Inactivation of Clostridium difficile spores in the healthcare environment using hydrogen peroxide vapour

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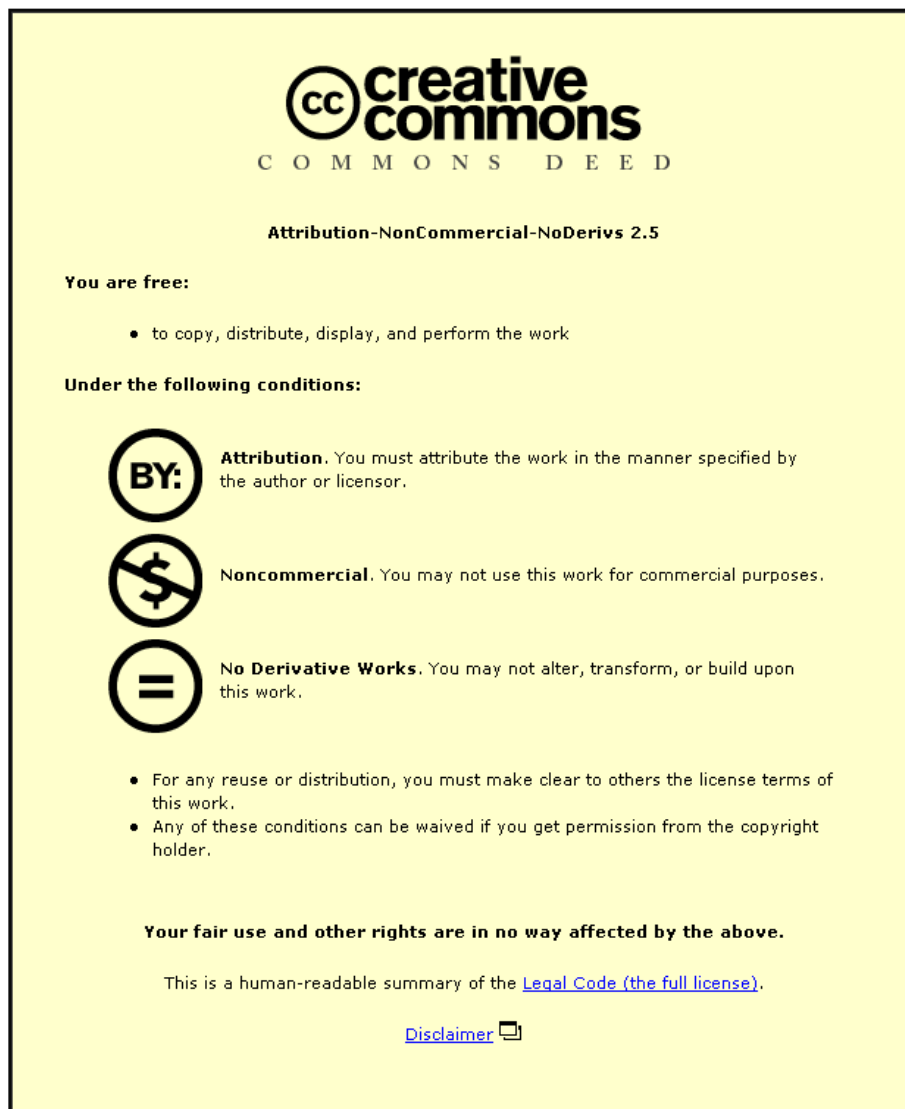
LICENCE

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Inactivation of *Clostridium difficile* spores in the Healthcare Environment using hydrogen peroxide vapour

by

Claire Marie Shaw, MEng

A Doctoral Thesis submitted in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in Chemical Engineering

Abstract

Healthcare-acquired infections (HAIs) cost the National Health Service (NHS) in England in excess of £1 billion per year. One of the main HAIs is caused by the endospore-forming bacterium *Clostridium difficile.* The most common cause of healthcare-acquired diarrhoea in the developed world, *C. difficile* was responsible for around 850 deaths in England and Wales in 2011. To help reduce the spread of the HAI-causing bacteria, terminal disinfection of isolation rooms and wards using hydrogen peroxide vapour is actively promoted. The key advantages of hydrogen peroxide vapour are its high oxidation potential which has been reported to inactivate bacteria, fungi and spores. An additional advantage of hydrogen peroxide vapour is that it is relatively environmentally friendly, breaking down into oxygen and water.

Investigation into bacterial inactivation kinetics was undertaken at controlled, steady concentrations of hydrogen peroxide vapour in the range of 10 ppm to 90 ppm. An exposure chamber was designed whereby the bacterial spores could be exposed to constant concentrations of hydrogen peroxide for various exposure times. Bacterial spores (1-log_{10} to 8-log_{10} cfu) were filter deposited onto membranes to achieve an even layer for consistent exposure of the hydrogen peroxide vapour to the spores. *Bacillus subtilis* is often used for method development in bacterial studies; advantages are it has been shown to be highly resistant to hydrogen peroxide vapour and is not a human pathogen. Following the method development, different strains of *C. difficile* (ribotypes 014, 027, 103 and 220) were exposed to identify differences in resistance. Inactivation models (Chick-Watson, Series-Event, Weibull and Baranyi) were used to fit the data generated using the environmental chamber. Decimal reduction values (*D*-values) were calculated from the models for comparative studies regarding the inactivation achieved for the different bacteria and different hydrogen peroxide concentrations.

The findings from this thesis revealed the Weibull model provides the best fit for most of the data. An initial shoulder period was identified for *B. subtilis* which was absent for *C. difficile* inactivation by hydrogen peroxide

vapour; *B. subtilis* is therefore more resistant to hydrogen peroxide disinfection than *C. difficile*. Typical *D*-values for *B. subtilis* and *C. difficile* when exposed to hydrogen peroxide vapour at a concentration of 90 ppm were 140 and 1 min, respectively. *C. difficile* inactivation data were used to develop a model to estimate the log reduction that could be achieved during an inactivation cycle based on the concentration-time integral ($\int C_p dt$). This model could be used to estimate the log reduction of commercially available hydrogen peroxide decontamination systems; these release a fixed amount of hydrogen peroxide into the room resulting in a peak concentration before decomposition to oxygen and water. Releasing the hydrogen peroxide into the room in this manner results in spatial and temporal variation; this could result in differences in bacterial inactivation in different areas within the room. Using the aforementioned regression model, the inactivation achieved at all locations within the room could be predicted, which could be used to optimise the current hydrogen peroxide decontamination cycles.

