes

The sterilization marketplace can be subdivided into three broad categories: (i) hospital sterilization, such as point

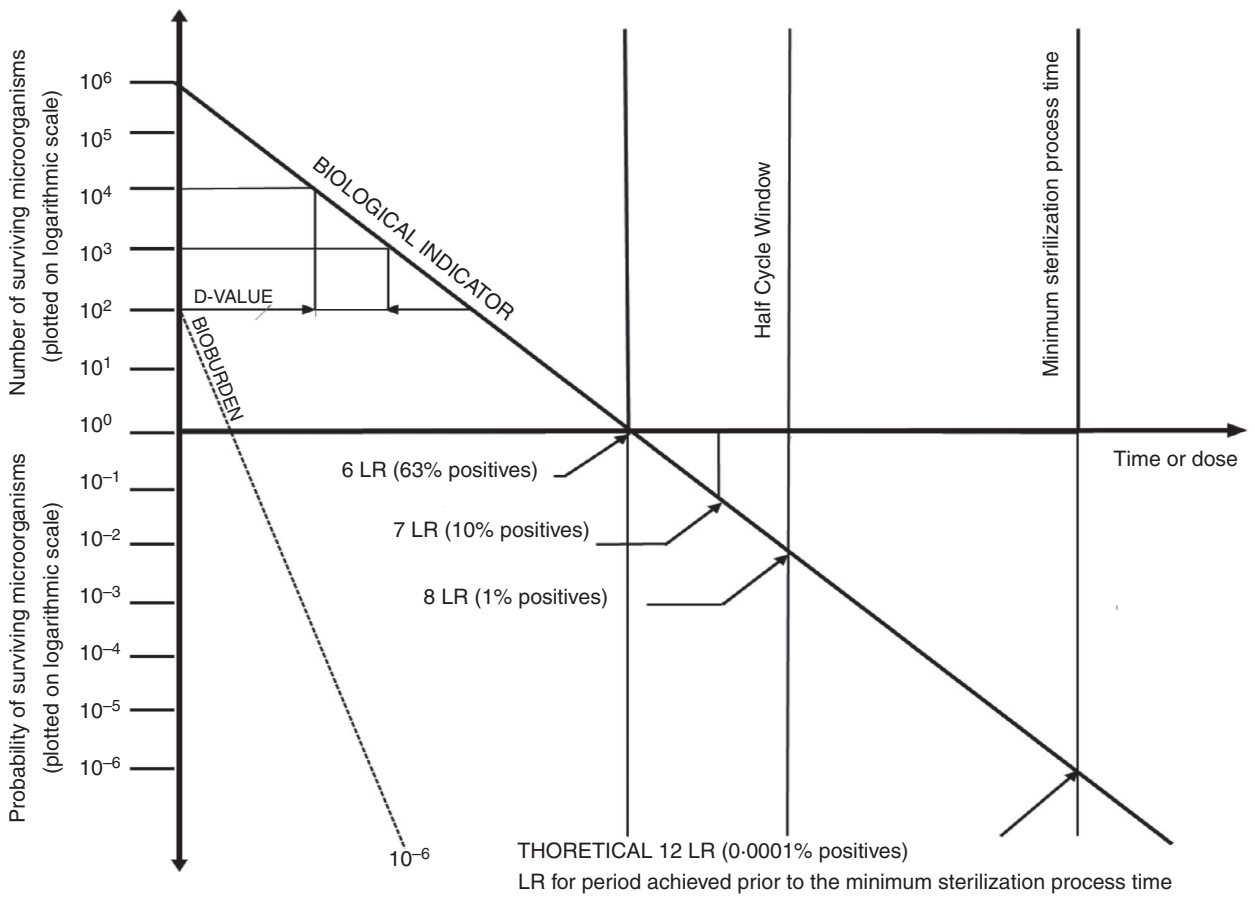

Figure 1 Sterility assurance level and example of the relationship between biological indicator and product bioburden. For illustration purposes, this graphical representation has been obtained from AAMI TIR16:2017. A BI (denoted by full line) has been selected that has a higher population and resistance (*D*-value) than that of the medical device product bioburden (denoted by dashed line). Using an overkill validation method, a sterilization process has been applied at 'half-cycle' parameters with full lethality on the BI. (In EO processing, half-cycle parameter is half of EO exposure time). In order to deliver the required sterility assurance level of $\leq 10^6$, a further 6 log reduction (LR) is applied by doubling the exposure period in the routine process. Note: Microbiological death in EO sterilization follows first-order kinetics and can be approximated by a straight line on a semi-logarithmic plot when the sterilizing conditions (i.e., process temperature, RH, and EO concentration) remain consistent for the duration of the exposure time. (AAMI, 2017)

Table 1 Global contract sterilization market by processing technology (source of Market share data: iia/GIPA (2017); source of other information: STERIS-AST.com (https://www.steris-ast.com/services/technology-comparison/)

	Ethylene Oxide	Gamma	E-beam	X-ray
Market share (%)	50	40·5	4·5	<5
Methodology	Penetration of sterilant gas into packaged product	Irradiation of product using photons from radioisotope	Product sterilized using ionizing energy from electron beam	Products sterilized using high energy X-ray photons
Sterilizaing Agent Penetration	Requires gas-permeable packaging and product design	Good penetration complete even at high densities (>0·4 g per cc)	Efficient penetration at bulk densities between 0·05–0·30 g per cc	Similar to gamma, excellent penetration for all product types
Material Compatibility	Very few material compatibility concerns. Liquids are generally not recommended	Compatible with most materials; plastics need to be evaluated Avoid acetals, PTFE (teflon), unstable polypropylene	Negative effects are frequently less pronounced or eliminated	Similar to gamma, however, negative effects are frequently less pronounced or eliminated
Turnaround time	Days: conventional = 5–10 days. All-in-one processing = 1 day	Hours: time varies based on dose requirements	Minutes: time varies based on dose requirements	Hours: time varies based on dose requirements
Process	Complex process: Variables include exposure time, temperature, humidity and EO concentration. Nonprocess variables impacting lethality include load density, packaging	Simple process: Variables include time in the cell and isotope load	Complex process: Variables include scan height, processing speed, number of passes and product orientation to the beam	Moderately complex process: Variables include processing speed, number of passes and number of pallets on conveyor
Process monitor	Biological indicators and process monitoring	Process parameters confirmed by dosimetry	Process parameters confirmed by dosimetry	Process parameters confirmed by dosimetry
Typical medical devices processed	Wide ranging compatibility: Bulk devices such as surgical procedure packs and trays Drug combination products such as Cardiovascular stents; devices with electronic/ electrical componentry	Some polymer incompatibility but suitable for many medical devices: Orthopaedic polymer and metal implants; Healthcare consumables such as sprays and wipes; labware; complex geometry devices	Wider polymer compatibility and suitable for many medical device types such as: Cardio and neurovascular devices; tissues; biologics	Similar to gamma

of use; (ii) in-house manufacturing sterilization, such as at point of medical device manufacture, often in-line applications; and (iii) contract sterilization, provided by contractors where medical device manufacturers obtain sterilization services along the supply chain after manufacturing. This review focuses on technologies employed in both the contract sterilization and in-house sterilization, specifically the terminal sterilization of medical devices. Terminal sterilization processes are delivered by the medical device manufacturers themselves or by specialist contractors, with a market estimated at over $4bn and growing at a CAGR over 8% a year (Gamma Irradiation Processing Alliance and International Irradiation Association, 2017). As highlighted in Table 1, the selection of terminal sterilization technologies is based on a number of factors, mainly market acceptance, product compatibility and availability. For further insights, see the whitepaper 'A Comparison of Gamma, E-beam, X-ray and Ethylene Oxide Technologies for the Industrial Sterilization of Medical Devices and Healthcare Products' (Gamma Irradiation Processing Alliance and International Irradiation Association 2017).

Use of gamma irradiation and ethylene oxide (EO) constitutes ca. 95% of the terminal sterilization market. In 2008, the US National Research Council determined that there were some 63 panoramic gamma irradiators in the United States, using some 198 million curies of cobalt-60 as a radiation source. It is currently estimated that there are some 400 million curies installed globally for use in gamma radiation facilities (Gamma Irradiation

Processing Alliance and International Irradiation Association 2017). While not all cobalt is dedicated to medical device sterilization, it may be estimated that such a loading of 400 million curies equates to the processing of some 10 million cubic metres of products, with a similar volume in EO processed globally. Although these two sterilization processes are the most widely used, hazards associated with their use require careful organization and control, reflecting the need to address transportation, installation, use and storage of radiation sources. For EO gas, necessary control and environmental abatement measures must be deployed, as it is deemed a carcinogen by the International Agency for Research on Cancer (IARC 2012).

In the field of radiation, much effort is currently underway to further the deployment of alternative accelerator-based technologies to reduce the reliance on isotope processing. Progress has been slow for reasons highlighted by Kroc *et al.* (2017), who offer a comprehensive review of the factors impeding device sterilization using irradiation with accelerator based technology. In gas sterilization technology, while there has been considerable effort by the terminal sterilization industry to control, manage and reduce the required inputs of EO gas, a viable gaseous alternative with similar sterilization efficacy and material compatibility remains elusive.

In the 1990s, a number of authors highlighted the pressing need for an alternative nonhazardous, noncarcinogenic gaseous industrial terminal sterilization (Klapes and Vesley 1990; Rutala and Weber 1996). Rutala and Weber (1998) described the characteristics of an ideal low-temperature industrial sterilization process, which is summarized in Table 3. As much as identifying the need for alternative gaseous sterilization solutions, these authors also proposed alternative methods. Rutala (1996) discussed the use of liquid peracetic acid, hydrogen peroxide (H_2O_2) plasma and a dual gas plasma system sterilization and disinfection in hospitals that potentially fit the criteria for an 'ideal low-temperature sterilization process'. Vaporized hydrogen peroxide (VH2O2) presents a viable alternative to EO that is already widely used in other healthcare applications. VH2O2 has a history of being an effective disinfectant and sterilant: it delivers sterilization in a fast, effective manner without generating carcinogenic or toxic residuals. The IARC has determined that H_2O_2 is not classifiable as to its carcinogenicity to humans (Agency for Toxic Substances and Disease Registry 2019). The advantages of using gaseous H_2O_2 are described by Hultman, Hill and McDonnell (2007): (i) it will have uniform contact with all exposed surfaces, including those with complex topographies; (ii) it may be safely maintained in a chamber environment and (iii) it may be efficiently and quickly removed from a chamber.

