Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

REVIEW ARTICLE

Opportunities for the application of real-time bacterial cell analysis using flow cytometry for the advancement of sterilization microbiology

B. McEvoy[1], M. Lynch[2] and N.J. Rowan[2]

1 STERIS Applied Sterilization Technologies, IDA Business and Technology Park, Tullamore, Ireland
2 Centre for Disinfection, Sterilization and Biosecurity, Athlone Institute of Technology, Athlone, Ireland

Keywords
bacterial endospores, flow cytometry, medical devices, real-time monitoring, sterilization, terminal gaseous sterilization, vaporized hydrogen peroxide, VH2O2, VHP.

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

2020/1089: received 28 May 2020, revised 10 September 2020 and accepted 21 September 2020

doi:10.1111/jam.14876

Abstract

Medical devices provide critical care and diagnostic applications through patient contact. Sterility assurance level (SAL) may be defined as the probability of a single viable micro-organism occurring on an item after a sterilization process. Sterilization microbiology often relies upon using an overkill validation method where a 12-log reduction in recalcitrant bacterial endospore population occurs during the process that exploits conventional laboratory-based culture media for enumeration. This timely review explores key assumptions underpinning use of conventional culture-based methods in sterilization microbiology. Consideration is given to how such methods may limit the ability to fully appreciate the inactivation kinetics of a sterilization process such as vaporized hydrogen peroxide (VH2O2) sterilization, and consequently design efficient sterilization processes. Specific use of the real-time flow cytometry (FCM) is described by way of elucidating the practical relevance of these limitation factors with implications and opportunities for the sterilization industry discussed. Application of FCM to address these culture-based limitation factors will inform real-time kinetic inactivation modelling and unlock potential to embrace emerging opportunities for pharma, medical device and sterilization industries including potentially disruptive applications that may involve reduced usage of sterilant.

Introduction

Background to sterilization microbiology and the industry

The sterilization marketplace can be subdivided into hospital sterilization (such as point of use), in-house manufacturing sterilization (such as at point of medical device manufacture, often inline applications) and contract sterilization, provided by contractors where medical device manufacturers obtain sterilization services along the supply chain after manufacturing (McEvoy and Rowan 2019). Central to sterilization microbiology is the treatment of medical devices where associated global market is estimated to exceed $400bn (International Trade Administration 2016). An important consideration is the safe use of sterile medical devices that provide critical care and diagnostic applications, whereby patient infection does not arise as a consequence of such patient contact. Addressing hospital-acquired infections (HAIs) and sepsis is a global challenge with an estimated annual cost to healthcare in the United States at $9.8bn (Hensley and Monson 2015) and $20bn (Guirgis *et al.* 2017), respectively. HAIs and sepsis are estimated to affect 1.7 m and 700 000 cases in the United States, respectively. While factors associated with the occurrence of these serious infections are varied, terminal sterilization modalities are unlikely to be point-of-infection due to comprehensive sterilization and validation processes (cited McEvoy and Rowan 2019).

Sterilization microbiology underpinning these processes delivers sterile assurance far above minimum requirements to achieve sterilization for patient safety. To achieve a required sterility assurance level (SAL), a

sterilization process is applied to medical devices prior to patient use. Sterilization validation typically relies upon using an overkill validation method, as shown in Fig. 1, where the process demonstrates a 12-log reduction in recalcitrant bacterial endospore population enumerated on artificial laboratory-based media, where it is assumed that the shape of the inactivation kinetic plot is a straight line and semi-logarithmic when the sterilizing conditions remain consistent for the duration of the exposure time (AAMI 2017). Thus, conventional microbiology assumes the process is predicable based upon probability of linear inactivation kinetic death rate plot. Terminal sterilization processes such as Gamma irradiation, Electron-beam irradiation, X-ray irradiation, gaseous ethylene oxide (EO) and hydrogen peroxide in vapour state are discussed by McEvoy and Rowan (2019).

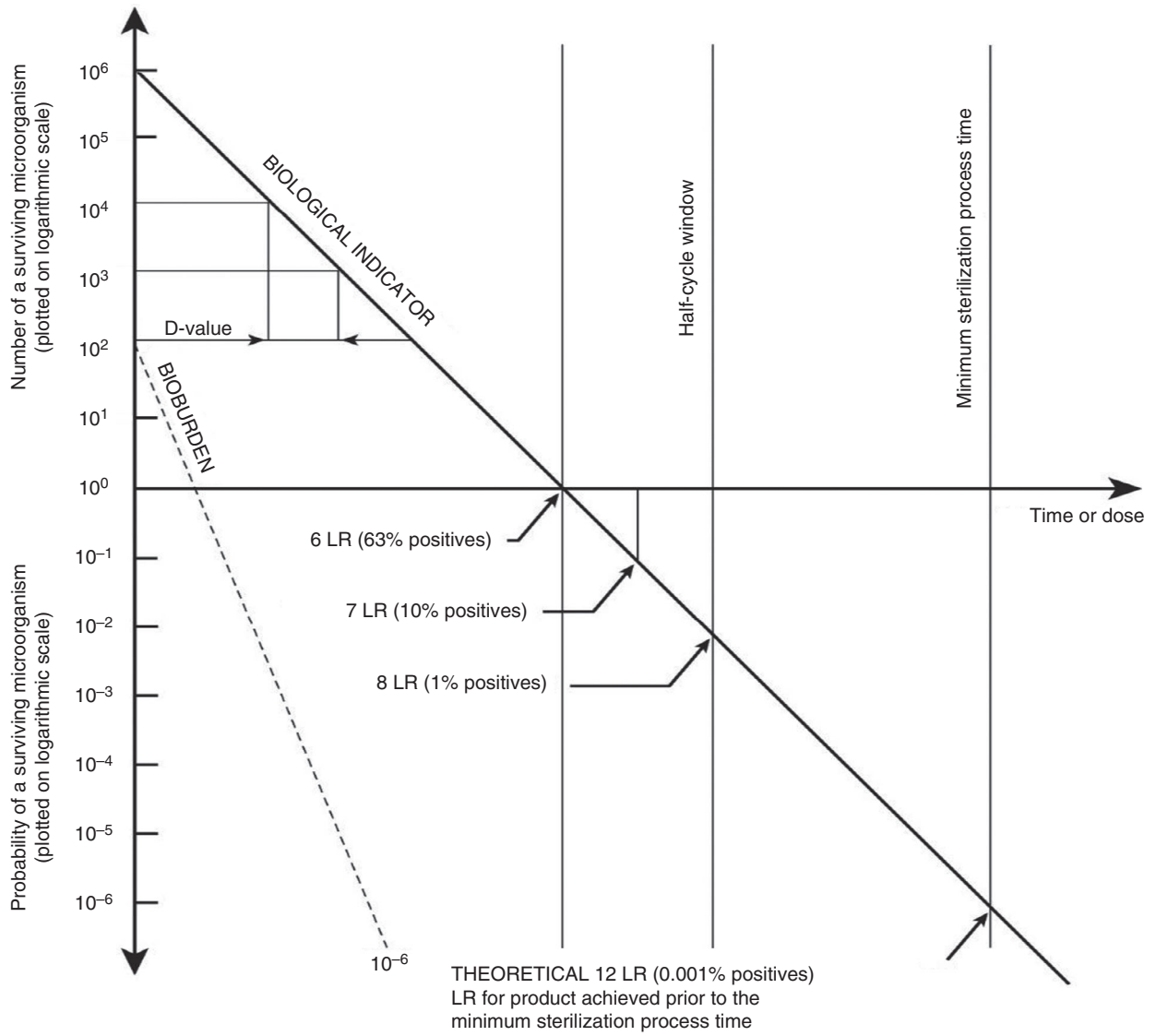

Figure 1 Sterility assurance level and example of the relationship between biological indicator and product bioburden. For illustration purposes, this graphical representation has been obtained from AAMI TIR16:2017. A BI (denoted by full line) has been selected that has a higher population and resistance (*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). To deliver the required sterility assurance level of $\leq 10^{-6}$, a further 6 log reduction is applied by doubling the exposure period in the routine process. Microbiological death generally 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, relative humidity and EO concentration) remain consistent for the duration of the exposure time (AAMI 2017).

SAL is defined as the 'probability of a single viable micro-organism occurring on an item after sterilization' (International Organization for Standardization [ISO] 2018a), and a sterilization process defined as a 'series of actions or operations needed to achieve the specified requirements for sterility' (ISO 2018b). Traditional technologies used in terminal sterilization include EO sterilization and radiation processing using Gamma, Electron beam or X-ray irradiation and are described as Category A sterilization processes by 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 ISO11135:2014 help (ISO 2014) inform manufacturers and users of the key aspects to be evaluated in defining the sterilization process and subsequent qualification and validation. Underpinning the validation of a sterilization process, in accordance with an ISO standard, is the microbiology assessment of the challenge presented by the medical device and the microbicidal effect of that process on the measured challenge and or where a biological indicator (BI) is chosen to represent such a challenge.

Limitations of current techniques in sterilization microbiology

Uniformity of microbial distribution, bioburden and predictability

Many of the sterilization standards are underpinned by the associated microbiological testing standards such as ISO11737-1 and ISO11737-2, which govern the testing of bioburden and sterility, respectively (ISO 2018a, 2019). Bioburden testing refers to the quantification of natural microflora residing on a medical device product. During a sterilization, a validation process is applied and qualified. As part of the qualification exercise, 'Tests of Sterility' may be applied as part of the demonstration of the process inactivation. Upon completion of such tests, it may be possible to plot an inactivation curve such as that demonstrated in Fig. 1 (McEvoy and Rowan 2019). However, conventional sterilization processes such as EO often require use of a BI population of reasonable uniformity that are reliably predictable in an end-point analysis such as a fractional sterilization process performed during validation.