Acknowledgements

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Abbreviations

ABHR	Alcohol-based hand rubs
aHP	Aerosolized hydrogen peroxide
ASP	Advanced Sterilization Products
ATP	Adenosine triphosphate
BHI	Brain-heart Infusion
BSA	Bovine serum albumin
CB	Columbia broth
CDAD	*C. difficile*-associated disease
Cdt	Concentration/time differential
cfu	Colony forming units
CI	Confidence interval
FFA	Fastidious anaerobic agar
HMDS	Hexamethyldisilazane
HPV	Hydrogen peroxide vapour
IMS	Industrial methylated sprits
MRSA	Methicillin-resistant *Staphylococcus aureus*
NR	Not recorded
PBS	Phosphate-Buffered Saline
ppm	Parts per million
RLU	Relative light units
SEM	Scanning electron microscope
TSA	Tryptone Soya Agar
VHP	Vaporised hydrogen peroxide
w/v %	Weight/Volume percentage

Nomenclature

b	Reaction order
C	concentration
C_P	Hydrogen peroxide concentration
D	D-value
E	number of intervals
f_i	model value
h	length between the intevals
i	number of hits or event level
k	Inactivation constant
K_C	Michaelis constant
m	mass flow-rate of the liquid
m_w	mass flow-rate of the water (kg/s)
$(m_A)_{wet}$	mass flow-rate of the air (kg/s)
n	number of hits or event level
N	Number of viable organisms
N_0	Initial number of viable organisms
N_{res}	Residual population density
p	number fitted parameters
q	sample size
r^2	Coefficient of multiple determination
t	Time
V	volume
x_h	mass fraction of hydrogen peroxide in the gas phase
y_h	mass fraction of hydrogen peroxide in the liquid phase
Y_I	absolute humidity at the inlet
y_i	experimental inactivation data
Y_O	absolute humidity at the outlet
$\bar{y}$	mean value of the experimental inactivation data

Greek Letters

α	Scale parameter
β	Shape parameter

Chapter 1
Introduction

1.1 Background

Healthcare acquired infections (HAIs) are of major concern within the NHS (National Health Service), affecting over 300,000 patients and costing in excess of £1 billion per year (Davies, 2013). Most of these additional costs are associated with the increased hospital stay required for treatment. The longer stay can also increase the rate of transmission of HAIs from patient to patient (Health Protection Agency, 2005). Although some significant progress has been made in recent years to reduce the number of cases of HAIs, they still pose a significant threat and warrant further investigation (Health Protection Agency, 2012a).

Fumigation or gaseous disinfection has been used for over a hundred years to decontaminate rooms or buildings contaminated with bacteria. Formaldehyde was one of the first chemicals used from the early 1900's (Kline, 1919; Ackland, Hinton & Denmeade, 1980). However, since the classification of formaldehyde as a carcinogen in 2006 there has been an increased interest in possible alternative disinfection agents (World Health Organization, 2006).

A procedure that has attracted some interest is hydrogen peroxide decontamination for use in healthcare facilities. Three main types of hydrogen peroxide disinfection process have been developed in recent years; (i) hydrogen peroxide vapour (HPV), (ii) vaporised hydrogen peroxide (VHP) and (iii) aerosolized hydrogen peroxide (aHP). The key differences in the processes are the peak hydrogen peroxide concentration achieved and the method by which the hydrogen peroxide is delivered into the room. The peak hydrogen peroxide concentrations achieved range between 30 and 1000 ppm (Otter & Yezli, 2011; Fu, Gent & Kumar, 2012; Pottage *et al.*, 2012). As with previous decontamination cycles, such as formaldehyde fumigation, the room or ward to be decontaminated is required to be vacated by the patients and staff as hydrogen peroxide is toxic to humans at concentrations above 1 ppm (Rogers

et al., 2005). As the room needs to be vacated before starting the decontamination, hydrogen peroxide vapour can only ever be used as a supplement to standard bleach cleaning and not as a replacement.

The main issue surrounding hydrogen peroxide decontamination devices in current use is that there is very little published research in which the inactivation kinetics of different bacteria has been investigated. Where the inactivation kinetics are studied there is little or no data given about the hydrogen peroxide concentration-time profile; most studies either report the type of hydrogen peroxide vapour decontamination system used or the peak hydrogen peroxide concentration reached during the exposure (Johnston, Lawson & Otter, 2005; Rastogi *et al.*, 2009; Bentley *et al.*, 2012a). There is currently a lack of information surrounding the design rationale behind the commercial hydrogen peroxide process cycles, with previous studies utilising commercially available *Geobacillus stearothermophilus* bioindicators to validate the efficiency of such decontamination cycles (Otter & Yezli, 2012). However, the use of these bioindicators has been called into question recently with Pottage *et al.* (2012) suggesting that MRSA (Meticillin-resistant Staphylococcus aureus) may be more resistant to hydrogen peroxide than commercially available bioindicators. Therefore, it has become necessary to investigate the inactivation kinetics of the different agents of HAIs when exposed to hydrogen peroxide vapour.

1.2 Research Aims and Objectives

The overall aim of this research was to develop a method for measuring the inactivation rates of bacterial spores exposed to a constant concentration of hydrogen peroxide. Using this data, regression could be used to identify the best kinetic model, thus allowing the estimation of spore inactivation in a commercial cycle.

The specific aims and objectives for the work presented here were to:

i.   Develop apparatus and operating procedures for the exposure of microorganisms to a constant concentration of hydrogen peroxide vapour

ii. Investigate the inactivation kinetics of *Bacillus subtilis* and *Clostridium difficile* spores at steady concentrations of hydrogen peroxide vapour within the range 10 to 90 ppm.

iii. Identify relevant inactivation models previously published in literature (Chick-Watson, Series-Event, Weibull and Baranyi) and compare the fit of inactivation data of *B. subtilis* and *C. difficile* spores when exposed to a constant concentration of hydrogen peroxide vapour.

iv. Use the inactivation data generated in the study to develop a model that can be used to estimate the inactivation efficiency ($\log_{10}$ reduction of bacteria) of routinely used commercial hydrogen peroxide vapour decontamination cycles where the concentration of the hydrogen peroxide varies spatially and temporally.

v. Compare the model developed to hydrogen peroxide profiles obtained from within a room using a commercial hydrogen peroxide decontamination cycle in an attempt to predict efficiency of such cycles.

1.3 Structure of Thesis

Brief descriptions of each chapter have been summarised below:

Chapter 1: Introduction: Outlined in this chapter are the aims and objectives of the work as well as introducing the structure of the thesis.

Chapter 2: Literature Review: A review is presented of the published literature to provide background and context to the research. The chapter is broken down into four main parts. Initially why disinfection is important is investigated, with the main emphasis on the healthcare environment. *C. difficile* is subsequently introduced with information being provided on its prevalence in the environment and also the nature of *C. difficile*-associated disease and its resistance to conventional cleaning. The third section provides an overview of the different vapour phase disinfection methods that have been used. Detailing the pros and cons associated with each method. Finally, different inactivation

models are presented that can be used to model the inactivation kinetics of bacteria. The strengths and weaknesses of the models are explored, in addition to previous instances of their use.

Chapter 3: Materials and Methods: Outlines all the methods including the bacteria, media, and chemicals utilised in this thesis. Provided are details of how the hydrogen peroxide sensors were calibrated and their response characteristics to ensure that the spores were exposed to the same hydrogen peroxide vapour concentrations during the experiment. The environmental chamber used to expose the spores is described in detail including the commissioning and operating procedures. The method for the production of the spore preparations and the method by which they were exposed inside the chamber are also set out.