Vaporized hydrogen peroxide

History of vaporized hydrogen peroxide in sterilization

The use of H_2O_2 as a biocidal agent originates from the early 1800s following its discovery by French chemist Louis-Jacques Thénard (1777–1857). The work of early pioneers in disinfection, such as Benjamin Ward Richardson (1828–96), helped advance the applications of H_2O_2 (McDonnell 2014). Today, its use as a disinfectant is widespread, with applications including wound antiseptic, general surface disinfectant and dental disinfectant. Hydrogen peroxide is also employed in disinfectant formulations used in contact lens treatment and food postharvesting, and as a packing sterilant in aseptic processing (Linley *et al.* 2012).

The origins of the use of vaporized H_2O_2 in industrial sterilization may be traced back to 1977, when US patent #4 169 123 was granted to Francis Moore and Leon Perkinson for their 'Hydrogen Peroxide Vapor Sterilization Method', which describes a 'cold sterilization process' alternative to EO sterilization and radiation sterilization (Moore and Perkinson 1979). The patent also describes, with examples, the efficacy of the process in delivering a 1×10^{-6} SAL, in line with the FDA recommendation for sterile medical and surgical products.

Subsequently, AMSCO (now part of STERIS) secured rights to use VH2O2 as a sterilant, and further developments of the equipment and process ensued. After the development of the flash vaporizer, AMSCO built the first prototype sterilizer in 1983. Throughout the 1980s, AMSCO worked with researchers, pharmaceutical clients and equipment clients to develop applications for VH2O2. One output of such activities was the release of the 'VHP 1000' Biodecontamination System that was launched to the pharmaceutical customer base in 1991 (AMSCO, 1992). Early work by Rickloff (1989) reported success with sterilization of intravenous sets using a 4·5-h deep vacuum VH2O2 process with *Bacillus subtilis* as an indicator organism (AMSCO, 1992).

From those early years, the application of VH2O2 has evolved into atmospheric and vacuum processes: atmospheric pressure conditions, as is the case for VH2O2 room, isolator, vehicle and building decontamination; or vacuum conditions in low-temperature sterilization applications, such as reusable medical device sterilization (McDonnell, 2014).

Mode of action

Hydrogen peroxide is a clear, water-soluble weak acid that, when concentrated, acts as a strong oxidizing agent. Its properties are described in Table 4 and contrasted

Table 2 Key consideration in process validation (adapted from ISO 2016a)

Consideration	Details
Sterilization equipment	Equipment will be suitable for processing with the sterilizing agent being used (in this case VH2O2) and will do so in a safe and consistent manner. The equipment will contain process control equipment, such as programmable logic controllers and instrumentation, that is capable of both controlling the process to defined parameters and reporting on the data outcomes of the delivered process in such a manner that the user can be confident that the sterilization process was delivered as intended
Sterilization process	A sterilization process is one comprising a 'series of actions or operations needed to achieve the specified requirements for sterility' (ISO 2018). Process parameters with their tolerances are defined in such a manner that they qualify the effectiveness of the sterilization process, and demonstrate the achievement of specification. The process may comprise such things as the sterilizer equipment, measurement devices, biological indicators, chemical indicators and testing, all of which combined are employed to define, control and report upon a sterilization process
Sterilizing agent	ISO14937:2009 requires that the sterilizing agent be described regarding safety, environmental aspects, material effects and microbicidal effectiveness. Regarding the microbicidal effectiveness, a number of key items must be demonstrated: i Demonstration of the lethal action against a range of representative challenge micro-organisms ii Establishment of an 'empirical mathematical relationship defining the microbial inactivation kinetics of identified resistant micro-organisms so that the probability of a micro-organism surviving exposure to a defined treatment can be predicted' per methods defined in Annexes B,C,D of ISO14937:2009) iii Identification of a reference micro-organism that has known high resistance to the sterilizing agent iv Characterization of the process variables that affect the lethal action of the sterilizing agent v Assessment of those factors that can adversely influence the effectiveness of the sterilizing agent based upon physical and/or chemical interactions vi Assessment of those factors that can adversely affect the delivery and/or distribution of the sterilizing agent vii Identification of a means for terminating the activity of the sterilizing agent, if applicable (ISO 2016a)
Process validation	The purpose of validation is to 'demonstrate that the sterilization process established in the process definition can be delivered effectively and reproducibly to the sterilization load (ISO 2016a). The validation process comprises a number of key stages: i installation qualification (IQ), which is performed to demonstrate that equipment is installed in accordance with specified requirements ii operational qualification (OQ), which is performed to demonstrate the capability of the equipment to deliver the specified sterilization process iii performance qualification (PQ), where the process is qualified using product samples and process indicators to demonstrate that the required lethality and subsequent sterility assurance are achieved

with those of EO; note that the boiling point of H_2O_2 is above that of water, and there is currently no evidence of carcinogenicity in humans as attested by assignment of zero classification by IARC. Hydrogen peroxide is an oxidizing agent and, as such, one would expect deleterious effects on biological constituents of micro-organisms: Finnegan *et al.* (2010), in their examination of H_2O_2, peracetic acid and chlorine dioxide, demonstrated that as redox potentials increase in biocides, the level of oxidation of amino acids also increases. While there are direct oxidation effects, there may also be secondary effects such as free-radical generation (McDonnell 2014). The disinfection and sterilization efficacy of H_2O_2 in both aqueous and gas forms from the formation of hydroxyl radicals is well documented (Block 1991; McDonnell 2014). Hydrogen peroxide shows effective antimicrobial activity against a wide range of organisms, including vegetative and bacterial endospores, fungi and viruses (Linley *et al.* 2012; McDonnell 2014); concentrations as low as 25 parts per million have been observed as bacteriostatic, with 3% solution being 'rapidly bactericidal' (Block 1991). However, the mode of inactivation and efficacy differ from gaseous to aqueous form, with gaseous being significantly more effective (Finnegan *et al.* 2010; McDonnell 2014). Finnegan *et al.* (2010) demonstrated how VH2O2 'outperformed' aqueous biocide in the denaturation of bovine serum albumin and postulated that vapour has higher kinetic energies and can more readily penetrate the protein. Furthermore, the work of Fichet *et al.* (2007) observed the formation of monomers (clumping) with the use of liquid biocide for the inactivation of prions and described how such clumping could affect the penetration of liquid sterilant into protein structures, unlike the vapour form.

Some common modes of action are also found between vapour and liquid biocide action. For example, DNA suffers single-strand breaks at phosphodiester bonds (McDonnell 2014). Gaseous H_2O_2 has been shown to

Table 3 Characteristics of an ideal low-temperature industrial sterilization process and VH2O2 sterilization. Criteria for ideal process adapted from Rutala and Weber (1998) into a current context for industrial sterilization process

Characteristic	Definition	VH2O2 sterilization
Efficacious	Being virucidal, bactericidal, tuberculocidal, fungicidal and sporocidal in semi-logarithmic manner, where *D*-values may be determined and hold true for calculation to a defined sterility assurance level	Efficacious disinfection and sterilization process killing microbes (Klapes and Vesley 1990; Cortezzo and Setlow 2005; Meszaros *et al.* 2005), viruses (Heckert *et al.* 1997) and prions (Fichet *et al.* 2004). Sterility assurance may be demonstrated and validated in accordance with ISO14937:2009
Rapid	Achieve sterilization quickly and predictably	'Peroxide processes are significantly faster (than EO) for the overall sterilization times' (McDonnell 2014)
Strong penetrability	Able to penetrate medical device materials and packaging such that surfaces required to be deemed sterile can achieve such a state	'Hydrogen peroxide is less stable and therefore less penetrating than EO' (McDonnell 2007). Number of studies showing penetrability in lumen devices (AMSCO 1992; Alfa *et al.* 1996; Rutala *et al.* 1998; Rutala *et al.* 1999; Penna *et al.* 1999)
Material compatibility	Ideally negligible or at least acceptable changes to the materials such that product functionality characteristics are not negatively impacted	Good compatibility but depends on 'concentration, exposure time and delivery mechanisms' (McDonnell 2014). Information on material compatibility described in AAMI TIR17 (2008) or STERIS Material compatibility factsheet (2002)
Nontoxic and safe	Should be both safe to operate and pose no health risk to those along the manufacturing supply chain or the end user in the hospital environment	See Table 4 for EO *vs* VHP comparison. Biocide readily degrades to water and oxygen (McDonnell 2014)
Process repeatability and monitoring	Sterilization process must be repeatable to ensure validation holds true. Process should also be capable of being monitored routinely to verify compliance to original validation activity	Equipment design, commissioning, validation and operation in compliance with ISO14937:2009, in particular clause 6 (Process and Equipment Characterization) (ISO 2016a)
Compliance	Process must meet requirements of ISO standards (typically ISO14937:2009)	Processes validated in accordance with ISO14937:2009
Cost-effectiveness	Process must be cost-effective with respect to overall manufacturing costs of the product and selling price to end consumers	Equipment and process costs similar to other gaseous modalities such as EO and steam sterilization

denature proteins, whereas in certain test conditions amino acids were not seen to oxidize (McDonnell 2014). In the presence of metals, such as iron, oxidation of amino acids was observed. Less is known about lipids and carbohydrates, but given the known effect of oxidation on lipids, it is expected that there is a similar deleterious oxidation from exposure to H_2O_2. However, additional research is merited to establish proper the oxidative nature and effects of H_2O_2 on the modification and destruction of lipids and proteins that will aid the understanding of the mechanistic mode of lethal action for treated micro-organisms including biological indicators (BI). Previous studies report that aggregated proteins and peptides associated with neurodegenerative disease can generate H_2O_2, apparently through interactions with redox-active metal ions (Tabner *et al.* 2005). Fichet *et al.* (2007) reported use of VH2O2 for prion inactivation. Isotopically coded H_2O_2 has been studied for its contribution to allowing quantitative comparison of cellular prion protein (PrP(C)) and aggregated oligomeric (PrP (β)) forms of prion protein through surface modification approaches (Serpa *et al.* 2014). Duerkop *et al.* (2018) dismissed the role of H_2O_2 in HSA aggregation where the main factors were reported to be cavitation and high shear stress.