Microbial diversity, heterogenicity and environmental-stress adaptation

Micro-organisms range in size, form, complexity and diversity including variety of physicochemical and environmental growth requirements for those capable of sustaining independent life, all with individual phenotypic identities. Some micro-organisms are highly complex, such as viruses and parasites, which do not grow on standard laboratory-based media. These fastidious micro-organisms require mammalian cell culture or an appropriate host to propagate, where molecular biology tools are typically used as indirect means of enumerating their number (Garvey *et al.* 2013, 2015). Micro-organisms can respond to changes in the environment where prior exposure to sub-lethal amount of stress has been shown to temper or harden the same micro-organisms against subsequent lethal levels of the same of different stresses (Rowan 1999, 2019). Micro-organisms can communicate to each other via quorum sensing (Wu *et al.* 2015) that further illustrates the importance of each single cell in any population. The heterogenicity of bacterial cells in an evenly distributed homologous culture may be viewed as a consequence of gene expression and cell-to-cell interactions, such as quorum sensing. Consequently, the need for analysis at a single cell level is often required for greater insight that includes both mechanistic and cellular responses to applied or lethal stresses (Farrell *et al.* 2011; Wu *et al.* 2015). As highlighted by Geier *et al.* (2008), 'social interactions among bacteria are more specific than interactions with the environment', through signalling compounds called auto-inducers. In addition, research by García-Contreras *et al.* (2015) highlights the role for quorum sensing in protecting cells from a wide range of environmental stresses. The phenotypic outcomes of such responses to stress environments, further add to the complexity of micro-organism populations, particular those in stressed state as would be expected during sterilization inactivation. The importance of phenotypic heterogenicity can manifest in the inactivation kinetics of a population to an environmental stress such as a disinfectant or sterilization process: Stone *et al.* (2009) offered an explanation for nonlinear inactivation kinetics due to 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 resistance to a lethal agent is not uniform'. Furthermore, when one considers superdormant spores as a microbial defence mechanism, the importance of heterogenicity in phenotypic composition with varying germinant receptors is apparent (Dembek *et al.* 2013; Ghosh and Setlow 2009; Zhang *et al.* 2012). Hence, such phenotypic variation in a population should be considered in sterilization microbiology. Most sterilization methods rely on 'overkill', or in other words a conservative over-processing with sterilant to provide the greatest level of assurance. However, as a consequence of that over-processing is the over

application of a hazardous sterilant like EO gas or a material affecting radiation such as gamma. If in the future, workers may seek to deliver reduced and targeted processes, and therefore the need to fully appreciate the behaviours of the microbiological target becomes even more pressing. As researchers strive for greater levels of understanding of inactivation kinetics to deliver more measured and potentially reduced processes, understanding of the aforementioned microbial mechanisms and behaviours in sterilization microbiology must be considered.

Binary growth of micro-organism and culture conditions post-treatment

Cell culture techniques in sterilization microbiology provide binary information, namely 'growth' and 'no-growth'. Microbial viability is determined by counting live cells based upon their ability to grow on artificial-laboratory-based media such as agars or broths under aerobic cultivation over a typical 48-h incubation at optimal temperature, where failure of similarly treated populations of micro-organisms to grow are presumed dead (Hayouni *et al.* 2008; Rowan *et al.* 2015; Léonard *et al.* 2016). Furthermore, culture-based techniques assume that one colony forming unit has emanated from a single cell, which may not be the case as a colony can arise from one or more cells. This limitation has been highlighted by many authors such as Ou *et al.* (2017); Laflamme *et al.* (2005); Mtimet *et al.* (2017); Reis *et al.* (2005) and therefore has the potential to underestimate the microbiological population being examined. Furthermore, as identified by both Ou *et al.* (2017) and Laflamme *et al.* (2005), culturability is inherently dependent on culture conditions. The binary nature of traditional culture techniques in sterilization microbiology provides little information regarding the heterogenicity of a population, phenotypic states as a micro-organism transitions from live to dead, or environmental conditions such as presence or absence of oxygen (Rowan 1999; Rowan *et al.* 2015).

Ability to recover and understand intermediate microbial states post-treatment

The ability to recover or resuscitate micro-organisms following a sterilization treatment is a key consideration, in that it may influence the ability to quantify and/or verify the efficacy of sterilization treatment (McEvoy and Rowan 2019). The risks associated with non-recovery of viable micro-organisms may be minimized with the use of reference micro-organisms, such as BIs, as recovery may be well characterized and prescribed with culture conditions. Investigations into BI grow-out times by Gillis *et al.* (2010) highlight some of the considerations with recovery of damaged micro-organisms. The authors identify that out-growth of a non-sterile BI is a function of (i) initiation of spore germination, (ii) conversion to the cell form and (iii) cellular metabolism and cell division and multiplication (Gillis *et al.* 2010). Therefore, if one considers a traditional sterility test where a BI is immersed in test media and the goal is to be able to recover a single viable micro-organism, then these multiple events must occur, successfully. The authors postulate that delayed outgrowth of BIs is likely to be a consequence of the delayed germination time of a damaged spore. If this is a correct assumption, then the significance of the test media and its ability to provide required conditions for the germination apparatus to operate effectively, should not be underestimated. If, however, natural bioburden population is being examined, an understanding of the identity of the micro-organisms is necessary to ensure culture conditions for recovery are appropriate. If one considers the proposal of Keller and Zengler (2004); 'As we now understand, >98% of the micro-organisms in our environment cannot be kept in culture, culture-independent technologies are required that can characterize (micro-organisms) precisely' (Müller and Nebe-Von-Caron 2010). These and other authors (Taimur Khan *et al.* 2010; Rowan 2011; Wilkinson 2018) highlighted that some micro-organisms may fail to grow in culture media simply due to fact that they may be missing a special requirement such as a growth factor or a symbiotic support.

Micro-organisms may exist in a viable but not culturable (VBNC) state, which has been observed as a consequence of applying a lethal or sub-lethal stress, such as that experienced during a decontamination or process technology (Rowan *et al.* 2015). The authors postulated that 'evidence suggests that these harsh environment cues (operational parameters of the pulsed light treatment and biological factors) may trigger a switch to the adaptive survival VBNC state in PL (pulsed light) treatments'. The concept of VBNC has been observed in many micro-organism species (Rowan 2004; Oliver 2005; Rowan *et al.* 2015) 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. Stress conditions result in other subpopulations and phenotypes such as VBNC which have lost their ability to grow on culture media, but continue to demonstrate metabolic activity (Léonard *et al.* 2016). Therefore, aside from the significance of not being capable of recovering a viable fraction of the treated population, this limitation also hinders the understanding of inactivation kinetics and the true mechanisms of the lethal agent in the life cycle of the micro-organism and cellular responses to stress.

Bacterial 'Viability' versus 'Vitality'

Bacterial viability may be defined as the ability to grow whereby key properties of structural, genetic, metabolic function to facilitate growth (Wilkinson 2018). Traditional culture techniques are able to detect such viability, for a given set of culture conditions. As already stated, many micro-organisms cannot be recovered or maintained in culture due to fastidious nutrient and condition needs. Hence, as stated by Wilkinson (2018), we arrive at a new term of 'Vitality'. The work of Kramer and Thielmann (2016) investigated the cellular activity associated with vitality in food relevant bacteria being treated with mild heat: Using non-culture techniques such as flow cytometry (FCM), the researchers were able to monitor cellular functions and structures such as membrane potential or respiratory activity. Hence, measurement of such cellular vitality indicators at a single cell level can elucidate detailed information regarding microbial inactivation.

Ability to examine mixed cultures

Traditional cell culture techniques seek to segregate and purify culture through the use of selective media and creation of distinct colonies for counting purposes. This unfortunately eliminates possibilities to understand the micro-organism type to micro-organism type interactions, including quorum sensing that may be happening *in situ*. The ability to understand such interactions has been shown to be particularly relevant in bioprocessing such as that described by Rodriguez and Thornton (2008).

Speed of analysis: lack of real-time monitoring and assessment

Traditional culture techniques are a 'time-consuming multiday process', that 'does not provide timely information that is required in applications such as industrial manufacturing, research and medical diagnoses' (Ou *et al.* 2017). This time lag between point of sampling and obtaining any results or information regarding the micro-organism population often renders the information as retrospective. However, the more significant issue is that the information gained from the recovery of a cell or population of cells on a culture plate is not representative of the state of the micro-organism at the point of sampling, as the micro-organisms more often have been recovered and artificially grown on nutrient-enriched media. In industrial bioprocesses, having real-time data allow decision-making during the real-time operation of the bioprocess (Díaz *et al.* 2010). Thus, as a consequence of such limitations, the need for more advanced real-time tools and methods that provide detailed phenotypic insights is merited. One such method with growing application is flow cytometry.

Flow Cytometry

History of Flow Cytometry

If one considers the optics developed by Leeuwenhoek in 1600s, and combine with the experimental apparatus of John Tyndall in the 1800s, the early design of flow cytometry can be imagined (Müller and Nebe-Von-Caron 2010). In the late 1940s, flow cytometers were generated to identify bacterial aerosols in warfare. Through the 1970s, the technology was further developed to a point of being able to detect large viruses. Since the 1980s, flow cytometry has been deployed to many applications with extensive publication of its use (Müller and Nebe-Von-Caron 2010). The review paper by Picot *et al.* (2012) offers a very detailed chronological history of the development of FCM.

As described by Picot *et al.* (2012) and Kalina *et al.* (2020), while many early applications focussed on mammalian cells, flow cytometry has extended to many clinical applications (Brown and Wittwer 2000; Suo *et al.* 2020). Furthermore, there is an increasing evidence that flow cytometry has been extensively used to advance routine microbiology laboratory analysis (Mejuto *et al.* 2017) real-time in vivo clinical determinations (Brown and Wittwer 2000; Kalina *et al.* 2020; Suo *et al.* 2020), and in field environmental monitoring (Safford and Bischel 2019). For example, Kalina *et al.* (2020) have reported on the effective use of FCM in primary immunodeficiencies, immunophenotyping, diagnostics and functional studies. Mejuto *et al.* (2017) have reported on the use of FCM as an effective alternative approach for urine culture in routine clinical microbiology laboratory. Álvarez-Barrientos *et al.* (2000) describe the application of FCM for parasite and virus detection in clinical specimens. Similarly, Glier and Holada (2012) describe how anti-prion monoclonal antibodies conjugates may enable FCM screening of clinical blood samples for prion disease. Safford and Bischel (2019) reviewed the potential of FCM to radically inform applications in waste treatment and reuse. Léonard *et al.* (2016) have reviewed the potential opportunities presented by FCM in the examination of the effect of antimicrobials on sub-populations of micro-organisms.