Chapter 4: Exposure of *Bacillus subtilis* spores to Hydrogen Peroxide Vapour: The results of experiments in which spores of *B. subtilis* were exposed to hydrogen peroxide are presented in this chapter. Detailed are the inactivation kinetics of *B. subtilis* obtained when exposed to different concentrations of hydrogen peroxide vapour. Using the experimental data inactivation rate constants were calculated using a variety of inactivation models. The inactivation data evaluated in this study for low hydrogen peroxide vapour concentrations (10 ppm to 90 ppm) was then compared to inactivation data for high hydrogen peroxide vapour concentrations from literature (275 ppm to 3879 ppm) using the same inactivation models.

Chapter 5: Exposure of *Clostridium difficile* spores to Hydrogen Peroxide Vapour: In this chapter, the results of the hydrogen peroxide inactivation of spores of *C. difficile* are presented. Demonstrated is the effect that the spore production method and the *C. difficile* strain have on the inactivation kinetics. Inactivation models were used to explore the time taken to achieve different log reductions; these results were then used to identify a model that uses the integral of the concentration-time data ($\int C_P dt$) to estimate the achieved log reduction.

Chapter 6: Spore Inactivation compared to Hydrogen Peroxide Cycle Data: This chapter validates the concentration-time integral inactivation model developed in the previous chapter by comparison to hydrogen peroxide vapour concentration histories from commercially available hydrogen peroxide decontamination systems. It demonstrates that it is possible to predict the inactivation likely to be achieved within a room or ward providing the hydrogen peroxide history is provided.

Chapter 7: Conclusions and Further Work: This chapter presents the main conclusions arising from all the work presented within the thesis. It also outlines proposals for future work.

Chapter 2
Literature Review

2.1 Introduction

Decontamination of the healthcare environment using vaporised hydrogen peroxide as the disinfection agent is receiving increased attention in the scientific literature as well as by national health protection agencies e.g. Department of Health (United Kingdom). There are a number of reasons that hydrogen peroxide has been investigated as the disinfectant of choice, which include:

- broad spectrum disinfection efficacy, with literature showing its efficacy against bacterial cell and spores, fungi, viruses, prions and protozoa and their cysts (Rij & Forney, 1995; Rogers *et al.*, 2005; Fichet *et al.*, 2007; Hall *et al.*, 2008; Coulon *et al.*, 2010; Pottage *et al.*, 2010)
- ease of application, as the hydrogen peroxide is delivered into the room via a predetermined cycle (Andersen *et al.*, 2006; Hall *et al.*, 2007)
- naturally decomposes to benign by-products oxygen and water (Johnston, Lawson & Otter, 2005)
- as it is a vapour it is able to diffuse and penetrate difficult-to-clean areas, e.g. behind radiators, under beds, grills etc. (Unger-Bimczok *et al.*, 2011)
- it has the best safety profile of all the gaseous decontamination methods available (McDonnell, Grignol & Antioga, 2002; Fu, Gent & Kumar, 2012)

This chapter covers the following areas: rates of nosocomial infections around the world (with emphasis on the UK) due to increased antibiotic resistance of microorganisms; the persistence of bacteria within the healthcare environment and how they are transmitted; the role of cleaning and hand hygiene in reducing environmental contamination and spread of HAIs

(healthcare-acquired infections); lapses in routine cleaning and the need for non-operator/automated disinfection systems for decontamination of large spaces e.g. isolation rooms, wards etc. and the types of disinfectants available; literature on the use of hydrogen peroxide vapour decontamination systems used in healthcare and other related fields; review of inactivation models to evaluate hydrogen peroxide inactivation kinetics.

2.2 Healthcare-Acquired Infections

A HAI is defined as an infection that arises as a result of healthcare intervention, with a patient not having tested positive for HAI-causing bacteria within the first three days of healthcare admission (Bengualid *et al*., 2011). The infections can arise in either the patient receiving care, or the healthcare workers administering the care (Health Protection Agency, 2005). There are many different types of HAI including those caused by methicillin-resistant *Staphylococcus aureus* (MRSA), *Clostridium difficile* (*C. difficile*), vancomycin-resistant Enterococci (VRE) and *Acinetobacter baumannii.* It has been estimated that 8 % to 12 % of hospital patients contract a HAI (Emmerson *et al*., 1995; Smyth *et al*., 2008). In order to try and control the spread of HAIs the number of infections are monitored, with one of the first studies into the prevalence of HAIs being carried out in 1980 (Dancer, 2008). Two of the most common HAIs that are investigated and monitored by the UK Health Protection Agency are MRSA and *C. difficile*. The latter is the leading cause of healthcare-acquired diarrhoea. *C. difficile* is a Gram positive, spore-forming obligately anaerobe; in its spore form it can survive harsh environments, high temperatures and common disinfection techniques (Heinlen & Ballard, 2010). MRSA, a non-spores-forming bacterium, is methicillin-resistant strain of *Staphylococcus aureus* and is responsible for many difficult-to-treat infections. It can infect open wounds and cause sepsis in patients, which can result in death (Gould, 2007).

2.2.1 Infection Rates

HAIs have become a major concern for hospital patients in recent years. The infections can often be complicated by antimicrobial and antibiotic

resistance and has been highly prioritised within the EU over the past few years (The Council of the European Union, 2009). It has been estimated HAIs cost the National Health Service (NHS) nearly £1 billion per year (Plowman *et al*., 2001; The Council of the European Union, 2009). The additional cost is mainly due to the extended stay in the hospital, reportedly up to two and a half times longer than uninfected patients, and the additional care required during this stay (Plowman *et al*., 2001). The number of people affected by HAIs each year is published by the Health Protection Agency and the Office for National Statistics. The reported numbers of *C. difficile* or MRSA -associated infection within England and Wales during hospitalisation are shown in Figure 2.1.
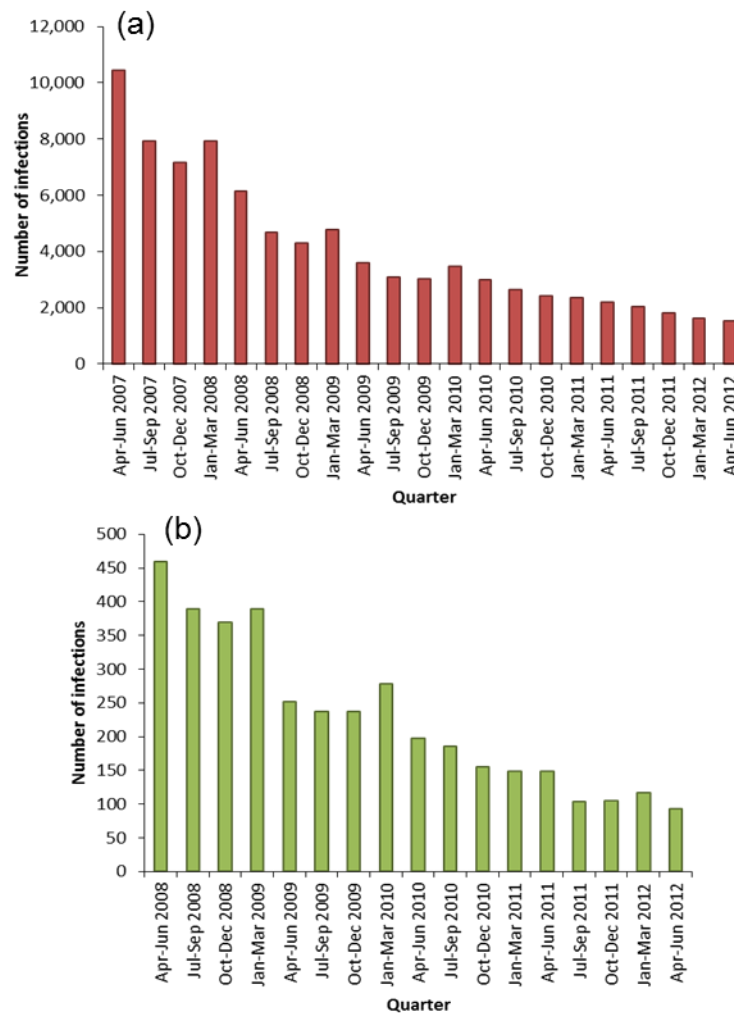
Figure 2.1 The number of trust apportioned cases of (a) *C. difficile* and (b) MRSA in England and Wales between 2008 and 2012.
Data obtained for the Office for National Statistics; MRSA results for 2008-2009 (Health Protection Agency, 2010b); *C. difficile* results for 2008-2009 (Health Protection Agency, 2010a) and *C. difficile* and MRSA results for 2010-2012 (Health Protection Agency, 2012a).