Although the focus of this review is the applicability of VH2O2 as a terminal sterilization process, there remains much discussion on the effect of aqueous and vapour phases and the condensation of vapour at material contact and sterilization. For further information on this topic, see Hultman *et al.* (2007), Unger-Bimczok *et al.* (2008), Pottage *et al.* (2010), and Agalloco and Akers (2013).

Vaporized hydrogen peroxide processes

Vapour-based processes may be subdivided into those performed at atmospheric conditions and those in a vacuum environment. One of the key applications of atmospheric VH2O2 is in the area of area or room disinfection: rooms and facilities including isolators and laminar air-flow cabinets, vehicles and aircraft are

disinfected with VH2O2. These applications use an atmospheric-based process (equipment ranges from handheld instruments to industrial-scale units integrated into heating, ventilation and air conditioning systems), where a VH2O2 'fog' is created in the environment being treated. This process has been deployed successfully for the last 30 years as a safer alternative to the use of humidified formaldehyde (McDonnell 2014). A comprehensive description of atmospheric-based processes can be found in McDonnell (2014).

However, it is mostly vacuum-based processes that have been developed and deployed for medical device product applications. As far back as 1988, James Rickloff of the American Sterilizer Company described how successful penetration and sterilization of materials were achieved with deep vacuum processes (AMSCO 1992). Akin to the EO process, a deep vacuum removes air that could impede vapour penetration to surfaces. Deep vacuum also facilitates the removal of ambient humidity, which creates the environment for injecting vaporized H_2O_2 to a maximum level, short of the saturation point after which condensation will occur. The importance of vacuum in drying the load is highlighted by Hultman *et al.* (2007): the 'maximum allowed concentration of peroxide vapour drops from 2·184 to 1·805 mg l^{-1} as moisture content in the carrier gas goes from 0% RH up to 10% RH, ... a 17·4% drop'. A typical vacuum-based process, similar to an EO cycle, comprises three distinct phases of (i) preconditioning, where a vacuum is applied to dry the load being sterilized; (ii) sterilization, where the sterilant vapour is injected to the sterilizer and allowed a period of contact with the product load; and (iii) aeration, where unwanted H_2O_2 is removed through a series of washes using air or steam. Some processes may include additional steps and variances. One is the Sterrad® low-temperature H_2O_2 gas plasma process, where a plasma is created by radiofrequency (RF) power (300W RF power in the case of Sterrad-100S®) and applied after the sterilant dwell phase (Crow and Smith 1995; Lerouge *et al.* 2000). The RF generates an electrical field, which reacts with the chemical sterilant to create a cloud of charged particles. Because the plasma is generated during the postconditioning phase, it is widely thought that its role is associated with the VH2O2 destruction and removal after sterilization (Krebs *et al.* 1998; Lerouge *et al.* 2000; McDonnell 2014).

Table 4 Key properties of EO gas (Linde 2011), and hydrogen peroxide (Seastar 2011); US National Library of Medicine 2018 (https://toxnet.nlm.nih.gov/cgi-bin/sis/search2/r?dbs+hsdb:@term+@DOCNO+547)

	Ethylene oxide gas	Vaporized hydrogen peroxide
Appearance/ Colour	Colourless	Colourless
Odour	Ethereal, poor warning properties at low concentrations	Slightly sharp, irritating
Molecular weight	44 g mol^{-1}	34 g mol^{-1}
Melting point	−112°C	−33°C (35%)
Boiling point	10·4°C	108°C (35%)
Autoignition temperature	440°C	N/A
Flammability range	3% (V)–100% (V)	Does not burn (20–35%)
Vapour pressure 20°C	1·4 bar (20°C)	0·0024 bar (30°C; 35%)
Acute toxicity	May cause irritation to the respiratory tract	Irritating to skin, eyes and respiratory system
LC50 (ppm)	2900 ppm/1 h	2g/m3/4h
Carcinogenic, mutagenic and teratogenic effects	May cause cancer. May cause heritable genetic damage. IARC Group 1 carcinogen	Inadequate evidence in humans for carcinogenicity. Not listed in IARC

Validation of VH2O2 processes

As with all terminal sterilization processes, 100% inspection of processed medical products is not possible. Prior validation of a measurable and repeatable process must, therefore, be performed. VH2O2 processes are qualified in accordance with ISO14937:2009 (ISO 2016a), 'Sterilization of health care products—General requirements for characterization of a sterilizing agent and the development, validation and routine control of a sterilization process for medical devices.' This standard requires the user to characterize the process by selecting an appropriate BI micro-organism of 'known high resistance' that is appropriate in demonstrating the 'microbial effectiveness of the sterilizing agent' (ISO 2016a). This standard is a general validation approach, applicable to many sterilization processes, whereas the more widely adopted traditional modalities of EO and radiation have internationally recognized and dedicated standards, namely ISO11135 (ISO 2014a) and ISO11137 (ISO 2015) respectively.

In the performance of validation activity and routine processing, BIs are employed as measures of microbiocidal efficacy. Previously, there have been investigation and debate on the choice of micro-organism as an appropriate BI. In previous studies, *Bacillus atrophaeus* showed greater resistance to lethal stresses when exposed to aqueous sterilant applications, whereas *Geobacillus stearothermophilus* showed greater tolerances to vapour-based approaches (McDonnell 2007). Considering where vaporized H_2O_2 is currently employed as a sterilization process: in hospital clinical applications regulations such as FDA 510k (FDA 2007) requirements dictate the use of 'the

most resistant organism' and go so far as to recommend *G. stearothermophilus* as appropriate for VH2O2 applications. However, ISO14937:2009 (which is appropriate for medical device terminal sterilization applications), like ISO11137 and ISO11135, requires the consideration of product bioburden, standard resistances and the use of a BI of 'known high resistance'. The selection of a BI as the microbiological challenge in the validation process is significant in that it defines the sterilization process required to meet that challenge. Hence, the appropriateness of the biological indicator challenge merits careful consideration in a validation process.

Opportunities for use of VH2O2 in terminal sterilization applications

When one examines the criteria for an ideal low-temperature gas process (Table 3) and examines the applications of VH2O2 in disinfection, decontamination and sterilization processes, it becomes apparent that VH2O2 offers a possible additional technology to the portfolio of terminal sterilization processes. Its limitations will be discussed later. A key proposition of low-temperature gaseous sterilization over irradiation processes is polymeric material compatibility. Researchers have examined the effect of VH2O2 on materials: various (STERIS 2002); polyurethane catheters, polyethylene tubing, polyvinylchloride (Lerouge *et al.* 2002); PU catheters (Ma *et al.* 2003); poly (D,L-lactic-co-glycolic acid) (Hee *et al.* 2008); steel (Sk *et al.* 2011); titanium dioxide (Junkar *et al.* 2016); and L-polylactic acid/gelatin(PLLA) (Hao *et al.* 2016). Consulting the AAMI TIR17 (2008) or STERIS Material compatibility factsheet (2002) reveals the extensive range of generally used materials employed in medical devices are suited to VH2O2 sterilization.

Where VH2O2 appears to offer many advantages is with the processing of novel materials. Researchers found comparability of VH2O2 with EO: (i) poly(lactic acid) (PLA)—which has been examined for use in sutures, tissue engineering support, orthopaedic applications and drug release—is a polymeric material with a relatively low melting temperature. Savaris *et al.* (2016) observed that sterilization modalities, such as EO, E-beam radiation, H_2O_2 plasma and gamma radiation, induced some variations in material properties, such as crystallinity, colour and contact angle, but were not significant, and therefore such modalities can be used in sterilization of PLA materials. (ii) Nuutinen *et al.* (2002) demonstrated how both EO and VH2O2 plasma offered sterilization options for bioabsorbable fibres that had 'limited effect on the mechanical properties and intrinsic viscosity of the materials tested'. Other researchers found distinct advantages to using VH2O2: Junkar *et al.* (2016), examining titanium nanotubes, found that the structure was preserved with VH2O2 due to lower temperature processing, unlike autoclaving. Hao *et al.* (2016), examining the effect of VH2O2 plasma and EO sterilization on PLLA/gelatin (PLLA/GA) scaffolds, reported that while both technologies were suitable sterilization processes for this material, predominantly due to lower temperature processing, improved cell proliferation was observed on scaffolds sterilized with VH2O2 due to lack of EO residuals and shorter sterilization cycles with VH2O2 processing.

The reduction in processing time, from a minimum 10–15 h in EO to <6 h in a typical VH2O2 process, has the additional benefit of less exposure of medical device materials and packaging to temperature, humidity and vacuum. Bi *et al.* (2013) showed how a VH2O2 process (30°C and six sterilant pulses, each with an exposure time of 5 min—for comparison, a typical EO exposure would be 2–4 h—offered an attractive sterilization technology for treatment of xenograph cancellous bone material ('No obvious changes of architecture or decrease in mechanical strength'), coupled with favourable cytotoxicity observed after treatment with VH2O2.

However, like any terminal sterilization process, while there are many benefits to using VH2O2 (highlighted in Table 3), there are also limitations that must be understood and addressed in process design and validation.

Limitations of VH2O2 in sterilization

Vaporized hydrogen peroxide has been developed and evaluated as an effective sterilization process for healthcare applications over many years (Alfa *et al.* 1996; Rutala and Weber 1998; Schneider 2013; Boiano and Steege 2015), as shown to be an effective agent at sterilizing bacteria (Crow and Smith 1995), prions (Fichet *et al.* 2007) and viruses (Cusinato *et al.* 2016). Klapes and Vesley (1990) reported:

> While aqueous hydrogen peroxide has a long history of use as a sterilant, the concept of vapour phase H2O2 (VPHP) sterilization has been developed within the past decade. VPHP represents a class of nontoxic cold gas sterilant which provides an opportunity to discontinue the use of such toxic or carcinogenic gaseous sterilants as EO and formaldehyde. VPHP technology could have immediate applications for the sterilization of speciality medical products, especially those which would be destroyed by steam sterilization or require lengthy aeration to reduce toxic EO residuals.