Principles of Flow Cytometry

Flow cytometry is best described as automated microscopy where thousands of cells can be analysed in a second. FCM quantitatively measures the optical characteristics of cells as they pass in single file in front of a focused light

beam (Veal *et al.* 2000). By measuring fluorescence, either natural or induced by use of fluorescent markers, cells may be differentiated based on size, shape or phenotypic characteristics. The flow cytometer consists of three main components, namely Fluidics, Optics and Electronics for measurement (Goure 2013).

The key aspect of flow cytometry is the measurement of scattered light following impact of the beam from a light source with the individual cells being analysed. Light that is scattered at acute angles, called 'forward scatter' gives an indication of the particle size, whereas light scattered at wider angles, called 'side scatter is proportional to the particles roughness and complexity' (Ambriz-Aviña *et al.* 2014). Even though measurement of light scatter can yield very useful information about bacterial cells, it is unfortunately non-specific to bacterial species. Therefore, fluorescent dyes must be employed in combination to achieve further specificity in the analysis. Cell viability, protein identity and enzymatic activity have been measured in such a manner (Ambriz-Aviña *et al.* 2014).

Another useful advancement of flow cytometry is 'Fluorescence Activated Cell Sorting (FACS)' whereby cells are differentiated and sorted by means of detection of a fluorescent marker using flow cytometry. For the past two decades, flow cytometry (FCM) has been used to investigate micro-organisms and 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). FCM is a sensitive technique operating in real time that can examine individual cells at rates of 100–1000 cells per second.

Light Scatter

Cell characterization using flow cytometry is achieved by means of measuring light scatter or fluorescence signals (Díaz *et al.* 2010). Examining cells with light scatter yields useful information regarding cell morphology and size: Forward scatter is normally assumed to be proportional to cell size, whereas 'side scatter light has been shown to be affected by intracellular structure and inclusion body formation' (Díaz *et al.* 2010).

Light scatter alone can yield important information about micro-organisms and can be an early differentiator of phenotypic populations. Reis and co-workers observed bimodal side scatter with vegetative cells of *Bacillus licheniformis* (Reis *et al.* 2005). Similarly, Hewitt *et al.* (1999) and Schenk *et al.* (2011) observed a bimodal distribution of side scatter light with *Escherichia coli*, which is characteristic of rod-shaped micro-organisms explained by Hewitt *et al.* (1999), as most likely due to 'an elongated cell's orientation as it travels through the laser beam can vary in range from the major to the minor axis'. Comas-Riu and Vives-Rego (2002) observed bimodal light distribution of forward scatter light from *Paenibacillus polymyxa* spores, and upon further investigation with a staining protocol using propidium iodide (PI) and Syto 13, observed two distinct phenotypic populations, one permeable to PI (and weakly permeable to Syto13) and the other sub-population impermeable to both dyes. Hence, light scatter in this case (unlike that in Reis and Hewitt studies) served as an early indication of phenotypic variation, which was then confirmed by further exploratory work.

FCM analysis of light scatter may also detect autofluorescence of micro-organisms. As highlighted by Magge *et al.* (2009), autofluorescence may occur in some species as a consequence of spore coat components such as dityrosine which cross-links through oxidation activities involving peroxidase and oxidase. Hence, in the design of a FCM experiment, it may be necessary to consider the addition of a negative control for autofluorescence as adopted by Zhang *et al.* (2020) in the execution of their research.

Use of Fluorescent probes

However, when looking at microbial cells, given their size, light scatter profiles alone may yield insufficient information. Hence, the use of fluorescent probes has proved to be a useful methodology when combined with flow cytometry. Fluorescent probes work from the principle that the wavelength of emission will be longer than that of excitation and the difference between the two, described as the Stokes shift, determines the effectiveness of the probe (Davey and Kell 1996; Adan *et al.* 2017).

Various fluorescent probes may be used to determine the physiological state of the organism and help differentiate a population by viability state. Probes such as those examples described in Table 1, may be used based on cell interaction characteristics to identify a population or may even be used to count micro-organisms. Probes used in flow cytometry studies may be divided into two broad groups: (i) nucleic-acid-binding dyes and (ii) metabolic/cellular/protein-binding dyes (Mathur *et al.* 2016).

Trevors (2003) describes the advantages and limitations of the use of fluorescent probes in bacterial research. Combinations of dyes may be used to generate 'multiparametric data' from individual cells in a heterogeneous population (Wilkinson 2018). The key properties favourable in a fluorescent probe include (i) biologically inert, (ii) a high extinction coefficient and a high quantum yield so that small concentrations of the stain can be detected within the cell, (iii) a narrow emission spectrum to avoid overlapping, (iv) photostability, (v) low toxicity and (vi) solubility in water (Díaz *et al.* 2010).

Table 1 Selection of Fluorescent stains commonly used with flow cytometry

Probe	Property	Cell fraction identified	References
Carboxyfluorescein diacetate (cFDA)	Intracellular esterase activity	Viable cell fraction	Majeed *et al*. (2018); Cronin and Wilkinson (2008); Wilkinson (2018)
DiBAC4	Anionic structure which enters cell and binds to intracellular proteins	Membrane potential	Buysschaert *et al*. (2016)
Dihydroethidium (HE)	Oxidation of HE results in formation of ethidium which, in turn, binds with DNA yielding fluorescence	Reactive oxygen species	Buysschaert *et al*. (2016)
Ethidium Bromide (EB)	Positively charged monovalent compound used to evaluate efflux pump system. Enters intact cells but is pumped out. However, where membrane and efflux pump is damaged, intracellular DNA is stained	Efflux pump and membrane activity	Léonard *et al*. (2016)
Fluorescein	Binds to proteins	Protein	Cronin and Wilkinson (2008)
Hoechst 33342	Binds to DNA	DNA	Cronin and Wilkinso (2008)
Propidium Iodide (PI)	PI is a commonly used dye for detection of dead or damaged cells as evidenced by permeabilization of the inner membrane. PI contains two positive charges normally excluded from the cells due to divalence. Therefore, PI can only enter permeabilized inner membranes	Dead or damaged cells	Leonard *et al*. (2016); Majeed *et al*. (2018)
Rhodamine 123	Cationic dye that only accumulates in cells with active membrane potential and reciprocally can be pumped back out of cells	Membrane potential	Buysschaert *et al*. (2016)
Sybr Green	DNA	Total cell counting	Buysschaert *et al*. (2016)
SYTO BC SYTO 9	Nucleic Acid stain (both DNA and RNA)	Total cells—intact and damaged cells	Majeed *et al*. (2018); Trevors (2003); Wilkinson (2018)
SYTO BC + polystyrene microspheres	Nucleic acid stain (both DNA and RNA)	Total cell counting	Majeed *et al*. (2018)

Fluorescent dyes used in flow cytometry

Membrane integrity and permeabilization

Membrane integrity is often employed as a definitive measure of cell viability. Thus, as a consequence, dyes typically fall into two categories, namely cell permeant and cell impermeant, depending on their ability to penetrate an intact cell membrane (Buysschaert *et al*. 2016). Membrane integrity indicates that cells continue to display metabolic activity by maintaining potential without guaranteeing cell replication (which is required for cell culturing techniques). Cells with damaged or compromised membranes are unable to maintain the electrochemical gradient across the membrane and are therefore considered as dead (Díaz *et al*. 2010). Dyes employed typically work on the principle of exclusion from a viable cell membrane and permeabilization through a damaged membrane. Propidium iodide is a commonly used dye for detection of dead cells as evidenced by permeabilization of the inner membrane. PI contains two positive charges and is 'normally excluded from the cells due to divalence'. Therefore, PI can only enter permeabilized inner membranes (Léonard*et al*. 2016). Viability may be determined through combination with dyes such as PI (membrane impermeant) where fluorescence indicates non-viability (Buysschaert *et al*. 2016).

Membrane permeant dyes may be used to combine with cellular nucleic acids: Sybr green binds to DNA while Syto9 binds to both DNA and RNA to fluoresce green (Buysschaert *et al*. 2016; Wilkinson 2018). Such dyes yield total counts as nucleic acids may be present in dead and damaged cells. Furthermore, the asymmetric cyanine-based dyes such as Syto are non-fluorescent until such time as they bind with nucleic acid, whereas DAPI or Sybr-based dyes enhance their fluorescence upon binding to nucleic acids (Díaz *et al*. 2010). This may be a consideration when trying to differentiate very small bacterial cells from background noise.

Cell structure and differences between gram-positive and gram-negative bacteria is an important consideration when design staining strategies (Berney *et al.* 2007; Buysschaert *et al.* 2016; Léonard *et al.* 2016). Gram-negative cells present greater challenge to staining, often requiring a time-dependant protocol due to presence of the outer membrane. Buysschaert *et al.* (2016) found that uptake of Syto dyes was immediate in gram-positive micro-organisms, whereas approximately 15-min incubation was required with Gram-negative bacteria. Even allowing sufficient time for dye uptake in gram negative may be insufficient to see homogeneous staining and therefore protocols may require addition of EDTA to chelate the LPS layer of Gram-negative bacteria (Berney *et al.* 2007; Buysschaert *et al.* 2016). As described by Buysschaert *et al.* (2016), work is required to determine the appropriate EDTA and dye concentration, and in their research they found that EDTA improved uptake in Gram-negative bacteria at lower dye concentrations. However, Davey and Kell (1996) highlight the impact of the EDTA sample preparation in terms of cell viability, change to side scatter profiling, cell-sorting and re-growth which should be considered.

Membrane potential/Energization

Membrane potential is often used to determine cell viability where viable cells maintain ion potential across the cell membrane, necessary in ATP synthesis and essential molecule transport (Buysschaert *et al.* 2016). Given the link between membrane potential and cell respiration and ATP synthesis, this may be considered a more appropriate measure of viability given the criticality of respiration (Hammes *et al.* 2011). When the difference between the ions inside and outside the cell decreases to zero, it serves as an indication that the membrane is structurally damaged (Díaz *et al.* 2010). Cationic dyes such as $DiOC_n$ are able to cross the cell membrane of viable cells and accumulate inside polarized cells. However, as advised by Magge *et al.* (2009), care should be taken with dyes such as $DiOC_6$, as non-specific binding to the spore coat was observed during their studies, further emphasizing the importance of understanding a selected dye and its site of action.