It can be inferred that there are over ten times more cases of healthcare-acquired fatalities caused by *C. difficile* than MRSA. Figure 2.1 also demonstrates there is a downward trend in both the number of MRSA and *C. difficile*-associated infections, which can be attributed to the increased awareness and initiatives established and implemented, such as encouraging hand washing. Although decreasing the number of infections still remain unacceptably high so further investigation and initiatives are still required. There is also a seasonal trend in the number of cases of *C. difficile*-associated infection, with a slight increase in the winter months, as observed by Polgreen *et al*. (2010).

Figures for the number of people that contract a *C. difficile* infection reveal that around 12 % of cases are fatal; *C. difficile* infection is reportedly the underlying cause of death in about half of cases as shown in Figure 2.2a. This compares to around 40 % of fatalities following contracting MRSA, with MRSA being named as the underlying cause of death in only a third of cases, as shown in Figure 2.2b. Plowman *et al*. (1999) estimated the additional cost that could be contributed to a patient contracting a HAI was around £3,200. However, a more recent study by the NHS found that the additional cost for a patient who contracts a *C. difficile* infection is closer to £7,000 (Robotham & Wilcox, 2012).
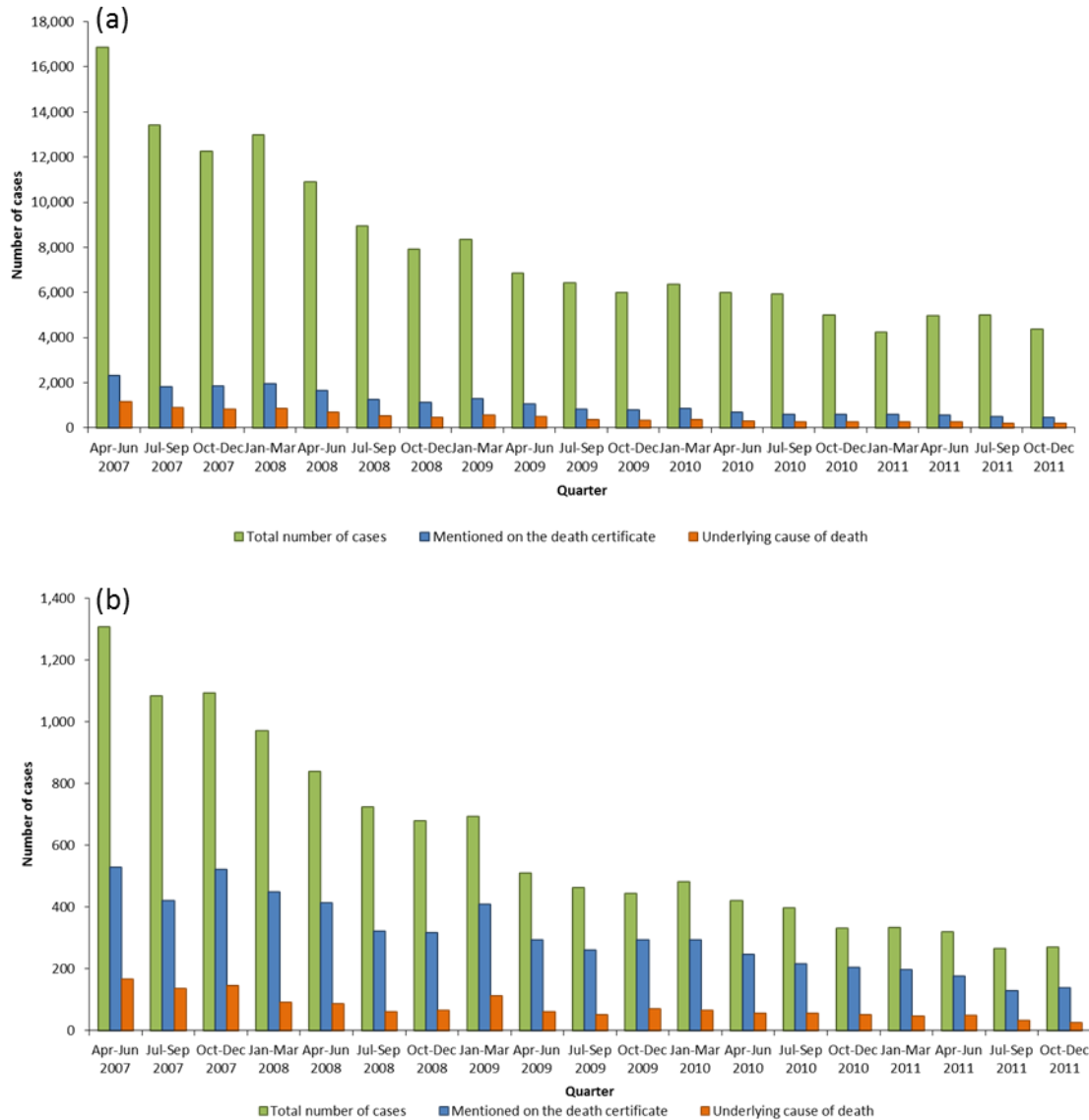
Figure 2.2 The total cases of (a) *C. difficile* (b) MRSA, with the number of deaths either when mentioned on the death certificate or as the underlying cause of death for England and Wales between 2007 and 2011.
Data obtained from the Office of National Statistics (Office for National Statistics, 2012a).