In healthcare, VH2O2 technology was successfully deployed into hospital environments with equipment such

as the 'VPro' range from STERIS and STERRAD equipment from Advanced Sterilization Products. Similarly, STERIS provides an extensive range of equipment and product applications for decontamination activities such as VPro hospital sterilizers (STERIS 2019a) and VHP Sterilization and Decontamination equipment (STERIS 2019b). As an example of such deployment, Wallace (2016) reviewed a selected number of 510ks submitted to FDA for sterilization equipment from January 2012 to June 2015, subdividing the sterilization into two categories: high-temperature steam and observed ten 510ks approved for new equipment (mostly for larger chamber sizes), and low-temperature sterilization, which could be subdivided into EO, H_2O_2+ozone and H_2O_2 (predominantly for endoscope processing). In the low-temperature applications, approvals for hydrogen peroxide-based processes outperform EO applications. A survey by Boiano and Steege (2015) of professional practice organizations found that of 373 respondents from healthcare facilities that deployed these gaseous sterilization approaches, 84% used VH2O2, 38% used EO and 22% used both. An extensive search of the literature highlights numerous studies reporting on the efficacy of developing and testing VH2O2 for disinfection and sterilization processes in healthcare settings. But there is a marked gap of published findings on applying VH2O2 terminal sterilization on medical devices, which may be attributed to the limitations described in Table 5. Material compatibility of cellulose-based materials, and ability to penetrate targeted surfaces, are two decisive limitations that have inhibited the advancement of VH2O2 for medical device terminal sterilization. Cellulose is known to degrade VH2O2 so much that it reduces the concentration of H_2O_2 in the vapour phase, rendering the sterilization process ineffective. Reich and Caputo (2005), examining isolator technology, found a 47% reduction in VH2O2 concentration in the presence of cellulose materials (compared to the same experiment in the absence of cellulose). A subsequent outcome of positive BIs dispersed randomly, indicating a resultant 'marginal nonuniform cycle'. Similarly, Corveleyn *et al.* (1997) examined the permeability of water vapour and VH2O2 across a number of packaging materials and observed marked differences in the permeation of VH2O2. Permeation across a Tyvek® package resulted in 87·7% of reference concentration being measured inside the package, compared to 30% with medical paper, most likely due to an 'absorption phenomenon or to the difference in the degradation rate caused by the presence of trace metals acting as catalysts of H_2O_2 breakdown'. With regard to penetration, this is very much linked to the chemistries associated with H_2O_2 in gaseous or liquid form. In the world of room decontamination, there has been much debate as to whether condensation of vapour occurs and the benefits to microbial inactivation.

Agalloco and Akers (2013) in their review of the use of H_2O_2 in room enclosure decontamination and disinfection highlight a number of limitations and associated mis-understandings: Understanding principles such as dew-point and liquid and vapour phases of a two phase system are critical. Hultman *et al.* (2007) provide useful insight into the chemistry associated with this two-phase system. Authors will differ in opinion as to whether condensation is necessary and beneficial to the desired inactivation of micro-organisms: Perspectives on the benefit associated with higher concentrations in the liquid (35% by weight H_2O_2 vapour condenses to approximately 78% by weight liquid at 25°C (Hultman *et al.* 2007)) *vs* the safety and material compatibility issues associated with creation of liquid H_2O_2 on surfaces are discussed.

As identified by Hultman *et al.* (2007), condensation of vapour may result in all surfaces not receiving sterilant

Table 5 Comparison of EO advantages and converse VH2O2 limitation against key sterilization characteristics

Characteristic	EO advantage	VH2O2 limitation	References
Material compatibility	Wide range of material compatibility, from device polymers and metals to packaging materials	Limitation with cellulose-based materials, with degradation of VH2O2	Corveleyn *et al.* (1997); Nuutinen *et al.* (2002); STERIS (2002); Meszaros *et al.* (2005); Reich and Caputo (2005); AAMI (2008); Bi *et al.* (2013); Bertoldi *et al.* (2015); Hao *et al.* (2016); Savaris *et al.* (2016)
Strong penetrability	EO, while a surface sterilant, is stable in gaseous phase sufficiently to reach required inner surfaces such as long lumen lengths, with appropriate cycle parameters of vacuum, temperature, sterilant concentration and time	Maintaining hydrogen peroxide in vapour form, while seeking to contact all required surfaces can be a challenge. Significant efficacy loss upon condensation	Alfa *et al.* (1996); Rutala *et al.* (1998); Penna *et al.* (1999); Rutala *et al.* (1999)
Compliance	In terminal sterilization, the use of EO is provided with extensive ISO guidance in accordance with ISO11135:2014	The application of VH2O2 in a terminal sterilization process demands that ISO14937:2009 be employed	ISO (2014a), ISO (2015), ISO (2016a)

contact: This would be of significant concern with a terminal sterilization application. Therefore, terminal sterilization processes are typically conducted under vacuum with a preconditioning step whereby air and natural humidity is removed and substituted with vapour at a defined temperature and pressure with the goal of maintaining vapour to such an extent that all required surfaces receive contact with the sterilant. Whilst temperature and pressure are important parameters, the calculations performed by Watling *et al.* (2002) demonstrate the importance of reducing the starting relative humidity: The authors found that peak H_2O_2 gas concentration reduces from a value in excess of 1500 ppm to less than 1000 ppm as initial relative humidity increases from 10 to 50%.

Another important consideration is the occurrence of nonlinear microbial inactivation kinetic data plots produced from VH2O2 treatment. This may influence the ability to standardize and validate a process, due to variability in sterilization efficacy.

Microbial inactivation with VH2O2

How VH2O2 affects micro-organisms, prions, protein molecules and other macromolecules is described by Klapes and Vesley (1990), Fichet *et al.* (2007), Finnegan *et al.* (2010) and Linley *et al.* (2012) respectively. The authors compare and contrast inactivation from aqueous H_2O_2 and VH2O2, and the consensus is of enhanced penetrability with VH2O2 and subsequently greater efficacy. Klapes and Vesley (1990), in their work on the use of VH2O2 to sterilize centrifuges, showed that *G. stearothermophilus* is more resistant than *B. subtilis* to VH2O2. Linley *et al.* (2012), in their review of VH2O2 applications, wrote 'The application of VPHP [VH2O2] as a potential sterilant is still clearly in its infancy: definitive knowledge of the mechanism(s) of microcidal action, and the factors which influence it, is lacking'. This re-affirmed Klapes and Vesley's (1990) assertion.

The methods of validation described in the ISO14937:2009 standard include a description of the 'Overkill Approach'. More often such methods are applied in a 'half-cycle approach', where a routine process sterilant dwell period is extrapolated (to 12 spore log reduction) from that used to achieve a 6 log reduction in a 10^6 BI, thus providing a SAL of $\leq 10^{-6}$. However, such an approach relies on achieving linear microbial inactivation kinetic plots, similar to that observed in EO sterilization of BIs of *B. atrophaeus*. A key underpinning assumption is that VH2O2-treated microbial population will show the same resistance to this applied lethal stress, which will be represented by a log linear inactivation kinetic plot (Stone *et al.* 2009). This assumption is frequently made when using homologous populations of *G. stearothermophilus* or *B. atrophaeus* as BIs.

However, as reported by Geeraerd *et al.* (2005) and shown in Fig. 2, microbial inactivation kinetics plots can take different forms. These researchers described nine variations in microbial plots that included both concave- and convex-shaped kinetic inactivation data, with a 'tailing' effect implying the possible occurrence of subpopulations of micro-organisms showing different resistance potentials, or variances under the same exposure conditions that may lead to uneven treatments due to things such as protective effects from microbial aggregation or clumping of cells or spores (Fig. 2).

As described by Stone *et al.* (2009), a commonly held explanation for nonlinear inactivation kinetics lies in the 'vitalistic theory, which holds that the resistance of individual cells in a population is not the same but follows a distribution'. Humpheson *et al.* (1998) further define the vitalistic theory, where in 'a genetically homogeneous population, phenotypic variation exists such that

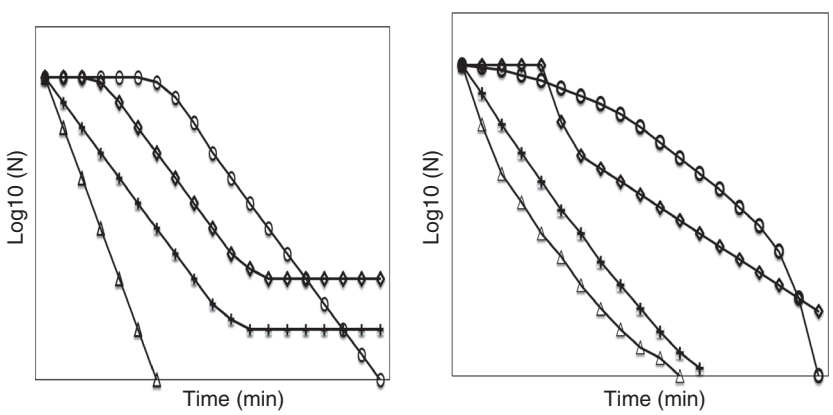

Figure 2 Commonly observed types of inactivation curves. Left plot: linear (r), linear with tailing (X), sigmoidal-like (□), linear with a preceding shoulder (m). Right plot: biphasic (r), concave (X), biphasic with a shoulder (□), and convex (m). (Geeraerd et al., 2005)

resistance to a lethal agent is not uniform'. Hideharu Shintani (Shintani 2014) attributes the biphasic shape of survivor curves to the mechanistic concept of clumping of micro-organisms on a BI, where the low-penetration processes (such as VH2O2) take additional time to reach the inner layers of micro-organisms in the clumps. Similarly, Kramer and Thielmann (2016), using flow cytometry (FCM) techniques, observed the agglomeration of *Listeria innocua* cells upon heat exposure, which then manifested in heat-resistant subpopulations. As described by Humpheson *et al.* (1998) in their review of inactivation of *Salmonella enteritidis*, a minor mutation event during growth is an unlikely source, and tailing may be more likely due to generation of cellular protective proteins (in their case, Heat Shock Proteins, or HSPs) during initial sublethal conditions. These protective proteins, where generated in a subpopulation, cause an increase in resistance. Allan *et al.* (1988) observed that 17 HSPs were generated in response to a heat shock treatment.

A known challenge in the use of VH2O2 is that it does not display linear inactivation kinetics with *G. stearothermophilus* (Agalloco and Akers 2013; Mcleod *et al.* 2017). As highlighted by McLeod *et al.* (2017), the inactivation 'was biphasic in the system used' and likely to be a result of different factors; the authors highlight the challenge of creating a spore monolayer on a small BI surface. Thus, a primary consideration is that the inactivation kinetics must be assessed in the total sterilization system being employed. Krebs *et al.* (1998) attributed the observed tailing to loss of efficiency in the process, with 'binding or consumption' of the sterilant gas.