Metabolic Activity

As part of metabolic activity, cells operate a suite of esterases and dehydrogenases whose activity may be detected and measured with dyes and flow cytometry. Like nucleic acids stains, enzymes may still be active after cell death (Buysschaert *et al.* 2016). Therefore, staining protocols should be carefully designed and more often involve multi-stain approach. Carboxfluorescein diacetate (cFDA) is cleaved by esterases to release the fluorescent fluorescein. cFDA is a viability stain that is effectively retained in the cell, but staining protocols should consider pH conditions as it may affect fluorescence emission intensity with pH 9 being most intense (Buysschaert *et al.* 2016). Another enzymatic activity of interest is that of superoxide dismutase in responding to reactive oxygen species (ROS) during oxidative stress. An increase in ROS may be detected using dihydroethidium (HE), where the oxidation of HE results in the cleavage of ethidium which fluoresces with the intercalation with DNA (Buysschaert *et al.* 2016).

Multi-staining strategies

Single staining strategies may overestimate populations or suggest incomplete determinations of phenotypic fractions whereby intermediate states are elucidated (Díaz *et al.* 2010; Léonard *et al.* 2016). Through the use of multiple fluorescent probes, different functional properties or morphological states may be assessed simultaneously and used to form a more complete understanding of the micro-organism (Buysschaert *et al.* 2016). An often used staining kit is Live/Dead® BacLight™ kit which uses a combination of SYTO9 and PI to distinguish intact live cells from permeabilized dead cells. Thus, multiple fluorochromes are often used in combination to label cellular components or properties and subsequently assist in differentiation. As shown by the work of Quirós *et al.* (2007) the dual staining of *Lactobacillus hilgardii* and *Saccharomyces cerevisiae* using CV6 and PI shows the value in elucidating cell fractions such as damaged and VBNC cells, not observed in cell culture plates. The authors were able to gain a fuller understanding of the culture kinetics in the bioprocess being examined.

As highlighted by Wilkinson (2018), micro-organism types may uptake stains differently and this should be considered in any protocol. The author highlighted some reliability challenges with PI. Hence, multi-stain strategies should be considered whereby fractions may be triangulated and validated. For example, a strategy might be to perform total stain with Syto, a non-viable stain with PI and a viable stain with cFDA. Majeed *et al.* (2018) utilized such a strategy whereby they were able to validate their staining strategy by comparing cFDA fraction to the Syto-PI fraction (each representing viable cell numbers).

In our own work, we have used multiple dyes to examine fractions of *Geobacillus stearothermophilus*. In this work, biological indicators of *G. stearothermophilus* were treated with vapourized hydrogen peroxide sterilization and examined with flow cytometry to elucidate fractions recovered in Tryptone Soya media broth. As shown in Fig. 2, the benefit of having a multi-stain strategy is apparent: Syto BC plots as expected show total count of cells due to the nucleic acid staining, while PI shows dead

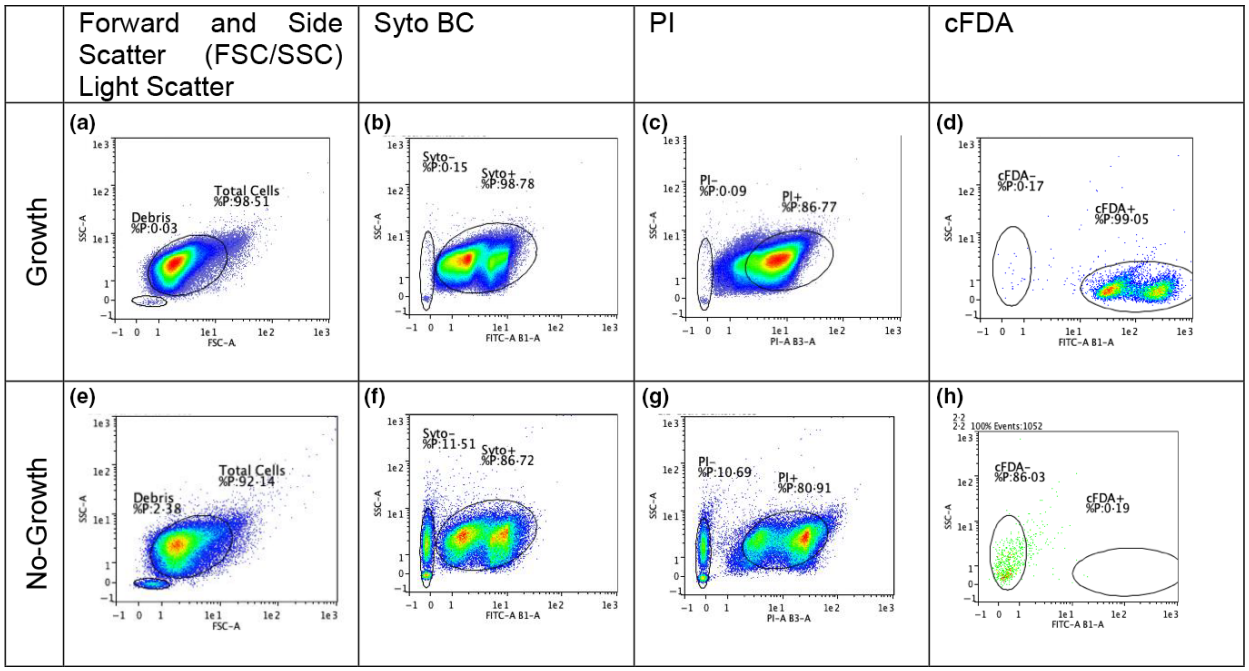

Figure 2 Flow cytometry of *Geobacillus stearothermophilus* biological indicators treated with vapourized hydrogen peroxide and incubated in TSB. Plots (a–d) are of from TSB tubes where growth was observed after 24 h. Plots (e–h) are from TSB tubes where no growth was observed after 48 h.

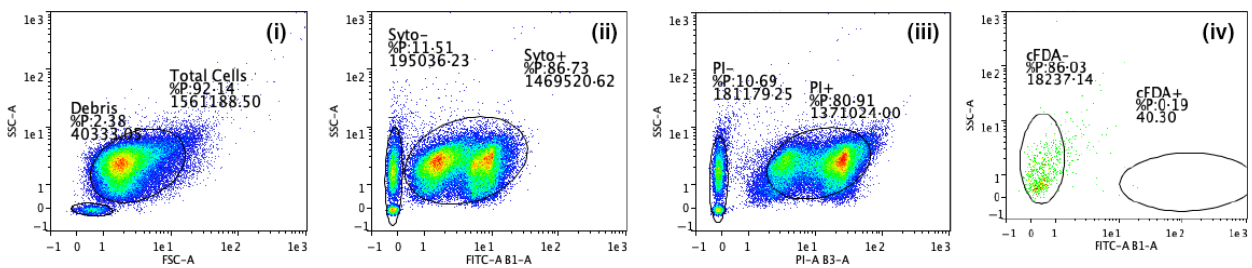

Figure 3 Flow cytometry of plates E–H (Fig. 2) unstained (i) and stained with Syto BC (ii); PI (iii); CFDA (iv) and including values for cells per ml. (i)–(iii) performed as same experimental test with Syto BC and PI applied as a dual stain. CFDA applied as separate experiment with differing dilution: When CFDA events are corrected to same dilution as Syto/PI experiment, a $CFDA^-$ cell count of $1{\cdot}47 \times 10^6$ is obtained.

and damaged cells due to the penetration of the dye through the inner membrane of the cell. In panel G of Fig. 2, PI stained positive as expected for a tube that yielded no viable growth and this was verified by the cFDA—population elucidated in panel H. However, in the TSB tube where growth was observed, PI-positive cells (Fig 2c) were also observed which was also somewhat expected to indicate damaged cells. Again, cFDA acted to confirm that albeit the cells were damaged, they were capable of metabolic activity.

As much as one can observe the ability of FCM and fluorescent probes to elucidate various fractions as shown in Fig. 2, the accuracy at which it elucidates such fractions is also a considerable benefit of using flow cytometry. Again, in our own research studies, a test tube of TSB containing a biological indicator of *G. stearothermophilus* was examined by flow cytometry with stains of CFDA, Syto BC and PI. The results of the examination of this tube are shown in Fig. 3.

When CFDA experiment is corrected for dilution, a similar population is observed for Syto BC^+, PI^+ and $CFDA^-$. This therefore indicates a population of total cells (Syto BC^+) of approximately $1{\cdot}5 \times 10^6$ cells of *G. stearothermophilus* that have damaged inner cell

membranes (PI^+) to such an extent that the cells are not viable ($CFDA^-$).

In defining a protocol for multiple staining, careful consideration should be given to (i) the individual staining protocols for each dye, (ii) the possibility for overspill of fluorescence and the need for compensation, (iii) the interference of one dye with another, for example, electron quenching, (iv) dye stability and (v) the potential for cellular metabolic activity to affect the staining protocol (Buysschaert *et al.* 2016; Kirchhoff and Cypionka 2017).

For example, Kirchhoff and Cypionka (2017) observed viable cells of *Dinoroseobacter shibae* and *Bacillus subtilis* being stained with PI and attributed this to a 'boosted membrane potential', that is, an amplified ion-motive force leading to permeability of the PI cation. In the case of *B. subtilis*, this phenomenon was observed when cells were in early growth phase (similar to our TSB culture stained after 24 h; Kirchhoff and Cypionka 2017). Again, this further highlights (i) the careful selection of appropriate stains, (ii) the definition of an appropriate protocol and (iii) the benefit of using multiple stains. In the case of our work, the use of CFDA acts as the ultimate arbitrator of viability, and subsequently, confirmation of the other fractions obtained.

Key advantages with Flow Cytometry

Key advantages may be summarized as (adapted from Wilkinson 2018) rapid test time and data generation (min); high numbers of test cells (in excess of 10 000); single cell analysis; real-time analysis; minimal sample volumes; significant data generation; detailed Data analysis tools; and multi-parameter analysis.

With regard to the examination of bacteria, the key advantage is appropriately summarized by Díaz *et al.* (2010), as 'the assessment of structural and/or functional cell properties such as metabolic activity, membrane potential and integrity or macro-molecules biosynthesis leads to a deeper characterization of cell populations'.

Challenges with FCM in sterilization microbiology

With regard to the examination of bacteria with flow cytometry, there are a number of challenges that must be considered and strategized for, particularly, bacterial size, staining protocols and creating a partial picture.