2.2.2 *Clostridium difficile in the Healthcare Environment*

Based on the above findings, it has been demonstrated that *C. difficile* is the most prevalent cause of HAIs in the UK, and therefore it will be the main focus of this work. *C. difficile* is a spore-forming anaerobic bacterium, found in the gut of 1 to 3 % of healthy adults (Heinlen & Ballard, 2010). The extent of colonisation increases to between 15 and 25 % in hospitalised antibiotic-treated or debilitated patients (Yassin *et al*., 2001; Jenkins, 2004). *C. difficile-*

associated infection is synonymous with antibiotic-associated diarrhoea and colitis and is recognised as the main cause of hospital-acquired diarrhoea in the developed world (Cleary, 1998; Wilcox, 2003). The normal gut flora in a healthy individual supresses the growth of *C. difficile*, however, the flora is reduced with the use of antibiotics, resulting in overgrowth of *C. difficile* (Cleary, 1998). Patients with *C. difficile*-associated infection can suffer from a variety of gastrointestinal infections, ranging from a mild infection, where discontinuing the use of antibiotics and keeping the patient hydrated can result in a cure, to much more severe pseudomembranous colitis, which can lead to toxic megacolon, colonic perforation and even death (Gerding *et al.*, 1995; Yassin *et al.*, 2001).

C. difficile is an anaerobic spore-forming bacterium, which in its spore form can persist in the healthcare environment for up to five months (Kim *et al.*, 1981; Yassin *et al.*, 2001). A spore is a dormant form of the bacteria, which helps protect the bacterium from the aerobic environment and chemical disinfectants (Leggett *et al.*, 2012). There are seven stages in the transition from vegetative cell to spore during which a thick dense coat is formed to protect DNA in the core of the spore (Leggett *et al.*, 2012). The condition under which the spores are formed also play a large role in how resistant the final spore will be. Melly *et al.* (2002) demonstrated that spores of *B. subtilis* produced at higher temperatures had lower water content within their core, making them more resistant to wet heat. This could be important as spores produced within the laboratory could show significantly difference in resistance to those produced within the environment; it also suggests that the atmospheric humidity may affect the disinfection kinetic rates.

There are many different strains of *C. difficile* with some being more prevalent in the hospital environment than others (Mutlu, *et al.* 2007; Health Protection Agency, 2012b; Patel, 2012). Research has shown that, although in decline, ribotype 027 is the most prevalent, accounting for around 15 % of all cases of *C. difficile*-associated infection (Ananthakrishnan, 2011). However, since it has started to decline other ribotypes such as 106 and 001 are being isolated with increasing frequency. The Health Protection Agency monitors the different strains throughout the NHS to monitor the spread and proportions of

the different ribotypes (Health Protection Agency, 2012b). Even though the number of cases of *C. difficile* strain 027 has started to decline it is still the most prevalent with double the number of cases of the second most common strain (106) (Health Protection Agency, 2012b). Ribotype 027 will be used as a model strain throughout this study as it is well-studied. Its increased pathogenicity and prevalence means it is a strain of interest making it a good model strain for study (Ananthakrishnan, 2011; Doan *et al.*, 2012; Patel, 2012).

2.2.3 Spread of Clostridium difficile in the Healthcare Environment

There are two main methods by which *C. difficile* is spread. The first is via the air as patients can suffer from explosive diarrhoea; it has been estimated that a gram of faeces from an infected patient can contain up to 5-$\log_{10}$ *C. difficile* spores. The second spreading mechanism is by hospital personnel who can transfer the spores from patient to patient with its presence on their hands, jewellery or medical devices, such as stethoscopes (Gerding *et al.*, 1995; Cleary, 1998; Wilcox, 2003). It has previously been shown that *C. difficile* spores can be recovered on 34 to 58 % of sites in hospital wards, which typically include bed frames, commodes and toilets; floors are the most commonly contaminated sites (Wilcox, 2003). Spores on contaminated sites are able to survive up to five months when the area is not properly disinfected (Kim *et al.*, 1981; Yassin *et al.*, 2001).

2.2.3.1 The effect of hand hygiene on the spread of HAIs

It has been widely accepted that the most common cause of the spread of HAIs is poor hand hygiene. Curtis & Cairncross (2003) demonstrated that increased hand hygiene can be linked to a decrease in the number of cases of diarrhoea. Kendall *et al.* (2012) advocate findings regarding hand hygiene by Sax *et al.* (2007), who identified the five key moments when healthcare staff should wash their hands as 1) before touching a patient, 2) before clean/aseptic procedures, 3) after body fluid exposure risk, 4) after touching a patient, and 5) after touching a patient's surroundings. Using the five key moments as a starting point, Steed *et al.* (2011) identified fifteen instances per hour for staff to wash their hands. As each hand wash should take a minimum of 15 s, in the case of 100 % compliance each healthcare worker would spend around 3 min

45 s each hour washing their hands (Boyce & Pittet, 2002). However, it has been shown that hand hygiene compliance is between 40 and 50 % and the actual time staff spend washing their hands is between 1.5 and 2 min per hour (Visscher & Randall Wickett, 2012).

Kaier *et al.* (2012) demonstrated that the spread of HAIs is increased when bed occupancy is at its highest. The study suggest that the main reason for this is that when healthcare staff are overworked the compliance with hand hygiene levels decreases. Another contributing factor to less frequent hand washing is that people are more likely to wash their hands when they are visibly soiled. For example hands are generally not visibly soiled following the taking of a pulse, yet it is still imperative for hands to be washed, although field observations have shown this not to be the case (Allegranzi & Pittet, 2009).

An increase in hand hygiene compliance has been seen since the introduction of alcohol-based hand rubs (ABHR) (Traore *et al.*, 2007). Although shown to control the spread of MRSA and VRE (non-spore-forming bacteria), there is some concern that the ABHR may not be effective against spore-forming bacteria. In fact, it has been demonstrated that the use of ABHR may increase the spread of *C. difficile* rather than aiding in its control (Oughton *et al.*, 2009; Ellingson & McDonald, 2010; Jabbar *et al.*, 2010). This is partly due to the lack of mechanical friction present when washing hands with water which helps physically remove spores, which does not occur with ABHR (Allegranzi & Pittet, 2009; The Research Committee of the Society of Healthcare Epidemiology of America, 2010) .

Most HAIs are spread via healthcare workers, a continued study of hand hygiene is important to reduce the spread. However, it has been discovered that ABHR used to disinfect the hands of healthcare staff are ineffective against *C. difficile*. These products are recommended for use after touching a patient or their surroundings if hands are not visibly soiled (Boyce & Pittet, 2002). Hand washing is also less likely after touching the environment as it is deemed to be clean, however *C. difficile* has been identified in many different locations including computer keyboards (Dancer, 2009; Wilson *et al.*, 2011). Therefore

enhanced cleaning methods are required alongside hand hygiene to inactivate or remove spores of *C. difficile* (and other agents of HAIs) from the environment.

2.2.3.2 Transmission of *C. difficile* through the environment

Walker *et al.* (2012) found that only between 15 and 25 % of *C. difficile*-associated infection within the healthcare setting could be identified as patient-to-patient transmission or transmission through the environment. However, the method transmission of the remaining 75 to 85 % of cases was identified, it was suggested that these cases were likely to be transferred by patient and relatives or through products brought into the hospital such as food. The study also showed that although transmission could be seen up to 8 weeks after the first onset of symptoms in a patient within the ward the majority of the transmissions would occur within the first week. Fawley & Wilcox (2001) also found that there was a link between the *C. difficile* strains found in the environment and infected patients. The study was carried out in two hospital wards over a 22-month period and found that a single endemic strain was responsible for 92.5 % of clinical isolate and 92 % of environmental isolates, suggesting that a single strain may be spread around the ward through patient or staff-to-patient transmission or through the environment.