Where biphasic inactivation is observed in a hydrogen peroxide-based system, it is difficult to negate the importance of VH2O2 in the redox activities and signalling in cells. Dickinson and Chang (2011) describe the extensive and important role fulfilled by H_2O_2 in cellular redox activities, and if one couples that with oxidative stress response (Lee and Helmann 2006; Mols and Abee 2011) and quorum-sensing mechanisms (Huillet *et al.* 2012), there may be many cellular and biochemical activities, yet to be defined, that could explain the biphasic lethality kinetics observed with H_2O_2. As Huillet *et al.* (2012) wrote, 'H_2O_2 stress induces the synthesis of many proteins and enzymes, such as catalase, thioredoxin reductase, ferroxidase and peroxidases, responsible for eliminating H_2O_2 from the cells.' As enzyme activation occurs in the germination and outgrowth phases, further understanding of the sequence of inactivation through the germination and outgrowth lifecycle stages could provide insight.

Another consideration, described by Rowan *et al.* (2015), is the concept of a viable but not culturable state (VBNC) that occurs because of stress in certain micro-organisms: 'evidence suggests that these harsh environment cues (operational parameters of the pulsed light (PL) treatment and biological factors) may trigger a switch to the adaptive survival VBNC state in PL treatments'. The concept of VBNC has been observed in many micro-organism species (Rowan 2004; Rowan *et al.* 2015; Rowan 2019), and one could therefore hypothesize that transitionary phases (rapid inactivation, slower inactivation and VBNC) in a homologous population may occur as a consequence of progressive cellular activity upon contact with a sterilizing agent. Understanding the cellular mechanisms of inactivation, the sequence of the inactivation from spore to outgrowth (and any intermediary states) and the spore defensive responses may also offer insights into why nonlinear inactivation kinetic plots are occasionally observed.

Implications of biphasic lethality with regard to process validation including VH2O2

A combination of factors influencing nonlinear microbial inactivation may also inform predictive microbiology, where decimal time required to achieve one-log reduction in microbial population under fixed lethal sterilizing conditions (or *D*-value), along with extrapolations from half-cycle parameters, are employed to determine SAL (Fig. 1). A problem potentially manifests in the overestimation of achieved SAL using a given challenge micro-organism, where biphasic microbial inactivation kinetic data show two different subpopulation plots. Yet the desired SAL is reached by extrapolation of treatment time in a half-cycle approach, where the *D*-value used is that which includes the less resistant subpopulation. Thus, where such a nonlinear inactivation is known, it needs to be understood and verified in the sterilization system being assessed, before a validation approach is determined. As ISO14937:2009 states, 'For sterilizing agents that do not exhibit linear inactivation kinetics, the nature of the inactivation kinetics should be investigated in order to derive a relationship from which it can be predicted that the specified probability of a microorganism surviving is not exceeded on applying the sterilization process' (ISO 2016a). A potentially important limitation in the above statement is that it appears to identify only the sterilizing agent as the source of the inactivation kinetic plots upon which decisions are made. One could contend that it is the overall sterilization system that contributes to and affects the observed inactivation kinetics. As shown by Humpheson *et al.* (1998), in their heat challenge of *S. enteridis* PT4, biphasic inactivation curves were observed at temperatures of 60°C, but these migrated to linear as temperatures were decreased to 51°C. Previous researchers have also reported on the heterogeneity of endospore germination and occurrence

of super dormant spores from genetically identical populations that can contribute to delayed germination which can also influence microbial inactivation kinetic models (Hong *et al.* 2010; Setlow *et al.* 2012; Zhang and Mathys 2019). Use of combined analytical techniques, such as FCM, phase contrast, fluorescent and transmission electron microscopy, along with use of Raman spectroscopy will help advance this important field of study.

Hence, one must consider the microbial response to the sterilizing agent in the sterilization system. One could expect the response to vary based on various conditions: sterilant concentration, sterilizing conditions (e.g. process temperature, vacuum), micro-organism type, micro-organism population, micro-organism carrier (e.g. BI material or bioburden surface). In their review of the inactivation kinetics of PL on micro-organism populations, Rowan *et al.* (2015) offer insights and review past work examining the vitalistic and mechanistic concepts and theories of microbial inactivation. Given the vitalistic phenotypic expression of subpopulations, and the key factors as cellular activity upon stress conditions, the micro-organism type is a significant factor. If the biphasic

Table 6 Key resistance mechanisms of bacterial spores and interaction with VH2O2

Spore component	Description of resistance mechanisms	Interaction with VH2O2	References
Spore coat resistance	Cross-linking in the coat layer may have some role in chemical and mechanical resistance	Protein structure cross-linked with disulphide bonds shown to house enzymes such as catalase and superoxide dismutase, which play significant roles in oxidative stress. Polycyclic terpenoids (termed sporulenes) as well as various pigments have also been identified in coats and contribute to spore resistance to hydrogen peroxide. Also, a permeability role in providing access to inner membrane	Henriques and Moran (2000); Riesenman and Nicholson (2000); Young and Setlow (2004a); Young and Setlow (2004b); Cortezzo and Setlow (2005); Cybulski *et al.* (2009); Setlow (2011); Leggett *et al.* (2012); Sella *et al.* (2014)
Inner membrane permeability	Acts as barrier in dormancy to small molecules including water, which is crucial to maintaining core conditions.	Cellular location of most significant interaction *Lipid component* Potential role for the phospholipid cardiolipin as a barrier for H_2O_2 access to spore core. As lipids may play a modulation role of important proteins such as germination receptors (GerA, GerB and GerK) as well as SpoVA proteins, any effect on the lipid content may indirectly affect spore germination *Protein–enzyme component* Cortezzo *et al.* (2004) describe how oxidizing agents can damage many proteins and enzymes, attacking both prosthetic groups such as iron–sulphur centres as well as amino acid residues, and such a damage can (i) result in a compromised metabolism during subsequent spore outgrowth, (ii) contribute to the osmotic stress sensitivity of the outgrowing spore and (iii) oxidatively damage proteins A number of authors identify potential damage to key germination proteins such as germinant receptors GerA, GerB and GerK (Young and Setlow 2004b), and GerD (Setlow *et al.* 2013) located in inner membrane As highlighted by McDonnell (2014), 'proteins appear to be an important target in the antimicrobial activity of hydrogen peroxide, both in gas and in liquid form'	Cortezzo *et al.* (2004); Young and Setlow (2004b); Griffiths and Setlow (2009); Setlow *et al.* (2013); Setlow *et al.* (2016)
Spore core	Three key molecules associated with resistance: i Water ii Dipicolinic acid iii Small acid-soluble proteins that saturate and protect DNA	A number of authors have concluded that the cellular target for VH2O2 is 'at or exterior to the spore's inner membrane' SASPs appear to eliminate DNA in the spore core as a site of inactivation with VH2O2 Low water content (27–55%) associated with DPA celated with divalent ions (Ca+) also appears to increase spore resistance to H_2O_2	Popham *et al.* (1995); Setlow (2006); Sella *et al.* (2014); Setlow (2014) Setlow and Setlow (1993); Loshon *et al.* (2001); Genest *et al.* (2002); Melly *et al.* (2002b); Leggett *et al.* (2012)

inactivation is a result of cellular responses, then this could vary by micro-organism type. Therefore, an established industry-standard BI exhibiting the most resistance to lethal sterilization processes may not be the most appropriate challenge micro-organism. Perhaps, greater consideration should be given to using a micro-organism that yields the closest fit to linear inactivation kinetics, while offering an appropriate challenge of 'known high resistance' to the sterilization process.

The purpose of the BI is to offer a higher level of resistance than the product bioburden being killed on a medical device being sterilized and a known challenge to the sterilization process, but also one that can be used in a predictive sense to arrive at the required SAL. While such characterization of the lethality kinetics in a sterilization system can provide clarity and direction on the most appropriate approach to validation and the required SAL, it does not answer the fundamental question of why such a nonlinear inactivation occurs. This is where molecular and cellular investigations on mechanistic activities underpinning sterilization processes, elucidated by microbiologists, can provide critical insights to help engineers design optimal processes as can be informed from adjacent industries (Farrell *et al.* 2011; Hayes *et al.* 2013; Garvey and Rowan 2019).

Interaction of VH2O2 with bacterial endospore cellular constituents

Evidence-based published literature on the effects of H_2O_2 on microbial cellular constituents is summarized in Table 6. However, all the work reviewed and described herein is based on the aqueous form of H_2O_2. Only the work of Fichet *et al.* (2007) examining the effect of VH2O2 gives insight into the vapour form. This lack of investigation into cellular effects from VH2O2 was also highlighted by Linley *et al.* (2012). As Table 6 shows, much of the research has elucidated the spore inner membrane as the likely site of interaction, with the inactivation consequences manifesting during germination (Popham *et al.* 1995; Melly *et al.* 2002a; Cortezzo *et al.* 2004; Cortezzo and Setlow 2005; Setlow *et al.* 2013). Leggett *et al.* (2016) reported that oxidizing agents are commonly used as sporicides and, given specific treatment conditions, can result in spore lysis. However, treatment with H_2O_2 does not necessarily result in spore lysis. Following exposure to this oxidizing agent, spores are left unable to form colonies even after neutralization of the microbiocide. Subsequent lysozyme treatment of such treated spores can often give rise to apparent spore germination, but these germinated spores exhibit little or no metabolic activity and do not outgrow (Setlow *et al.* 2013).

Given their importance as BIs, further work investigating the interaction of VH2O2 with spore components is warranted to understand the location(s) and nature of the sporicidal activity. While the lethality of spores is more often perceived as sporocidal, Leggett *et al.* (2016) have highlighted that manifestation of a cidal effect at outgrowth may be deemed bactericidal or bacteriostatic. Melly *et al.* (2002a), examining the inactivation of *B. subtilis* spores by H_2O_2, have determined that inactivation likely occurs during germination, when high-energy compounds such as ATP are not manufactured in the cell, mostly likely due to lack of core water content necessary for enzyme action. Investigative work by Loshon *et al.* (2001) used assays to try to determine the primary mechanism of spore killing by Sterilox (superoxide water disinfectant). The authors used BacLight stain with propidium iodide to report that inner membrane damage occurs only after endospore germination. Russell (2003) also highlighted that it is important to understand the reactions of different types of micro-organisms to biocidal agents from the point of view of cell structure and physiology, including (i) the mechanisms of action of biocides, (ii) the mechanisms whereby micro-organisms resist biocide action and (iii) the improved usage of biocides in clinical and environmental situations.