Bacterial Size

Bacteria are some three times smaller than mammalian cells in mass and therefore contain less cellular constituents, such as nucleic acids, where fluorescent probes are often targeted. As a consequence of this, the areas of light scatter of interest are often either close to the instrumentation noise or the cellular debris detected as events by the flow cytometer (Tracy *et al.* 2008; Ambriz-Aviña *et al.* 2014).

Staining Protocols

The importance and moreover the significance and impact of the staining protocol on the final result are highlighted by many researchers. For example, Nescerecka *et al.* (2016) identified that the use of solvents such as DMSO may affect the permeability of the cell and subsequently affect results. Other researchers have reported bleaching of probes over time, for example, Stiefel *et al.* (2015) observed loss of SYTO9 in *Staphylococcus aureus* and *Pseudomonas aeruginosa* cells over time of 10–130 min with a loss of 4–8% every 5 min. In some studies, cellular and metabolic interference was observed. As described already, ion motive force can potentially result in misleading results with PI. Similarly, Stiefel *et al.* (2015) describe how Gram-negative micro-organisms with two cell membranes may limit the permeabilization of SYTO9. Also, the same workers observed variances in uptake of SYTO9 in live and dead cell populations. Background fluorescence may also impact results. Again, related to the size of the bacterial cell, the impact of background fluorescence must be known and accounted for. For example, PI yields high fluorescence in the unbound form (Stiefel *et al.* 2015). Regarding the probes themselves, emission spectra wavelengths may overlap and therefore compensation must be applied to yield accurate determinations. Additionally, spores themselves have proved to be challenging to analyse. Bacterial endospores have many protective strategies deployed during dormancy including spore coat, dormant metabolic activity, nucleic acid protected with small acid soluble proteins. All of these mechanisms make it somewhat difficult to stain with FCM probes, consequently with mixed results being observed in the literature (Comas-Riu and Vives-Rego 2002; Cronin and Wilkinson 2007; Mathys *et al.* 2007; Majeed *et al.* 2018; Trunet *et al.* 2019; Young and Setlow 2004b). Some researchers were unable to stain endospores while others had some success with specific probes, staining protocols and use of germinants. As expected, an understanding of the action and target of the probes is imperative: Laflamme *et al.* (2004) found that while Syto9 penetrates free endospores, DiBAC did not work, not because the molecule could not pass the spore coat but because the mechanism it is testing namely efflux pump is dormant in a dormant endospore.

Creating a partial picture

In the research field of Food sterilization and preservation, flow cytometry has been used as an investigative

tool quite extensively (Borch-Pedersen *et al.* 2017; Lv *et al.* 2019; Zhang *et al.* 2020). The very recent work of Zhang *et al.* (2020) demonstrates how FCM can be employed to elucidate various phenotypic fractions observed during an inactivation treatment. FCM can also show the sequence of events from intact spores to germinated spores (high fluorescence with Syto) to an intermediate (moderate fluorescence) 'unknown state' to finally an inactivated state (PI positive). However, it can equally be observed that a limitation resides with FCM, where one fraction is often labelled 'unknown'—it is known from a stain fluorescence perspective but not fully resolved from a phenotypic or vitality state. Some authors determine this to be the VBNC fraction accounting for differences with cell counts from conventional plate count techniques. Zhang *et al.* (2020) examined the cultivability of each fraction to further inform, and arrived at the conclusion that this fraction is likely to be 'germinated with partial sublethal damage'. Our work to date, as shown in Figs 2 and 3, demonstrates elucidation of various fractions with Syto and PI stain and viability assessment using CFDA. However, like many others have identified, FCM is one of the necessary tools that may be used to inform but other investigative tools and approaches are needed to form the rich picture about bacterial state in a sterilization or decontamination process. Comas-Riu and Vives-Rego (2002) observed how FCM could differentiate culture fractions such as vegetative cells and spores, all of varying degrees of viability in a way that microscopic or electric particle analysis were not able to do. The authors, using cell sorting and examination under SEM, were able to clearly identify the various fractions in the culture of *P. polymyxa* being examined, hence demonstrating the complementarity of using multiple techniques. Table 2 describes some of the other tools and techniques that may be employed to help form such a rich picture.

As shown in Table 2, additional information can be obtained using complementary techniques such as quantitative PCR and image analysis techniques that have been successfully used in adjacent fields of environmental and food microbiology. Techniques such as RAMAN spectroscopy, Maldi time-of-flight (tof) Mass Spectrometry along with use of scanning and transmission electron microscopy can provide important cellular and mechanistic information on spore composition and destruction post-treatments (De Gelder *et al.* 2007; Lasch *et al.* 2008; Hutchison *et al.* 2014). Raman bands of calcium dipicolinate and amino acids such as phenylalanine and tyrosine are more intense in the spectra of sporulating bacteria compared with those of bacteria from earlier phases of growth and therefore can be used to detect sporulation of cells by a characteristic band at 1018 cm(−1) from calcium dipicolinate. The increase in amino acids could possibly be explained by the formation of small acid-soluble proteins that saturate the endospore DNA (De Gelder *et al.* 2007). These rapid approaches can also be used to detect intermediate morphological and physiological states and therefore complement FCM in providing a more complete picture.

Opportunities for real-time FCM in sterilization microbiology

As much as researchers should understand and address limitations, the benefits of flow cytometry in the examination of micro-organisms cannot be overlooked. As stated by Wilkinson (2018), 'FCM data may reflect differing properties such as the extent of cell membrane integrity, functionality of membrane potential, presence of intracellular enzyme activity and DNA base composition'.

As highlighted by Majeed *et al.* (2018) 'intermediate states between viable and dead bacteria like injured and stressed cells are difficult to detect by the plating method and are often termed as viable but nonculturable' (VBNC). Hence, we arrive at another classification based on the term 'vitality' or the degree to which a cell can perform various aspects of metabolic, physiological and genetic functionality and the extent of structural and morphological integrity (Kramer and Thielman 2016).

While significant investigative work has been conducted by a number of researchers regarding the effect of sterilants at a cellular level (Roth *et al.* 2010; Leggett *et al.* 2016; Setlow *et al.* 2016; Young and Setlow 2004a, 2004b), often this work is limited to the use of mutants to compare to wild-type micro-organisms to test such things as cellular permeability for instance. As identified by Cronin and Wilkinson (2008), the use of such mutants still fails to account for the inherent heterogenicity in large micro-organism populations. Some traditional polymerase chain reaction (PCR)-based methods may be limited in differentiating genetic material from viable and non-viable cells (Wilkinson 2018). As highlighted by Wang *et al.* (2017), quantitative-PCR (q-PCR) will not differentiate between viable and non-viable cells, whereas PCR in combination with a nucleic-acid-binding dye such as ethidium monoazide bromide offers potential for differentiation. EMA-q-PCR works on the same principle of membrane permeabilization where EMA intercalates into the DNA of permeabilized membranes preventing amplification by nucleic acids (Pisz *et al.* 2007). Therefore, while EMA-q-PCR offers potential assessment of viability, detection of injured (and viable cells) may be limited, similar to the use of PI in FCM. Hence, FCM using a combination of dyes not just those based on membrane permeabilization as an indicator of viability

Table 2 Potential use of real-time flow cytometry as a tool to address assumptions and limitations underpinning use of conventional culture-based media in sterilization microbiology. Complementary methods to FCM are also described

	Enumeration method for determining survivor populations		
Assumption or limitation	Gold standard culture-based method (Agar, Broth)	Flow cytometry	Other complementary methods
Binary growth of biological indicator	Conventional plate counts assume colonies occur from a single cell every time (Rowan 2019). Broth cultures, for end-point determinations, also assume culture are drawn from single cells. No information may be obtained from non-cultured states	While plate counts can only detect viable cells, FCM counts all cells as events, whether dead or alive. FCM staining strategy (e.g. PI and CFDA) stain will elucidate whether cells are alive, dead, or in an intermediate state, giving exact numbers for each sub population. Ability to elucidate non-culturable states	RAMAN spectroscopy (De Gelder *et al*. 2007)
Characterization of viable endospores and other complex micro-organisms	Endospores are dormant so assumptions is these forms would have all germinated and grown in broth or media—but little understanding of potential sub-lethal damage when an inactivation process is applied (McEvoy and Rowan 2019)	FCM can readily detect unstained viable spores. Spores that are damaged may be stained with PI and germinated spores with other viability stains such as CFDA	RAMAN spectroscopy (De Gelder *et al*. 2007) RNA transcription (Dembek *et al*. 2013) SEM (Rowan *et al*. 2008; Hutchison *et al*. 2014) TEM (Rowan *et al*. 2001; Hutchison *et al*. 2014) Maldi-Tof MS (Lasch *et al*. 2008)
Detection of intermediate morphological and physiological states	Standard culture-based approaches do not enumerate intermediate or viable non-culturable states (Bradley *et al*. 2012; Rowan *et al.* 2015)	PI stain can differentiate a gradient of live-damaged-dead cells depending on degree of PI uptake. Various stains can inform about the physiological state, for example, CFDA regarding metabolic activity	Redox stains (Yaqub *et al*. 2004) ATPase and PCR (Garvey *et al*. 2016) TEM (Sahin *et al*. 2012; Reineke *et al*. 2013) SEM (Reineke *et al*. 2013; Georget *et al*. 2014)
Environmental conditions and microbial stress-adaptation	Prior exposure to sub-lethal stresses may harden microbes to lethal levels of the same stress (Rowan 1999; Rowan *et al*. 2015); ; . Growth in oxygen-rich environment may cause metabolic suicide for some aerobic organisms where vital catalase and superoxide dismutase genes damaged, yet are capable of growth under reduced oxygen conditions (Rowan 1999)	FCM with targeted staining protocols will help elucidate environment and stress responses: PI stain, which stains damaged/dead cells, will give an indication of the ability of the organism to adapt to stress. CFDA may be employed to indicate vitality by demonstrating metabolic activity	Gene-chip microarray for microbial genotypes and species in communities, bioinformatics (Aspedon *et al*. 2006) Microfluidic technologies for microbial community monitoring (Dhar and Lee 2018). qPCR for gene expression under stress conditions (Rowan 2019)
Homogeneity versus Heterogeneity	Natural bioburden may be a mixed culture of different cell types and species. In sterilization microbiology, it is assumed that homogeneity of Biological Indicators (BIs) as a reference micro-organism, compensates for this (McEvoy and Rowan 2019)	Microbes of different size/population will separate out on dot plots making it possible to examine heterogenicity in microbial populations, including those from natural bioburden	Multiplex PCR assays (Diercks *et al*. 2009), MinION for microbial whole genome sequencing (Tyler *et al*. 2018) Immunoassays (Bartholomew *et al*. 2017) Maldi-TOF (Kryazhevskikh *et al* (2015); Li *et al* (2019)
Speed of enumeration post-sterilization treatment	Treated samples require up to 24- or 48-hr incubation before enumeration of survivors (McEvoy and Rowan 2019)	FCM gives total, and population, cell counts in real time, as the sample is analysed	PCR is more time-consuming, but can give complimentary data (Garvey *et al*. 2010)