As previously stated one of the main ways in which *C. difficile* can enter the environment is via a patient with explosive diarrhoea (Wilcox, 2003). Spores of *C. difficile* are approximately 2 μm in diameter and can remain airborne for up to 4 h with 53 to 426 cfu/m^3 being found in the air of an infected ward (Roberts *et al.*, 2008). The spores can then settle on to surfaces within the room and spread from patient to patient if the facilities are shared. The spores can also settle onto the bed sheets of patients or contaminate them during an episode of diarrhoea; these spores can then be disturbed and aerosolised again (Roberts *et al.*, 2008). In a study conducted by Best *et al.* (2012) it was shown that *C. difficile* spores were aerosolised when a toilet was flushed with an open lid. The risk of this is increased as most hospital toilets are without lids as they are considered a source of bacteria. There is therefore an increased probability of widespread spore contamination in an environment with an infected patient, even following their relocation or discharge.

2.2.4 Survival of Clostridium difficile in the Healthcare Environment

C. difficile is able to survive on hospital surfaces for an extended time period because of its ability to produce spores, which are hardy and difficult to eradicate with simple cleaning techniques (Kramer *et al.*, 2006; Fawley *et al.*, 2007). Spores on contaminated sites have been shown to survive for up to five months when the area is not properly disinfected (Yassin *et al.*, 2001). Unsurprisingly, the *C. difficile* strains considered to be the most prevalent are those capable of producing the greatest number of spores (Vohra & Poxton, 2011). The ability of *C. difficile* to produce spores has also been shown to increase upon exposure to non-chlorine-based cleaning agents (Wilcox & Fawley, 2000). No cleaning agent has been found to date that can inactivate *C. difficile* spores in less than a minute without possessing high toxicity and damage to the surface being disinfected (Leggett *et al.*, 2012). This means that *C. difficile* is able to survive for longer in the hospital environment than non-spore-forming bacteria such as MRSA (Neely & Maley, 2000).

Surveillance of *C. difficile* is recommended in the healthcare setting as it enables an increase in the cases to be detected at an early stage, as well as allowing the identification of any risk-factors (Vonberg *et al.*, 2008). Following surveillance it has been found that patients on wards that have housed patients suffering from a *C. difficile*-associated infection are at a greater risk of contracting the infection (Gerding, Muto & Owens, 2008). Chang & Nelson (2000) found an increased risk of 12 % of contracting a *C. difficile*-associated infection if an individual were in a ward that has previously been occupied by someone with the infection. One method employed by healthcare providers to minimise the spread of *C. difficile* is to move infected patients to single occupancy rooms if possible; in cases where this is not possible, patients suffering from the infection should be cohorted and treated by designated staff to avoid further transfer of the infection (Gerding, Muto & Owens, 2008; Vonberg *et al.*, 2008).

2.3 Current Cleaning Methods and Their Effectiveness

It is well established that cleaning of hospital wards is important when tackling HAIs, especially since the public often associate an unclean hospital ward with an increased risk of contracting such an infection (Dancer, 1999). Most hospitals use bleach as the disinfecting agent at concentrations greater than 1000 ppm as detailed in Table 2.1.

Dubberke *et al*. (2007) compared the number of surfaces contaminated with *C. difficile* in room housing both *C. difficile*- positive and negative patients. Of the rooms six rooms sampled housing *C. difficile*-positive 100 % were found to have at least one positive sample compared to 33 % (4/12) of rooms housing *C. difficile*-negative patients, the results from this study are summarised in Table 2.1. In another study by Eckstein *et al*. (2007) pre-moistened swabs were used to identify contaminated areas in nine private rooms containing *C. difficile*-positive patients before and after a bleach clean. They found that before disinfection all nine rooms contained at least one site positive for *C. difficile* and even after the disinfection seven out of the nine rooms remained positive. This failure of bleach to disinfect a room entirely was also evident when the number of patients contracting HAIs is considered, a patient in a room where the prior occupant had a HAI, has an increased risk of 73 % of contracting the same HAI (Carling & Bartley, 2010). A summary of the literature on the distribution of contaminated hospital surfaces and the sampling method used is presented in Table 2.1.

Table 2.1 Effectiveness of disinfection agents against *C. difficile* contamination of hospital surfaces.

C. difficile contaminated sites or cases before decontamination	Location within healthcare establishment	Environmental sites sampled	Sampling method	Disinfectant used	*C. difficile* contaminated sites or cases after decontamination	Study
30/54 (56 %) with 100 % (9/9) of rooms with at least one positive sample	9 private rooms of *C. difficile*-positive patients	Bedrail, bedside table, phone, call button, toilet and door handle	Pre-moistened cotton-tipped swabs	Freshly-prepared Sunstorm bleach	24/54 (44 %) with 7/9 (78 %) of the rooms with at least one positive sample	Eckstein *et al.*, 2007
13/48 (27 %) with 6/6 (*C. difficile*-positive) and 4/12 (*C. difficile*-negative)	18 private rooms; 6 contained *C. difficile*-positive patients	One sample from a "clean" surface (bedrail, bedside table, phone or call button) and one from a "dirty" surface (toilet or commode and surrounding floor)	Cellulose sponges pre-moistened with DE neutralising buffer	***	***	Dubberke *et al.*, 2007
9/120 (7.5 %)	8 different wards including: general, surgery and elderly care	Bedrails, call buttons, telephone and television control buttons, bathrooms and toilets	Polywipe cellulose sponges pre-moistened in neutralizing buffer	Chlorine dioxide-containing solution	17/212 (8 %)	Goldenberg *et al.* 2012
46/194 (24 %)	2 university hospitals	Toilet seat, bathroom sink, and floor, bedside and care tables, phone, door handle, chair, floor and bedrail	Swabs pre-moistened with Schaedler broth	Cloth soaked in 0.5% sodium hypochlorite solution (5,000 ppm available chlorine)	23/194 (12 %)	Barbut *et al.* 2009

C. difficile contaminated sites or cases before decontamination	Location within healthcare establishment	Environmental sites sampled	Sampling method	Disinfectant used	*C. difficile* contaminated sites or cases after decontamination	Study
11/78 (14 %)	One ward containing 7 patients	Floors, walls, windows, bathroom environments, bed frames, and doors	Rodac plates with cycloserine-cefoxitin fructose agar with 0.1 % sodium taurocholate	Hypochlorite solutions (1,600 ppm available chlorine)	1/78 (1.3 %)	Kaatz *et al*. 1988
0.85/1000 patient days	*C. difficile*-positive isolates from 3 different hospitals	***	*C. difficile*-positive faecal specimens 48 h after admission	Household bleach diluted to 5000 ppm hypochlorite	0.45/1000 patient days	Hacek *et al*. 2010
Overall contaminated sites 395/1128 (35 %) and 8.9/100 admissions	Elderly care ward	Floors, radiators, bedframes, toilet floors and sluice floors	Sterile cotton wool swabs moistened with 0.25% Ringer's solution; positive faecal samples diagnosed on request	Hypochlorite	5.3/100 admissions	Wilcox *et al*. 2003
8.6/1000 patient days	Bone marrow transplantation unit	***	*C. difficile*-positive faecal specimens 48 h after admission	1:10 hypochlorite solution	3.3/1000 patient days	Mayfield *et al*. 2000