Conclusion

The medical device market is one of great importance to healthcare and currently relies on sterilization technologies such as irradiation technologies or gaseous EO. For some time, it has been recognized that there is a need for other gaseous technologies as an alternative to EO. Vaporized hydrogen peroxide sterilization is a widely adopted method in the hospital setting, but it is very much in its infancy in the terminal sterilization of medical devices. VH2O2 has limitations, including cellulosic material compatibility and penetration, which may affect the efficacy of sterilization for established and emerging medical devices that are increasing in complexity. A key consideration in the use of VH2O2 is variance in microbial inactivation kinetics, indicating a need to further understand the mechanism of spore lethality from VH2O2.

While significant investigative work has been conducted into the effect of sterilants at a cellular level (Young and Setlow 2004a, 2004b; Roth *et al.* 2010; Leggett *et al.* 2016; Setlow *et al.* 2016), this work is frequently limited, for instance studying mutants to compare with wild-type micro-organisms to test such things as cellular permeability. Use of such mutants fails to account for the inherent heterogenicity in large micro-organism populations (Cronin and Wilkinson 2008). As Reis *et al.* (2005) write, '*Bacillus* sp. have been found to exhibit a variety of rich

dynamic behaviours including long-term oscillations, multiple steady-states, genetic instability and un-interpretable transients', the consequences of which make process (biotechnology cell culturing) optimization and process prediction 'a difficult task'.

For the past two decades, FCM has been used to investigate micro-organisms. It offers an 'accurate technique to identify spores, vegetative cells and the number of viable and dead cells in the given population' (Majeed *et al.* 2018) and to examine the stages of germination and outgrowth in spore formers (Trunet *et al.* 2017). Traditional techniques such as microbial enumeration and resuscitation approaches offer some insight, but it is expected that newer real-time nonculture-based methods —such as bioinformatics, next-generation sequencing and FCM—may provide greater understanding of cellular responses after VH2O2 processing. The significance and importance of such future work is timely, as the International Organization for Standardization, through the formation of Working Group 16 under Technical Committee 198, seeks to establish a consensus standard for the use of VH2O2 in terminal sterilization processes. Establishing such a standard may move the FDA classification of VH2O2 terminal sterilization from Category B to Category A and subsequently advance the adoption of VH2O2 processes. Investigative work to establish optimal process parameters, including BI selection and associated inactivation kinetics, will provide clear instruction to users on how to correctly validate a VH2O2 process for terminal sterilization applications.

Acknowledgements

The authors acknowledge Athlone Institute of Technology Doctoral Scholarship Programme and funding support from STERIS AST.

Conflict of Interest

There is no conflict of interest.

References

Agalloco, J.P. and Akers, J.E. (2013) Overcoming limitations of vapourised hydrogen peroxide. *Pharma Technol Eur* **37**, 54–65.

Agency for Toxic Substances and Disease Registry (2019). https://www.atsdr.cdc.gov/mmg/mmg.asp?xml:id=304&txml:id=55 (accessed 21 June, 2019).

Alfa, M.J., DeGagne, R.T., Olson, N. and Puchalski, T. (1996) Comparison of ion plasma, VA hydrogen peroxide, and 100% EO sterilizers to the 12/88 ethyle gas sterilizer. *Infect Cont Hosp Epidemiol* **17**, 92–100.

Allan, B., Linseman, M., Macdonald, L.A., Lam, J.S. and Kropinskil, A.M. (1988) Heat shock response of *Pseudomonas aeruginosa*. *J Bacteriol* **170**, 3668–3674.

AMSCO (1992) *VHP Technology: A Collection of Scientific Papers*, 2nd edn. Apex: AMSCO Scientific.

Association for the Advancement of Medical Instrumentation (2008) *AAMI TIR17:2008 Compatibility of Materials Subject to Sterilization*. ISBN 1-57020-321-0. 1110 N. Glebe Road, Suite 220 Arlington, VA 22201-4795: Association for the Advancement of Medical Instrumentation. Approved and published 26 August 2008

Association for the Advancement of Medical Instrumentation (2017) *AAMI TIR16:2017 Microbiological aspects of ethylene oxide sterilization*. ISBN 1-57020-363-6. 1110 N. Glebe Road, Suite 220 Arlington, VA 22201-4795: Association for the Advancement of Medical Instrumentation Approved and published 3 September 2009.

Bauld, T. (2016) A plea for help in reducing hospital-acquired infections. *Biomedical Instrum Technol* **50**, 391.

Bertoldi, S., Farè, S., Haugen, H.J. and Tanzi, M.C. (2015) Exploiting novel sterilization techniques for porous polyurethane scaffolds. *J Mater Sci: Mater Med* **26**, 1–12.

Bi, L., Li, D.-C., Huang, Z.-S. and Yuan, Z. (2013) Effects of sodium hydroxide, sodium hypochlorite, and gaseous hydrogen peroxide on the natural properties of cancellous bone. *Artif Organs* **37**, 629–636.

Block, S.S. (1991). Chapter 9, Peroxygen compounds. In *Disinfection, Sterilization and Preservation*. 4th edn. ed. Block, S.S. pp. 167–181. Philadelphia, PA: Lea & Febiger, ISBN 0-8121-1364-0.

Boiano, J.M. and Steege, A.L. (2015) Ethylene oxide and hydrogen peroxide gas plasma sterilization: precautionary practices in U.S. Hospitals. *Zentralsterilisation (Wiesb)*, **23**, 262–268. HHS Public Access.

Cortezzo, D.E. and Setlow, P. (2005) Analysis of factors that influence the sensitivity of spores of *Bacillus subtilis* to DNA-damaging chemicals. *J Appl Microbiol* **98**, 606–617.

Cortezzo, D.E., Koziol-Dube, K., Setlow, B. and Setlow, P. (2004) Treatment with oxidizing agents damages the inner membrane of spores of *Bacillus subtilis* and sensitizes spores to subsequent stress. *J Appl Microbiol* **97**, 838–852.

Corveleyn, S., Vandenbossche, G.M.R. and Remon, J.P. (1997) Near-Infrared (NIR) monitoring of H2O2 vapor concentration during vapor hydrogen peroxide (VHP) sterilisation. *Pharma Res* **14**, 294–298.

Cronin, U.P. and Wilkinson, M.G. (2008) Monitoring changes in germination and permeability of *Bacillus cereus* endospores following chemical, heat and enzymatic treatments using flow cytometry. *J Rapid Meth Automat Microbiol* **16**, 164–184.

Crow, S. and Smith, J.H. (1995) Gas plasma sterilization: application of space-age technology. *Infect Cont Hosp Epidemiol* **16**, 483–487.

Cusinato, R., Pacenti, M., Martello, T., Fattori, P., Morroni, M. and Palù, G. (2016) Effectiveness of hydrogen peroxide

and electron-beam irradiation treatment for removal and inactivation of viruses in equine-derived xenografts. *J Vir Methods* **232**, 39–46.

Cybulski, R.J., Sanz, P., Alem, F., Stibitz, S., Bull, R.L. and O'Brien, A.D. (2009) Four superoxide dismutases contribute to Bacillus anthracis virulence and provide spores with redundant protection from oxidative stress. *Infect Immun* **77**, 274–285.

Dasenbrock, H.H., Rudy, R.F., Smith, T.R., Guttieres, D., Frerichs, K.U., Gormley, W.B. and Du, R. (2016) Hospital-acquired infections after aneurysmal subarachnoid hemorrhage: a nationwide analysis. *World Neurosurg* **88**, 459–474.

Dickinson, B.C. and Chang, C.J. (2011) Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nat Chem Biol* **7**, 504–511.

Duerkop, M., Berger, E., Duraeur, A. and Jurgbauer, A. (2018) Influence of cavitation and high shear stress on HSA aggreation behaviour. *Eng Life Sci* **18**, 169–178.

Dugani, S., Veillard, J. and Kissoon, N. (2017) Reducing the global burden of sepsis. *Can Med Assoc J* **189**, E2–E3. https://doi.org/10.1503/cmaj.160798

Farrell, H., Hayes, J., Laffey, J.G. and Rowan, N.J. (2011) Studies on the relationship between pulsed UV light irradiation and the simultaneous occurrence of molecular and cellular damage in clinically-relevant *Candida albicans*. *J Microbiol Meth* **84**, 317–326.

Fichet, G., Comoy, E., Duval, C., Antloga, K., Dehen, C., Charbonnier, A. and Deslys, J.P. (2004) Novel methods for disinfection of prion-contaminated medical devices. *Lancet* **364**, 521–526.

Fichet, G., Antloga, K., Comoy, E., Deslys, J.P. and McDonnell, G. (2007) Prion inactivation using a new gaseous hydrogen peroxide sterilisation process. *J Hosp Infect* **67**, 278–286.

Finnegan, M., Linley, E., Denyer, S.P., McDonnell, G., Simons, C. and Maillard, J.Y. (2010) Mode of action of hydrogen peroxide and other oxidizing agents: differences between liquid and gas forms. *J Antimicro Chemother* **65**, 2108–2115.

Food and Drug Adminstration (USA) (2007) *Biological Indicator (BI) Premarket Notification [510(k)] Submissions*. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/biological-indicator-bi-premarket-notification-510k-submissions accessed 10 October 2017.

Gamma Irradiation Processing Alliance, and International Irradiation Association (2017) *A Comparison of Gamma, E-beam, X-ray and Ethylene Oxide Technologies for the Industrial Sterilization of Medical Devices and Healthcare Products, 1–49*. http://iiaglobal.com/wp-content/uploads/2018/01/White-Paper-Comparison-Gamma-Eb-Xray-and-EO-for-Sterilisation.pdf accessed 10 June 2018.

Garvey, M. and Rowan, N.J. (2019) Pulsed UV as a potential surface sanitizer in food production processes to ensure consumer safety. *Curr Opin Food Sci* **26**, 65–70.