(Continued)

Table 2 (Continued)

Assumption or limitation	Enumeration method for determining survivor populations		
	Gold standard culture-based method (Agar, Broth)	Flow cytometry	Other complementary methods
Uniformity of cell distribution	CFU counts on agar or broth cultures assume uniformity of cell distribution (Farrell *et al*. 2011; McEvoy and Rowan, 2019)	FCM will give indication of how uniform (size/granularity) cells with FSC indicating size and SSC the granularity of the cells. SSC-Height v SSC-Area to gate out duplets and aggregates (Zhang *et al*. 2020)	Microscopy, SEM, qPCR and combined cell culture (Garvey *et al*. 2013) Image analysis (Rowan *et al*. 2000; Wan-Mohtar *et al*. 2016)
Viability versus vitality	Treated micro-organisms may retain vital functions, but are not capable of growing on artificial laboratory media, thus underestimating population of potential survivors (Rowan 1999; Rowan *et al*. 2015)	Fluorescent probes may be used to target specific cell functions that only operate in alive cells. For example: EB is used to verify a functioning efflux pump (Léonard *et al*. 2016)	Quantitative PCR for specific genes related to vitality (Garvey *et al*. 2010, 2016)

and therefore offers complementary technological insights to addressing complex environmental microbiology challenges.

In their analysis of the effectiveness of disinfectants on nosocomial bacterial species, Massicotte *et al.* (2017) compared traditional culture techniques and FCM, and came to the conclusion that the ability of FCM to detect intermediary states between live and dead cells is a very important aspect given the hypothesis that some organisms such as *Enterococcus faecalis*, in a sub-lethal state had the potential to provide a 'biocharge of bacteria' for future nosocomial infection or food poisoning.

In our investigative work looking at the inactivation of biological indicators of *G. stearothermophilus* using vapourized hydrogen peroxide, the usefulness of FCM as an informative tool cannot be overstated. As shown in Fig. 2e–h, from a test tube of TSB showing no growth and therefore, no useful information from such a traditional technique, FCM has provided a rich picture of information regarding cell numbers, viability and damage. As described by McEvoy and Rowan (2019), investigation of the cellular location of lethality of VH2O2 is an important aspect of understanding sterilization kinetics and one which traditional cell culture techniques simply cannot inform. FCM is already providing valuable insights through the use of various fluorescent probes. However, it is equally recognized that the usefulness of FCM is further accelerated when used in combination with other investigative techniques.

Furthermore, sterilization microbiology often relies on the use of reference micro-organisms in the form of Biological indicators. BIs are often chosen as their resistance to a given sterilization or decontamination method is known and the method for micro-organism recovery is well described (McEvoy and Rowan 2019). However, as sterilization methods are designed to be more efficient and more tailored to the microbiology challenge of the healthcare product being processed, the reliance on BIs for process validation becomes less. As a consequence, the natural microbiology flora is used to define the minimum processing requirement. Use of traditional microbiology techniques that relies upon an understanding of different microbial species present may necessitate providing an enormity of different culture conditions for enumerating and resuscitating the variety of micro-organisms present that have either complex growth requirements or fail to grown in laboratory-based culture media (Tyler *et al.* 2018; Rowan 2019). There is evidence that some lethal or sub-lethally treated-bacteria may be adversely affected by environmental culture conditions (Rowan 2004; Rowan *et al.* 2015).

FCM can be used to discern microbial homogeneity through real-time dot plots of different cell populations. FCM combined with other techniques (Table 2) can effectively monitor, profile and investigate changes in microbial ecosystems. When assessing inactivation from a sterilization or decontamination process, FCM can rapidly assess survivors and those bacteria that may enter a viable but non-culturable state, thus yielding important information during the examination of the inactivation kinetics during process design.

Conclusion

Sterilization validation relies on the demonstration of a measured inactivation of micro-organisms, whether micro-organisms from the natural microflora of the device being sterilized or that of a reference BI (McEvoy

Box 1: Steps to be considered in the design of an FCM experiment.

a Understand the interaction of the probe and the micro-organism being studied and recognizing the growth stage of the micro-organism
b Include sufficient negative and positive control to subtract any background fluorescence, including autofluorescence from the micro-organism itself (Zhang *et al*. 2020).
c Have a multiple staining and counterstaining strategy to triangulate results.
d Assess the requirement for signal compensation (Alvarez *et al*. 2010; Adan *et al*. 2017).
e Design the protocol optimizing conditions, for example, temperature, probe concentration and appropriateness of materials, for example, TRIS buffers as opposed to DMSO (Nescerecka *et al*. 2016).
f Use other methods such as light microscopy, electron microscopy, Raman microscopy, PCR, etc. (to further inform the results obtained from FCM (see Table 2).

and Rowan 2019). To deliver more precise and efficient sterilization processes, a deeper understanding of the inactivation must be understood. On account of the heterogenicity and fastidiousness of micro-organisms, current traditional culture-based methods are limited: They offer a binary assessment of growth or no growth, not taking into account intermediate states that may exist during an inactivation process. As described by Reis *et al.* (2005), '*Bacillus* spp. have been found to exhibit a variety of rich dynamic behaviour including long-term oscillations, multiple steady-states, genetic instability and un-interpretable transients' and the consequences of which make process (Biotechnology cell culturing) optimization and process prediction 'a difficult task'. In addition, culture-based methods are time-consuming and often require a prior knowledge of the microflora species to design experimental recovery methods that are capable of recovering viable cells, in particular those that may be only sub-lethally impacted by the inactivation process (Rowan 2004; Rowan *et al.* 2015).

FCM can inform limitations associated with conventional sterilization microbiology. The use of FCM with appropriate fluorescent probes such as PI offers the opportunity to examine the heterogenous sub-populations including population transitions from viable to dead cells upon treatment with a sterilization process such as VH2O2. The use of FCM multi-parameter analysis using multiple fluorescent probes and alternative methods helps provide a rich picture of the 'vitality' states in heterogenous populations, and such a picture can potentially help inform the inactivation kinetics observed with VH2O2 treatment. However, FCM is not without its own limitations. Such limitations may be addressed by the researcher taking the appropriate steps in protocol design (see Box 1) and/or adding complementary investigative techniques and tools such as those described in Table 2.

Adjacent techniques described in Table 2 include Raman spectroscopy and new omics technologies, such as metagenomics and transcriptomics, which will advance efficacy and application of FCM analysis. Raman spectroscopy may complement FCM analysis of membrane permeability by providing insights into chemical composition (CaDPA) changes within the cell through measurement of Raman shifts (De Gelder *et al.* 2007; Piktel *et al.* 2017). Similarly, developments in omics (transcriptomics, proteomics and metabolomics) of micro-organisms may provide complementary mechanistic cellular information regarding stress resistance and genetic modifications in biomarkers during an inactivation treatment (den Besten *et al.* 2018). Zhang *et al.* (2010) describe how multi-omic strategies may help decipher complex microbial metabolism. Rowan (2019) described the benefits of omics technologies in advancing pulsed light as a potential disruptive decontamination technology for agri-food, water and healthcare applications, where use of FCM would provide valuable real-time determinations. For example, automated FCM analysis would offer considerable complementary determinations when used with metagenomics profiling and next-generation sequencing (Breitwieser *et al.* 2018). As described by Hammes and Egli (2010), 'cultivation-independent viability analysis on the single cell level is one of the finest uses of FCM', *and with* careful experimental design, coupled with complementary techniques such as Raman spectroscopy or omics, FCM may be employed to further inform sterilization microbiology and ultimately aid the improvement of decontamination and sterilization processes.

Acknowledgements

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

Conflict of Interest

There is no conflict of interest.

References

Adan, A., Alizada, G., Kiraz, Y., Baran, Y. and Nalbant, A. (2017) Flow cytometry: basic principles and applications. *Crit. Rev. Biotechol* **37**, 163–176.

Alvarez, D.F., Helm, K., DeGregori, J., Roederer, M. and Majka, S. (2010) Publishing flow cytometry data. *Am J Physiol – Lung Cell Mol Physiol* **298**, 127–130.

Álvarez-Barrientos, A., Arroyo, J., Cantón, R., Nombela, C. and Sánchez-Pérez, M. (2000) Applications of flow cytometry to clinical microbiology. *Clin Microbiol Rev.* **13**, 167–195.

Ambriz-Aviña, V., Contreras-Garduño, J.A. and Pedraza-Reyes, M. (2014) Applications of flow cytometry to characterize bacterial physiological responses. *BioMed Res Int* **2014**, 1–14.

Aspedon, A., Palmer, K. and Whiteley, M. (2006) Microarray analysis of the osmotic stress response in *Pseudomonas aeruginosa*. *J. Bacteriol* **188**, 2721–2725.

AAMI(2017) AAMI TIR16:2017 Microbiological aspects of ethylene oxide sterilization. ISBN 1-57020-363-6. Association for the Advancement of Medical Instrumentation. Approved and published 3 september 2009.

Bartholomew, R.A., Ozanich, R.M., Arce, J.S., Engelmann, H.E., Heredia-Langner, A., Hofstad, B.A., Hutchison, J.R., Jarman, K. *et al.* (2017) Evaluation of immunoassays and general biological indicator tests for field screening of *Bacillus anthracis* and Ricin. *Heal Secur* **15**, 81–96.

Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.U. and Egli, T. (2007) Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry. *Appl Environ Microbiol* **73**, 3283–3290.

den Besten H.M.W., Amézquita A., Bover-Cid S., Dagnas S, Ellouze M., Guillou S., Nychas G., O'Mahony C. *et al.* (2018) Next generation of microbiological risk assessment: Potential of omics data for exposure assessment. *International Journal of Food Microbiology*, **287**, 18–27. http://dx.doi.org/10.1016/j.ijfoodmicro.2017.10.006.

Borch-Pedersen, K., Mellegård, H., Reineke, K., Boysen, P., Sevenich, R., Lindbäck, T. and Aspholm, M. (2017) Effects of high pressure on *Bacillus licheniformis* spore germination and inactivation. *Appl Environ Microbiol* **83**, 1–13.

Bradley, D., McNeil, B., Laffey, J.G. and Rowan, N.J. (2012) Studies on the pathogenesis and survival of different culture forms of *Listeria monocytogenes* to pulsed UV-light irradiation after exposure to mild-food processing stresses. *Food Microbiol* **30**, 330–339.

Breitwieser, F.P., Lu, J. and Salzberg, S.L. (2018) A review of methods and databases for metagenomic classification and assembly. *Brief Bioinform* **20**, 1125–1139.

Brown, M. and Wittwer, C. (2000) Flow cytometry: principles and clinical applications in hematology'. *Clin Chem* **46**, 1221–1229.

Buysschaert, B., Byloos, B., Leys, N., Van Houdt, R. and Boon, N. (2016) Reevaluating multicolor flow cytometry to assess microbial viability. *Appl Microbiol Biotechnol* **100**, 9037–9051.

Comas-Riu, J. and Vives-Rego, J. (2002) Cytometric monitoring of growth, sporogenesis and spore cell sorting in *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*). *J Appl Microbiol* **92**, 475–481.

Cronin, U.P. and Wilkinson, M.G. (2007) The use of flow cytometry to study the germination of *Bacillus cereus* endospores. *Cytom Part A* **71**, 143–153.

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

Davey, H.M. and Kell, D.B. (1996) Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. *Microbiol Rev* **60**, 641–696.

De Gelder, J., Scheldeman, P., Leus, K., Heyndrickx, M., Vandenabeele, P., Moens, L. and De Vos, P. (2007) Raman spectroscopic study of bacterial endospores. *Anal Bioanal Chem* **389**, 2143–2151.

Dembek, M., Stabler, R.A., Witney, A.A., Wren, B.W. and Fairweather, N.F. (2013) Transcriptional analysis of temporal gene expression in germinating *Clostridium difficile* 630 endospores. *PLoS One* **8**, e64011

Dhar, B.C. and Lee, N.Y. (2018) Lab-on-a-chip technology for environmental monitoring of microorganisms. *Biochip J* **12**, 173–183.

Díaz, M., Herrero, M., García, L.A. and Quirós, C. (2010) Application of flow cytometry to industrial microbial bioprocesses. *Biochem Eng J* **48**, 385–407.

Diercks, A., Kostner, H. and Ozinsky, A. (2009) Resolving cell population heterogeneity: real-time PCR for simultaneous multiplexed gene detection in multiple single-cell samples. *PLoS One* **4**, e6326.

Farrell, H., Hayes, J., Laffey, J. and Rowan, N. (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 Methods* **84**, 317–326.

García-Contreras, R., Nuñez-López, L., Jasso-Chávez, R., Kwan, B.W., Belmont, J.A., Rangel-Vega, A., Maeda, T. and Wood, T.K. (2015) Quorum sensing enhancement of the stress response promotes resistance to quorum quenching and prevents social cheating. *ISME J* **9**, 115–125.

Garvey, M., Farrell, H., Cormican, M. and Rowan, N. (2010) Investigations of the relationship between use of in vitro cell culture-quantitative PCR and a mouse-based bioassay for evaluating critical factors affecting the disinfection performance of pulsed UV light for treating *Cryptosporidium parvum* oocysts in saline. *J Microbiol Methods* **80**, 267–273.

Garvey, M., Hayes, J., Clifford, E., Kirf, D. and Rowan, N. (2013) Efficacy of measuring cellular ATP levels to determine the inactivation of pulsed UV treated *Cryptosporidium parvum* oocysts suspended in water. *Water Sci. Technol. Water Supply* **13**, 202–213.

Garvey, M., Rabbitt, D., Stocca, A. and Rowan, N. (2015) Pulsed ultraviolet light inactivation of *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms. *Water Environ J* **29**, 36–42.

Garvey, M., Coughlan, G., Murphy, N. and Rowan, N. (2016) The pulsed light inactivation of veterinary relevant microbial biofilms and the use of a RTPCR assay to detect parasite species within biofilm structures. *Open Vet J* **6**, 15–22.

Geier, H., Mostowy, S., Cangelosi, G.A., Behr, M.A. and Ford, T.E. (2008) Autoinducer-2 triggers the oxidative stress response in *Mycobacterium avium*, leading to biofilm formation. *Appl Environ Microbiol* **74**, 1798–1804.

Georget, E., Kapoor, S., Winter, R., Reineke, K., Song, Y., Callanan, M., Ananta, E., Heinz, V. *et al.* (2014) In situ investigation of *Geobacillus stearothermophilus* spore germination and inactivation mechanisms under moderate high pressure. *Food Microbiol* **41**, 8–18.

Ghosh, S. and Setlow, P. (2009) Isolation and characterization of superdormant spores of *Bacillus* species. *J. Bacteriol* **191**, 1787–1797.

Gillis, J.R., Mosley, G.A., Kowalski, J.B., Krushefski, G., Nirgenau, P.T. and Mccauley, K.J. (2010) Understanding biological indicator grow-out times. *Pharm Tech Europe* **34**, 1–8.

Glier, H. and Holada, K. (2012) Blood storage affects the detection of cellular prion protein on peripheral blood leukocytes and circulating dendritic cells in part by promoting platelet satellitism. *J Immunol Methods* **380**, 65–72.

Goure, J.P. (ed.) (2013) Optics in Instruments: Applications in Biology and Medicine. New York: John Wiley and Sons Inc. ProQuest Ebook Central. Accessed 26 May 2020.

Guirgis, F.W., Jones, L., Esma, R., Weiss, A., McCurdy, K., Ferreira, J., Cannon, C., McLauchlin, L. *et al.* (2017) Managing sepsis: Electronic recognition, rapid response teams, and standardized care save lives. *J Crit Care* **40**, 296–302.

Hammes, F., Berney, M. and Egli, T. (2011) Cultivation-independent assessment of bacterial viability. In *High Resolution Microbial Single Cell Analytics* ed. Müller, S. and Bley, T., pp. 123–150. Berlin, Heidelberg: Springer, Berlin Heidelberg.

Hammes, F. and Egli, T. (2010) Cytometric methods for measuring bacteria in water: advantages, pitfalls and applications. *Anal Bioanal Chem* **397**, 1083–1095.

Hayouni, E.A., Bouix, M., Abedrabba, M., Leveau, J.Y. and Hamdi, M. (2008) Mechanism of action of *Melaleuca armillaris* (Sol. Ex Gaertu) Sm. essential oil on six LAB strains as assessed by multiparametric flow cytometry and automated microtiter-based assay. *Food Chem* **111**, 707–718.

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

Hewitt, C.J., Nebe-Von Caron, G., Nienow, A.W. and McFarlane, C.M. (1999) The use of multi-parameter flow cytometry to compare the physiological response of *Escherichia coli* W3110 to glucose limitation during batch, fed-batch and continuous culture cultivations. *J. Biotechnol* **75**, 251–264.

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.

Hutchison, E.A., Miller, D.A. and Angert, E.R. (2014) Sporulation in bacteria: beyond the standard model. *Microbiol. Spectr*, **2**, 1–15.

International Organization for Standardization (ISO) (2014) *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 (2018a) *ISO11737-1:2018 Sterilization of Health Care Products—Microbiological Methods—Part 1: Determination of a Population of Microorganisms on Products*. Geneva: ISO.

International Organization for Standardization (2018b) *ISO11139:2018 Sterilization of Health Care Products—Vocabulary of terms used in sterilization and related equipment and process standards*. Geneva: ISO.

International Organization for Standardization (2019) *ISO11737-2:2019 Sterilization of health care products—Microbiological methods— art 2: Tests of sterility performed in the definition, validation and maintenance of a sterilization process*. Geneva: ISO.

International Trade Administration (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).

Kalina, T., Abraham, R.S., Rizzi, M. and van der Burg, M. (2020) Editorial: application of cytometry in primary immunodeficiencies. *Front Immun* **11**, 1–4.

Keller, M. and Zengler, K. (2004) Tapping into microbial diversity. *Nat Rev Microbiol* **2**, 141–150.

Kirchhoff, C. and Cypionka, H. (2017) Propidium ion enters viable cells with high membrane potential during live-dead staining. *J. Microbiol Methods* **142**, 79–82.

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

Kryazhevskikh, N.A., Loiko, N.G., Demkina, E.V., Mulyukin, A.L., Lebedev, A.T., Gaponov, A.M., and El'-Registan, G. I.(2015) Applicability of MALDI mass spectrometry for diagnostics of phase variants in bacterial populations. *Microbiology* **84**, 328–346.

Laflamme, C., Lavigne, S., Ho, J. and Duchaine, C. (2004) Assessment of bacterial endospore viability with fluorescent dyes. *J Appl Microbiol* **96**, 684–692.

Laflamme, C., Ho, J., Veillette, M., De Latrémoille, M.C., Verreault, D., Mériaux, A. and Duchaine, C. (2005) Flow cytometry analysis of germinating *Bacillus* spores, using membrane potential dye. *Arch Microbiol* **183**, 107–112.

Lasch, P., Nattermann, H., Erhard, M., Stämmler, M., Grunow, R., Bannert, N., Appel, B. and Naumann, D. (2008) MALDI-TOF mass spectrometry compatible inactivation method for highly pathogenic microbial cells and spores. *Anal Chem* **80**, 2026–2034.

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.

Léonard, L., Chibane, L.B., Bouhedda, B.O., Degraeve, P. and Oulahal, N. (2016) Recent advances on multi-parameter flow cytometry to characterize antimicrobial treatments. *Front Microbiol* **7**, 1–16.