2.3.1 Contamination Levels of High Touch Areas

So-called high touch areas are those most likely to be contaminated and therefore most commonly studied. These are likely to be areas close to the patient, such as the curtains, bed rails, over-bed table, locker and the nurses' call button, as these are the most frequently touched sites by both the patients and staff. These sites are frequently sources of contamination for example, in daily routine cleaning bed rails are often omitted as they are time consuming to clean and the over-bed tables can be missed as they contain personal items which cleaning staff are not permitted to touch or move (Dancer *et al*., 2009; Dumigan *et al*., 2010).

The number of high touch areas contaminated within the healthcare environment has been shown to shown to vary considerably depending on the type of patient being treated. Eckstein *et al*. (2007) found 30/54 to be positive for *C. difficile* when a room containing patients positive for *C. difficile* was sampled, compared to 11/78 and 9/120 positive when general wards were sampled by Kaatz *et al*. (1988) and Goldenberg *et al*. (2012) respectively (results presented in Table 2.1). This unsurprising result confirms that when a patient is positive for *C. difficile* the room is far more likely to be contaminated.

2.3.2 Efficacy of Hospital Cleaning Agents

Chlorine-releasing cleaning products such as hypochlorites, for example bleach, have been shown to be an effective sporicidal agent (Foegeding & Busta, 1983; Mayfield *et al*., 2000; Wilcox & Fawley, 2000). They are currently the most commonly used agents in the hospital environment following a recommendation by the Department of Health (2009). A study by Goldenberg *et al*. (2012) compared *C. difficile* survival when using a "standard cleaning" method and a chlorine dioxide-based cleaning regimen. The results of an environmental cleaning audit, an NHS-approved computerized audit tool for measuring and monitoring a range of cleaning standards, showed no statistical difference between the two techniques, with a score of 96.6 % for the "standard clean" and 97.1 % for the chlorine dioxide-based cleaning. However, sampling was conducted randomly and therefore the times between cleaning and

sampling could differ, which allowed time for recontamination (Patel, 2012). It was not disclosed in the study what "standard cleaning", was taken to consist of making it impossible to compare the results to others within literature.

A number of *in vitro* studies have also been carried out to investigate the sporicidal effect of chlorine-based cleaning agents. Speight *et al.* (2011) showed there are huge discrepancies in the bacterial inactivation that can be achieved by different disinfectants, whether used as per the manufacturers' instructions or at higher concentrations. The results suggests that chlorine dioxide is generally the most effective disinfectant although it was not always effective at low concentrations or for low contact times. However, only the overall concentration of the disinfectant is given, meaning that the concentration of the active ingredient cannot be calculated, making it impossible to identify the most efficient one. In a study by Maillard (2011), it was shown that the concentration of sodium hypochlorite bleach can have a substantial effect on its efficiency with only a 2.2-$\log_{10}$ reduction of *B. subtilis* after 30 min exposure to 500 ppm compared to a ≥6-$\log_{10}$ reduction after 10 min exposure to 5,000 ppm. Further *in situ* investigations were conducted by Mayfield *et al.* (2000) who compared a 1:10 hypochlorite solution (1 part hypochlorite to 9 parts water) to the standard ammonium solution, reporting a reduction from 8.6 to 3.3 cases per 1,000 patient-days when using the hypochlorite solution. However, there can are disadvantages with the effectiveness of any chlorine-based disinfectants as there is a specific minimum exposure times of several minutes required in order to induce disinfection and reducing this would therefore reduce their efficacy (Fraise, 2010; Maillard, 2011).

2.3.3 Methods for Monitoring Cleaning Efficiency

Cleaning within hospitals is routinely judged by a visual audit rather than a more scientific approach. This method of monitoring is not robust enough as the bacteria causing these infections are not visible to the naked eye (Dancer, 2009). More Objective methods currently being tried include the use of fluorescent gels and ATP (Adenosine triphosphate) swabs (Carling & Bartley, 2010). These offer very different approaches with the fluorescent gels being used during cleaning to identify areas that are likely to be missed. In contrast

ATP swabs are used to take a sample from an area which can then be tested using a portable handheld luminometer (Carling *et al.*, 2006; Carling & Bartley, 2010).

2.3.3.1 Fluorescent gels

Fluorescent gels are used to identify the areas with a healthcare environment that are frequently missed during terminal cleaning (after patient death or discharge). For this a gel is spread onto the high touch areas previous discussed; the gel used is non-toxic and can remain on a surface for several weeks unless removed using a liquid detergent and minimum abrasion. The gel is invisible on the surface under normal conditions but fluoresces brightly under ultraviolet light. Therefore, any surfaces that fluoresces after cleaning have been missed during a standard clean (Carling *et al.*, 2006, Carling *et al.*, 2008; Goodman *et al.*, 2008). All of the studies identified that the most likely area to be missed included the toilet and main doors and the light switch, whereas the areas which received most attention were the bedside table and tray and the sink. Both Carling *et al.* (2008) and Goodman *et al.* (2008) used a pre-intervention step where the cleaning staff were unaware that the fluorescent gel was been used before an intervention step where the cleaning staff received training on the areas highlighted as the most like to be missed during the pre-intervention step. As a result, the percentage of sites which were effectively cleaned increased from 22 to 66 % (door knob) and 5 to 36 % (light switch) for the Carling *et al.* (2008) and Goodman *et al.* (2008) studies respectively.

2.3.3.2 ATP swabs

Adenosine triphosphate (ATP) bioluminescence swabs have been used for 30 years as a rapid (15 min) test to quantify the amount of microbial and non-microbial material on a surface, by applying a specialized swab to sample an area and analysing using a handheld luminometer (Thore, Lundin & Anséhn, 1983). The amount of ATP is quantified by the luminometer and expressed as relative light units (RLU) (Carling & Bartley, 2010). ATP swabs can be used to identify well cleaned surfaces with low levels of organic contamination or readings of 250-300 RLU, as well as highly contaminated surfaced with a high

loading of organic material present which yield readings of greater than 1000 RLU (Boyce *et al.*, 2009).