Geeraerd, A.H., Valdramidis, V.P. and Van Impe, J.F. (2005) GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. *Inter J Food Microbiol* **102**, 95–105.

Genest, P.C., Setlow, B., Melly, E. and Setlow, P. (2002) Killing of spores of *Bacillus subtilis* by peroxynitrite appears to be caused by membrane damage. *Microbiology* **148**, 307–314.

Griffiths, K.K. and Setlow, P. (2009) Effects of modification of membrane lipid composition on *Bacillus subtilis* sporulation and spore properties. *Journal of Applied Micro* **106**, 2064–2078.

Guirgis, F.W., Jones, L., Esma, R., Weiss, A., McCurdy, K., Ferreira, J., Gray-Eurom, K. (2017) Managing sepsis: electronic recognition, rapid response teams, and standardized care save lives. *J Crit Care* **40**, 296–302.

Hao, H., Xiaoli, Z., Nian, Q.I.N., Xiaoyan, H.E., Qing, Z. and Qian, H.E. (2016) Effect of ETO and hydrogen peroxide plasma sterilization methods on cell proliferation ability in PLLA/GA scaffolds. *Oxid Comm* **7**, 1–7.

Hayes, J., Kirf, D., Garvey, M. and Rowan, N.J. (2013) Disinfection and toxicological assessment of pulsed UV and pulsed-plasma gas-discharge treated water containing the waterborne enteroparasite *Cryptosporidium pavum*. *J Microbiol Method* **94**, 325–337.

Heckert, R.A., Best, M., Jordan, L.T., Dulac, G.C., Eddington, D.L. and Sterritt, W.G. (1997) Efficacy of vaporized hydrogen peroxide against exotic animal viruses ducted with a variety of viral agents, which included representatives of several virus families. *Orthomyxoviridae* **63**, 3916–3918.

Hee, M., Kim, H., Hwa, C., Ho, S., Koo, J., Jin, S. and Park, J. (2008) Surface and coatings technology effects of low-temperature hydrogen peroxide gas on sterilization and cytocompatibility of porous poly (D, L-lactic-co-glycolic acid) scaffolds. *Surf Coat Technol* **202**, 5762–5767.

Henriques, A.O. and Moran, C.P. (2000) Structure and assembly of the bacterial endospore coat. *Methods* **20**, 95–110.

Hensley, B.J. and Monson, J.R.T. (2015) Hospital-acquired infections. *Surgery* **33**, 528–533.

Hong, L., Zhang, P., Setlow, P. and Li, Y.Q. (2010) Characterization of bacterial spore germination using integrted phase contrast microscopy, Raman spectroscopy, and optical tweezers. *Anal Chem* **82**, 3840–3847.

Huillet, E., Tempelaars, M.H., André-Leroux, G., Wanapaisan, P., Bridoux, L., Makhzami, S., Lereclus, D. (2012) PlcRa, a new quorum-sensing regulator from *Bacillus cereus*, plays a role in oxidative stress responses and cysteine metabolism in stationary phase. *PLoS ONE* **7**, 1–19.

Hultman, C., Hill, A. and McDonnell, G. (2007) The physical chemistry of decontamination with gaseous hydrogen peroxide. *Pharma Eng* **27**, 22–32.

Humpheson, L., Adams, M.R., Anderson, W.A. and Cole, M.B. (1998) Biphasic thermal inactivation kinetics in *Salmonella enteritidis* PT4. *Appl Environ Microbiol* **64**, 459–464.

IARC (2012) Chemical agents and related occupations. IARC Monographs 100F, pp. 379–400. http://monographs.iarc.fr/ENG/Monographs/vol100F/mono100F-28.pdf. Accessed 3 Jan 2018.

International Organization for Standardization (ISO) (2014a) *ISO11135:2014 Sterilization of Health-care Products — Ethylene Oxide — Requirements for the Development, Validation and Routine Control of a Sterilization Process for Medical Devices*. Geneva: ISO.

International Organization for Standardization (ISO) (2015) *ISO 11137–1:2015 Sterilization of Health Care Products — Radiation —Part 1: Requirements for Development, Validation and Routine Control of a Sterilization Process for Medical Devices*. Geneva: ISO.

International Organization for Standardization (ISO) (2016a) *ISO 14937:2009 Sterilization of Health Care Products - General Requirements for Characterization of a Sterilizing Agent and the Development, Validation and Routine Control of a Sterilization Process for Medical Devices*. Geneva: ISO.

International Organization for Standardization (ISO) (2016b) *ISO13485:2016 Medical Devices — Quality Management Systems — Requirements for Regulatory Purposes*. Geneva: ISO.

International Organization for Standardization (ISO) (2018) *ISO11139:2018 Sterilization of Health Care Products — Vocabulary of Terms Used in Sterilization and Related Equipment and Process Standards*. Geneva: ISO.

International Trade Administration (2016) 2016 Top Markets Report Medical Devices: A Market Assessment Tool for U.S. Exporters. https://www.trade.gov/topmarkets/pdf/Medical_Devices_Top_Markets_Report.pdf (accessed 16 Jan 2018)

Junkar, I., Kulkarni, M., Drašler, B., Rugelj, N., Mazare, A., Flašker, A., Iglič, A. (2016) Influence of various sterilization procedures on TiO2 nanotubes used for biomedical devices. *Bioelectrochem* **109**, 79–86.

Klapes, N.A. and Vesley, D. (1990) Vapour-phase hydrogen-peroxide as a surface decontaminant and sterilant. *Appl Environ Microbiol* **56**, 503–506.

Kramer, B. and Thielmann, J. (2016) Monitoring the live to dead transition of bacteria during thermal stress by a multi-method approach. *J Microbiol Meth* **123**, 24–30.

Krebs, M.C., Bécasse, P., Verjat, D. and Darbord, J.C. (1998) Gas-plasma sterilization: relative efficacy of the hydrogen peroxide phase compared with that of the plasma phase. *Inter J Pharma* **160**, 75–81.

Kroc, T.K., Thangaraj, J.C.T., Penning, R.T. and Kephart, R.D. (2017) Accelerator-driven medical sterilization to replace Co-60 sources. Retrieved from http://lss.fnal.gov/archive/2017/pub/fermilab-pub-17-314-di.pdf (accessed 26 Jan 2019).

Lee, J.W. and Helmann, J.D. (2006) The PerR transcription factor senses H_2O_2 by metal-catalysed histidine oxidation. *Nature* **440**, 363–367.

Leggett, M.J., Mcdonnell, G., Denyer, S.P., Setlow, P. and Maillard, J.Y. (2012) Bacterial spore structures and their protective role in biocide resistance. *J Appl Microbiol* **113**, 485–498.

Leggett, M.J., Setlow, P., Sattar, S.A. and Maillard, J.Y. (2016) Assessing the activity of microbicides against bacterial spores: knowledge and pitfalls. *J Appl Microbiol* **120**, 1174–1180.

Lerouge, S., Wertheimer, M.R., Marchand, R., Tabrizian, M. and Yahia, L. (2000) Effect of gas composition on spore mortality and etching during low-pressure plasma sterilization. *J Biomed Mater Res* **51**, 128–135.

Lerouge, S., Tabrizian, M., Wertheimer, M.R., Marchand, R. and Yahia, L. (2002) Safety of plasma-based sterilization: surface modifications of polymeric medical devices induced by Sterrad and Plazlyte processes. *Bio-Med Mater Engin* **12**, 3–13.

Linde (2011) *Safety data sheet – ethylene oxide*. http://hiq.linde-gas.com/en/images/Ethylene%20Oxide%20SDS%20Linde%20EU%20format%20HiQ%20Jan%202011_tcm899-92356.pdf accessed 23 Jan 2019.

Linley, E., Denyer, S.P., McDonnell, G., Simons, C. and Maillard, J.Y. (2012) Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action. *J Antimicrob Chemother* **67**, 1589–1596.

Loshon, C.A., Melly, E., Setlow, B. and Setlow, P. (2001) Analysis of the killing of spores of *Bacillus subtilis* by a new disinfectant, Sterilox. *J Appl Microbiol* **91**, 1051–1058.

Ma, N., Petit, A., Huk, O.L., Yahia, L. and Tabrizian, M. (2003) Safety issue of re-sterilization of polyurethane electrophysiology catheters: a cytotoxicity study. *J Biomater Sci, Polymer Edition* **14**, 213–226.

Majeed, M., Majeed, S., Nagabhushanam, K., Punnapuzha, A., Philip, S. and Mundkur, L. (2018) Rapid assessment of viable but non-culturable *Bacillus coagulans* MTCC 5856 in commercial formulations using flow cytometry. *PLoS ONE* **13**, 1–14.

Masterson, C.H., Murphy, E., Major, I., González, H., O'Toole, D., McCarthy, S., Laffey, J.G. and Rowan, N.J. (2019) Purified beta-glucan from the *Lentinus edodes* mushroom attenuates antibiotic resistant *Klebsiella pneumoniae*-induced pulmonary sepsis. *Am Thoracic Soc.* A28. BACTERIAL AND VIRAL LUNG INFECTIONS AND PATHOGENESIS, A1222–A1222.

McDonnell, G. (2007) Chemical sterilization. In *Antisepsis, Disinfection and Sterilization: Types, Action and Resistance* ed. McDonnell, G. pp. 191–216. Washington, DC: ASM Press.

McDonnell, G. (2014) The use of hydrogen peroxide for disinfection and sterilization applications. In *PATAI'S Chemistry of Functional Groups* ed. Rappoport, Z. https://doi.org/10.1002/9780470682531.pat0885

Mcleod, N.P., Clifford, M., Sutton, J.M. and Mcleod, N. (2017) Evaluation of novel process indicators for rapid monitoring of hydrogen peroxide decontamination processes. *PDA J Pharma Sci Technol* **71**, 393–404.

Melly, E., Cowan, A.E. and Setlow, P. (2002a) Studies on the mechanism of killing of *Bacillus subtilis* spores by hydrogen peroxide. *J Appl Microbiol* **93**, 316–325.

Melly, E., Genest, P.C., Gilmore, M.E., Little, S., Popham, D.L., Driks, A. and Setlow, P. (2002b) Analysis of the properties of spores of *Bacillus subtilis* prepared at different temperatures. *J Appl Microbiol* **92**, 1105–1115.