Li, Y., Shan, M., Zhu, Z., Mao, X., Yan, M., Chen, Y., Gu, B. (2019) Application of MALDI-TOF MS to rapid identification of anaerobic bacteria. *BMC Infect Dis* **19**, 1–11.

Lv, R., Wang, D., Zou, M., Wang, W., Ma, X., Chen, W., Liu, D. (2019) Analysis of *Bacillus cereus* cell viability, sublethal injury, and death induced by mild thermal treatment. *J. Food Saf* **39**, 1–8.

Magge, A., Setlow, B., Cowan, A.E. and Setlow, P. (2009) Analysis of dye binding by and membrane potential in spores of *Bacillus* species. *J Appl Microbiol* **106**, 814–824.

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.

Massicotte, R., Mafu, A.A., Ahmad, D., Deshaies, F., Pichette, G. and Belhumeur, P. (2017) Comparison between flow cytometry and traditional culture methods for efficacy assessment of six disinfectant agents against nosocomial bacterial species. *Front Microbiol* **8**, 1–14.

Mathur, H., Rea, C.M., Fallico, V., Cotter, P.D., Hill, C. and Ross, R.P.(2016) Flow cytometry as a tool to study the effects of bacteriocins on prokaryotic and eukaryotic Cells. *J Mol Biomark Diagn* **S8**, 1–8.

Mathys, A., Chapman, B., Bull, M., Heinz, V. and Knorr, D. (2007) Flow cytometric assessment of *Bacillus* spore response to high pressure and heat. *Innov Food Sci Emerg Technol* **8**, 519–527.

Mejuto, P., Luengo, M. and Díaz-Gigante, J. (2017) Automated flow cytometry: an alternative to urine culture in a routine clinical microbiology laboratory? *Int. J Microbiol* **2017**, 1–8.

McEvoy, B. and Rowan, N.J. (2019) Terminal sterilization of medical devices using vaporized hydrogen peroxide: a review of current methods and emerging opportunities. *J Appl Microbiol* **127**, 1403–1420.

Mtimet, N., Trunet, C., Mathot, A.G., Venaille, L., Leguérinel, I., Coroller, L. and Couvert, O. (2017) Walking dead: permeabilization of heat-treated *Geobacillus stearothermophilus* ATCC 12980 spores under growth-preventing conditions. *Food Microbiol* **64**, 126–134.

Müller, S. and Nebe-Von-Caron, G. (2010) Functional single-cell analyses: flow cytometry and cell sorting of microbial populations and communities. *FEMS Microbiol Rev* **34**, 554–587.

Nescerecka, A., Hammes, F. and Juhna, T. (2016) A pipeline for developing and testing staining protocols for flow cytometry, demonstrated with SYBR Green I and propidium iodide viability staining. *J Microbiol Methods* **131**, 172–180.

Oliver, J.D. (2005) The viable but nonculturable state in bacteria. *J Microbiol* **43**, 93–100.

Ou, F., McGoverin, C., Swift, S. and Vanholsbeeck, F. (2017) Absolute bacterial cell enumeration using flow cytometry. *J Appl Microbiol* **123**, 464–477.

Picot, J., Guerin, C.L., Le Van Kim, C. and Boulanger, C.M. (2012) Flow cytometry: Retrospective, fundamentals and recent instrumentation. *Cytotechnology* **64**, 109–130.

Piktel, E., Pogoda, K., Roman, M., Niemirowicz, K., Tokajuk, G., Wróblewska, M., Szynaka, B., Kwiatek, W.M. *et al.*(2017) Sporicidal activity of ceragenin CSA-13 against *Bacillus subtilis*. *Sci Rep* **7**, 1–12.

Pisz, J.M., Lawrence, J.R., Schafer, A.N. and Siciliano, S.D. (2007) Differentiation of genes extracted from non-viable versus viable micro-organisms in environmental samples using ethidium monoazide bromide. *J Microbiol Methods* **71**, 312–318.

Quirós, C., Herrero, M., García, L.A. and Díaz, M. (2007) Application of flow cytometry to segregated kinetic modeling based on the physiological states of microorganisms. *Appl Environ Microbiol* **73**, 3993–4000.

Reineke, K., Ellinger, N., Berger, D., Baier, D., Mathys, A., Setlow, P. and Knorr, D. (2013) Structural analysis of high pressure treated *Bacillus subtilis* spores. *Innov Food Sci Emerg Technol* **17**, 43–53.

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.

Rodriguez, S.B. and Thornton, R.J. (2008) Use of flow cytometry with fluorescent antibodies in real-time monitoring of simultaneously inoculated alcoholic-malolactic fermentation of Chardonnay. *Lett Appl Microbiol* **46**, 38–42.

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. (1999) Evidence that inimical food-preservation barriers alter microbial resistance, cell morphology and virulence. *Trends Food Sci Technol* **10**, 261–270.

Rowan, N.J., Candlish, A.A.G., Bubert, A., Anderson, J.G., Kramer, K. and Mclauchlin, J. (2000) Virulent rough filaments of *Listeria monocytogenes* from clinical and food samples secreting wild-type levels of cell-free p60 protein. *J Clin Microbiol* **38**, 2643–2648.

Rowan, N.J., MacGregor, S.J., Anderson, J.G., Cameron, D. and Farish, O. (2001) Inactivation of *Mycobacterium paratuberculosis* by Pulsed Electric Fields. *Appl Environ Microbiol* **67**, 2833–2836.

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

Rowan, N.J., Espie, S., Harrower, J., Farrell, H., Marsili, L., Anderson, J.G. and MacGregor, S.J. (2008) Evidence of lethal and sublethal injury in food-borne bacterial pathogens exposed to high-intensity pulsed-plasma gas discharges. *Lett Appl Microbiol* **46**, 80–86.

Rowan, N.J. (2011) Defining established and emerging microbial risks in the aquatic environment: current knowledge, implications, and outlooks. *Int J Microbiol* **2011**, 1–15.

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.

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**, 316–322.

Safford, H.R. and Bischel, H.N. (2019) Flow cytometry applications in water treatment, distribution, and reuse: A review. *Water Res* **151**, 110–133.

Sahin, O., Yong, E.H., Driks, A. and Mahadevan, L. (2012) Physical basis for the adaptive flexibility of *Bacillus* spore coats. *J R Soc Interface* **9**, 3156–3160.

Suo, Y., Gu, Z. and Wei, X. (2020) Advances of in vivo flow cytometry on cancer studies. *Cytom Part A* **97**, 15–23.

Schenk, M., Raffellini, S., Guerrero, S., Blanco, G.A. and Alzamora, S.M. (2011) Inactivation of *Escherichia coli*, *Listeria innocua* and *Saccharomyces cerevisiae* by UV-C light: study of cell injury by flow cytometry. *LWT – Food Sci Technol* **44**, 191–198.

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 CO_2 plus peracetic acid. *J Appl Microbiol* **120**, 57–69.

Stiefel, P., Schmidt-Emrich, S., Maniura-Weber, K. and Ren, Q. (2015) Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. *BMC Microbiol* **15**, 36.

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.

Taimur Khan, M.M., Pyle, B.H. and Camper, A.K. (2010) Specific and rapid enumeration of viable but nonculturable and viable-culturable Gram-negative bacteria by using flow cytometry. *Appl Environ Microbiol* **76**, 5088–5096.

Tracy, B.P., Gaida, S.M. and Papoutsakis, E.T. (2008) Development and application of flow-cytometric techniques for analyzing and sorting endospore-forming clostridia. *Appl Environ Microbiol* **74**, 7497–7506.

Trevors, J.T. (2003) Fluorescent probes for bacterial cytoplasmic membrane research. *J Biochem Biophysical Meth* **57**, 87–103.

Trunet, C., Ngo, H. and Coroller, L. (2019) Quantifying permeabilization and activity recovery of *Bacillus* spores in adverse conditions for growth. *Food Microbiol* **81**, 115–120.

Tyler, A.D., Mataseje, L., Urfano, C.J., Schmidt, L., Antonation, K.S., Mulvey, M.R. and Corbett, C.R. (2018) Evaluation of Oxford Nanopore's MinION sequencing device for microbial whole genome sequencing applications. *Sci Rep*, **8**, 1–12.

Veal, D.A., Deere, D., Ferrari, B., Piper, J. and Attfield, P.V. (2000) Fluorescence staining and flow cytometry for monitoring microbial cells. *J. Immunol Methods* **243**, 191–210.

Wang, H., Bédard, E., Prévost, M., Camper, A.K., Hill, V.R. and Pruden, A. (2017) Methodological approaches for monitoring opportunistic pathogens in premise plumbing: a review. *Water Res* **117**, 68–86.

Wan-Mohtar, W.A.A.Q.I., Ab Kadir, S. and Saari, N. (2016) The morphology of *Ganoderma lucidum* mycelium in a repeated-batch fermentation for exopolysaccharide production. *Biotechnol Reports* **11**, 2–11.

Wilkinson, M.G. (2018) Flow cytometry as a potential method of measuring bacterial viability in probiotic products: a review. *Trends Food Sci. Technol* **78**, 1–10.

Wu, L., Wang, S., Song, Y., Wang, X. and Yan, X. (2015) Applications and challenges for single-bacteria analysis by flow cytometry. *Sci China Chem* **59**, 30–39.

Yaqub, S., Anderson, J.G., MacGregor, S.J. and Rowan, N.J. (2004) Use of a fluorescent viability stain to assess lethal and sublethal injury in food-borne bacteria exposed to high-intensity pulsed electric fields. *Lett Appl. Microbiol* **39**, 246–251.

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, P., Kong, L., Wang, G., Scotland, M., Ghosh, S., Setlow, B., Setlow, P. and Li, Y.Q. (2012) Analysis of the slow germination of multiple individual superdormant *Bacillus subtilis* spores using multifocus Raman microspectroscopy and differential interference contrast microscopy. *J Appl Microbiol* **112**, 526–536.

Zhang, W., Li, F. and Nie, L. (2010) Integrating multiple "omics" analysis for microbial biology: Application and methodologies. *Microbiology* **156**, 287–301.

Zhang, Y., Delbrück, A.I., Off, C.L., Benke, S. and Mathys, A. (2020) Flow cytometry combined with single cell sorting to study heterogeneous germination of bacillus spores under high pressure. *Front Microbiol* **10**, 1–13.