Meszaros, J.E., Antloga, K., Justi, C., Plesnicher, C. and McDonnell, G. (2005) Area fumigation with hydrogen peroxide vapor. *Appl Biosafety* **10**, 91–100.

Mols, M. and Abee, T. (2011) Primary and secondary oxidative stress in *Bacillus*. *Environ Microbiol* **13**, 1387–1394.

Moore, F. and Perkinson, L. (1979) Hydrogen peroxide vapor sterilization method. US Patent 4,169,123. https://patents.google.com/patent/US4169123A/en (accessed 8 Feb 2019).

Nuutinen, J., Clerc, C. and Virta, T. (2002) Effect of gamma, ethylene oxide, electron beam and plasma sterilization on behaviour of SR-PLLA bres in vitro. *J Biomater Sci Polym Ed* **13**, 1325–1336.

Penna, T.C.V., Ferraz, C.A.M. and Cassola, M.A. (1999) The presterilization microbial load on used medical devices and the effectiveness of hydrogen peroxide gas plasma against *Bacillus subtilis* spores. *Infect Cont Hosp Epidemiol* **20**, 1–20.

Popham, D.L., Sengupta, S. and Setlow, P. (1995) Heat, hydrogen peroxide, and UV resistance of *Bacillus subtilis* spores with increased core water content and with or without major DNA-binding proteins. *Appl Environ Microbiol* **61**, 3633–3638.

Pottage, T., Richardson, C., Parks, S., Walker, J.T. and Bennett, A.M. (2010) Evaluation of hydrogen peroxide gaseous disinfection systems to decontaminate viruses. *J Hosp Infect* **74**, 55–61.

RAISIN Working Group (2009) "RAISIN" – a national programme for early warning, investigation and surveillance of healthcare-associated infection in France. *Euro Surveill* **14**:pii=19408. Available: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19408

Reich, R. and Caputo, R.A. (2005) The adverse effects of cellulosic material in isolator barrier technology. *Pharma Technol* **29**, 52–58.

Reis, A., Da Silva, T.L., Kent, C.A., Kosseva, M., Roseiro, J.C. and Hewitt, C.J. (2005) Monitoring population dynamics of the thermophilic *Bacillus licheniformis* CCMI 1034 in batch and continuous cultures using multi-parameter flow cytometry. *J Biotechnol* **115**, 199–210.

Riesenman, P.J. and Nicholson, W.L. (2000) Role of the spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. *Appl Environ Microbiol* **66**, 620–626.

Roth, S., Feichtinger, J. and Hertel, C. (2010) Characterization of *Bacillus subtilis* spore inactivation in low-pressure, low-temperature gas plasma sterilization processes. *J Appl Microbiol* **108**, 521–531.

Rowan, N.J. (2004) Viable but non-culturable forms of food and waterborne bacteria: Quo Vadis? *Trend Food Sci Technol* **15**, 462–467.

Rowan, N.J. (2019) Pulsed light as an emerging technology to cause disruption for food and adjacent industries - Quo Vadis? *Trends Food Sci Technol* **88**, 316–332.

Rowan, N.J. and Anderson, J.G. (1998) Growth and enterotoxin production by diarrhogenic *Bacillus cereus* in dietary supplements prepared for hospitalized HIV patients. *J Hosp Infect* **2**, 139–146.

Rowan, N.J., Valdramidis, V.P. and Gómez-López, V.M. (2015) A review of quantitative methods to describe efficacy of pulsed light generated inactivation data that embraces the occurrence of viable but non culturable state microorganisms. *Trends Food Sci Technol* **44**, 79–92.

Russell, A.D. (2003) Similarities and differences in the responses of microorganisms to biocides. *J Antimicrob Chemother* **52**, 750–763.

Rutala, W.A. (1996) Disinfection and sterilization of patient-care items. *Infect Cont Hosp Epidemiol* **17**, 377–384.

Rutala, W.A. and Weber, D.J. (1996) Low-temperature sterilization technologies: Do we need to redefine "sterilization"? *Infect Cont Hosp Epidemiol* **17**, 87–91.

Rutala, W.A. and Weber, D.J. (1998) Clinical effectiveness of low-temperature sterilization technologies. *Infect Cont Hosp Epidemiol* **19**, 798–804.

Rutala, W.A., Gergen, M.F. and Weber, D.J. (1998) Comparative evaluation of the sporicidal activity of new low-temperature sterilization technologies: Ethylene oxide, 2 plasma sterilization systems, and liquid peracetic acid. *Am J Infect Cont* **26**, 393–398.

Rutala, W.A., Gergen, M.F. and Weber, D.J. (1999) Sporicidal activity of a new low-temperature sterilization technology: the Sterrad 50 sterilizer. *Infect Cont Hos Epidemiol* **20**, 514–516.

Savaris, M., dos Santos, V. and Brandalise, R.N. (2016) Influence of different sterilization processes on the properties of commercial poly(lactic acid). *Mater Sci Engin C* **69**, 661–667.

Schneider, P.M. (2013) New technologies and trends in sterilization and disinfection. *Am J Infection Control* **41**, S81–S86.

Seastar (2011) *Material Safety Data Sheet – Hydrogen Peroxide Solution*. http://www.seastarchemicals.com/wpcontent/uploads/2015/03/SSNSSE_10HydrogenPeroxideSDSRev201407.pdf accessed 23 January 2019.

Sella, S.R.B.R., Vandenberghe, L.P.S. and Soccol, C.R. (2014) Life cycle and spore resistance of spore-forming *Bacillus atrophaeus*. *Microbiol Res* **169**, 931–939.

Serpa, J.J., Makepeace, K.A., Borchers, T.H., Wishart, D.S., Pertochencnko, E.U. and Borchers, T.H. (2014) Using isotopically-coded hydrogen peroxide as a surface modification reagent for the structural characterization of prion protein aggregates. *J. Proteomics* **100**, 160–166.

Setlow, P. (2006) Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *J Appl Microbiol* **101**, 514–525.

Setlow, P. (2011) Resistance of bacterial spores. In *Bacterial Stress Responses*, 2nd Edition ed. Storz, G. and Hengge, R. pp. 319–332. Washington, DC: ASM Press.

Setlow, P. (2014) Spore resistance properties. Microbiol. *Spectrum* **2**, 1–14. https://doi.org/10.1128/microbiolspec.TBS-0003-2012

Setlow, B. and Setlow, P. (1993) Binding of small, acid-soluble spore proteins to DNA plays a significant role in the resistance of *Bacillus subtilis* spores to hydrogen peroxide. *Appl Environ Microbiol* **59**, 3418–3423.

Setlow, P., Liu, J. and Faeder, J.R. (2012) Heterogenity of bacterial spore populations. In *Bacterial Spores: Current Research and Applications* ed. Abel-Santos, E. pp. 199–214. Norfolk: Caister Academic Press.

Setlow, B., Yu, J., Li, Y.Q. and Setlow, P. (2013) Analysis of the germination kinetics of individual *Bacillus subtilis* spores treated with hydrogen peroxide or sodium hypochlorite. *Lett Appl Microbiol* **57**, 259–265.

Setlow, B., Korza, G., Blatt, K.M.S., Fey, J.P. and Setlow, P. (2016) Mechanism of *Bacillus subtilis* spore inactivation by and resistance to supercritical CO2 plus peracetic Acid. *J Appl Microbiol* **120**, 57–69.

Shintani, H. (2014) Important points to attain reproducible sterility assurance. *Biochem Physiol* **3**, 1–4.

Sk, M.H., Overfelt, R.A., Haney, R.L. and Fergus, J.W. (2011) Hydrogen embrittlement of 4340 steel due to condensation during vaporized hydrogen peroxide treatment. *Mater Sci Engin A* **528**, 3639–3645.

STERIS (2002) Material compatibility with vaporized hydrogen peroxide (VHP®) sterilization: a new option for point-of-manufacture sterilization. Retrieved from www.sterislifesciences.com/~/media/Files/LifeSciences_com/PDF/BiodecontaminationServices/MaterialCompatibilitywithVaporizedHydrogenPeroxide.ashx

STERIS (2019a) *V-PRO® Low-temperature Sterilization Systems*. Retrieved from www.steris.com/healthcare/products/v-pro-sterilizers/

STERIS (2019b) *VHP Sterilization & Biodecontamination*. Retrieved from www.sterislifesciences.com/products/equipment/vhp-sterilization-and-biodecontamination

Stone, G., Chapman, B. and Lovell, D. (2009) Development of a log-quadratic model to describe microbial inactivation, illustrated by thermal inactivation of *Clostridium botulinum*. *Appl Environ Microbiol* **75**, 6998–7005.

Tabner, B.J., El-Agnaf, O.M.A., German, M.J., Fullwood, M.J. and Allsop, D. (2005) Protein aggregation, metals and oxidative stress in neurodegenerative diseases. *Biochem Soc Trans* **33**, 1082–1086.

Trunet, C., Carlin, F. and Coroller, L. (2017) Investigating germination and outgrowth of bacterial spores at several scales. *Trends Food Sci Technol* **64**, 60–68.

Unger-Bimczok, B., Kottke, V., Hertel, C. and Rauschnabel, J. (2008) The influence of humidity, hydrogen peroxide concentration, and condensation on the inactivation of geobacillus stearothermophilus spores with hydrogen peroxide vapor. *J Pharma Innov* **3**, 123–133.

Wallace, C.A. (2016) New developments in disinfection and sterilization. *Am J Infect Con* **44**, e23–e27. https://doi.org/10.1016/j.ajic.2016.02.022.

Watling, D., Ryle, C., Parks, M. and Christopher, M. (2002) Theorethical analysis of the condensation of hydrogen peroxide gas and water vapour as used in surface decontamination. *PDA J Pharma Sci* **56**, 291–299.

Young, S.B. and Setlow, P. (2004a) Mechanisms of *Bacillus subtilis* spore resistance to and killing by aqueous ozone. *J Appl Microbiol* **96**, 1133–1142.

Young, S.B. and Setlow, P. (2004b) Mechanisms of killing of *Bacillus subtilis* spores by Decon and Oxone, two general decontaminants for biological agents. *J Appl Microbiol* **96**, 289–301.

Zhang, Y. and Mathys, A. (2019) Superdomant spores as a hurdle for gentle germination-inactivation based spore control strategies. *Front Microbiol* **9**, 3163. https://doi.org/10.3389/fmicb.2018.0163.