There are two main issues with the use of ATP (i) the system cannot differentiate between viable bacteria and organic debris and (ii) ATP cannot be used in areas cleaned using bleach as it can quench the bioluminescence reaction (Boyce *et al.*, 2009; Carling & Bartley, 2010). This is a severe limitation as bleach is the most common cleaning agent used within hospital wards due to it being cheap and well understood as it has been used for many years. Griffith *et al.* (2000) claimed that only 33 % of the ATP reading accounted for viable bacteria with the rest being debris which can include dead bacteria. This is confirmed by studies which compare culture-based methods to the ATP measurement, which shows a 70 % agreement in the sites that pass or fail after cleaning. Of the remaining 30 % that demonstrate a variance between the ATP measurement and the microbiological count most demonstrate a high ATP reading, which could be caused by presence of food residue (Shama & Malik, 2013). A false positive result being the most common failure is good as although it may result in unnecessary cleaning it does not compromise safety, whereas a false negative would imply the room was clean when in reality it required extra cleaning.

2.4 Vapour Phase Disinfectants

Vapour phase or gaseous decontamination is defined as the process whereby a vapour or gaseous form of a chemical disinfectant is generated to decontaminate a specific area or room (Davies *et al.*, 2011). In gaseous decontamination, the room is generally sealed during the procedure as at the concentration required for the disinfection process, the agents used are toxic. The main advantage is that the chemical agent can reach all surfaces, some of which may be missed or hard to reach with manual cleaning. Nevertheless, with all gaseous cleaning methods it has been found that manual cleaning is needed at least before the decontamination cycle to remove any surface dirt or biological matter that could reduce disinfection efficacy (Otter *et al.*, 2009). Some of the chemicals used may also require a further clean following the cycle as they can leave residues on surfaces (Johnston, Lawson & Otter, 2005).

2.4.1 Formaldehyde

Formaldehyde has been used for about one hundred years since the discovery of the antiseptic and disinfecting properties of formalin (Burrage, 1897; Kline, 1919; Meszaros *et al.*, 2005). Ackland *et al.* (1980) produced one of the most recent papers investigating the decontamination of areas using formaldehyde. The work emphasises that all the doors and windows require sealing before the disinfection process could be carried out. For the process, a formaldehyde concentration of approximately 1,100 ppm to 1,500 ppm was released over a 10 h period. The conditions within the room were also important with the temperature needing to be maintained at around 20 °C. After the exposure, a ventilation period of around 8 h was required for the room to drop below the accepted level for formaldehyde (1 ppm). There was also the possibility of paraformaldehyde condensing on the surfaces within the room, and therefore it was important for the room to be cleaned after the decontamination process. This study shows that the time required for inactivation using formaldehyde is very long (around 10 h), the room needs to be vacated by patients and staff and sealed for the process making it an impractical procedure.

There are also significant hazards with the use of formaldehyde: it was identified a possible carcinogen in the 1980s and finally classified as a carcinogen in 2006 (World Health Organization, 2006; Salthammer, Mentese & Marutzky, 2010). Further issues with the use of formaldehyde are given by Chenier (2003), who stated that it forms a colourless gas with a pungent irritating odour that can also be highly flammable and form explosive mixtures in air if not carefully monitored. Therefore, a modern alternative to the use of formaldehyde is required that has short cycle times, high disinfection properties and is environmentally-friendly.

2.4.2 Chlorine Dioxide

Chlorine dioxide was recognised as an effective disinfectant in the 1900s (Knapp & Battisti 2000). However, it is only in the last 10 years that its use as a gaseous disinfectant has attracted significant attention (Jin *et al.*, 2009). The

interest in chlorine dioxide as a gaseous disinfectant is due to the fact it has a relatively low toxicity to humans as well as having good, broad spectrum biocidal and sporicidal properties (Jeng & Woodworth, 1990; Wintner, Contino & Neill, 2005). It also has lower oxidation properties than other gaseous disinfectants, such as hydrogen peroxide and ozone and is therefore less likely to corrode the commonly used materials of construction. This makes it easier to use in a hospital environment (Jeng & Woodworth, 1990).

Chlorine dioxide has to be produced on site due to its explosive nature which makes it hard to store and transport (Jin *et al.* 2009). It is most commonly produced by passing a 2 % chlorine-nitrogen gas mixture over sodium chlorite granules, producing a yellow-green gas that has a similar odour to chlorine (Knapp & Battisti 2000; Jin *et al.* 2009; Davies *et al.* 2011). Before decontamination can be conducted with this gas, a humidification stage is required to reach a 70 % relative humidity, as an aid to disinfection. After decontamination, the chlorine dioxide needs removing, which is most commonly achieved by breaking down the gas using UV (ultraviolet) light (Jeng & Woodworth 1990; Knapp & Battisti 2000; Wintner *et al.* 2005; Davies *et al.* 2011).

Jeng & Woodworth (1990) have shown that even at relatively low concentrations of 7 ppm, a 6-$\log_{10}$ reduction of *B. subtilis* can be achieved after 120 min. However, this time is reduced to 15 min when the chlorine dioxide concentration is increased to 30 ppm. Rastogi *et al.* (2009) investigated the inactivation of *Bacillus anthracis* dried on six different building materials: carpet, ceiling tile, cinder tile, steel I-beam, wallboard and wood. There researchers found that after a 3 h exposure to 3,600 ppm chlorine dioxide gas there was a significant difference in the log reduction achieved varying between 2.5 for wood and 6.6 for the ceiling tiles, with an initial concentration of 6-$\log_{10}$ spores per bioindicator. However, when the initial spore concentration was increased to 8-$\log_{10}$ spores per bioindicator, the reduction achieved was greatly reduced to 1.2 for wood and 3.9 for ceiling tiles. This may be related to the fact that the spores were deposited onto the surfaces by pipette and as the liquid evaporated spores may have become stacked upon one another (Bayliss *et al.*, 2012)

As with all decontamination methods there are a number of disadvantages. A particular disadvantage with chlorine dioxide gas is that it is explosive in concentrations above 10 % (Jin *et al.*, 2009). Another major disadvantage is that a by-product of the gas production is chlorine gas, which means the production needs to be heavily monitored to ensure that there is no accidental exposure. The chlorine dioxide gas can also penetrate certain plastics, including the polyvinyl used in medical device containers. Its high solubility in water and the fact that it breaks down when exposed to UV light could reduce its efficiency (Davies *et al.*, 2011).

2.4.3 Hydrogen Peroxide

Hydrogen peroxide disinfection is considered to be a low toxicity decontamination process as it ultimately breaks down into oxygen and water (Kahnert *et al.*, 2005). However, due to its mode of action, it will not only react with oxidisable organic matter, such as faeces, but also with all surfaces and materials. This can reduce the efficiency of the decontamination process because if it reacts with a catalase-containing substance, such as blood, the hydrogen peroxide can be degraded (Pottage *et al.*, 2010; Davies *et al.*, 2011). As with all of the gaseous decontamination systems, a strong advantage is its ability to reach and disinfect all the surfaces that would be hard to reach or easily missed with standard bleach cleaning (Shapey *et al.*, 2008). However, it does have some disadvantages gaseous disinfectants namely increased downtime; a pre-clean of the room is required to remove any organic material which would otherwise reduce the efficiency (Otter *et al.*, 2009).

One of the main uses of hydrogen peroxide is the terminal cleaning or decontamination of a side room after a patient suffering from a HAI, is discharged or transferred to another ward, rather than for regular cleaning. This is important as routinely used detergents do not always eradicate *C. difficile* and MRSA, which may remain viable for weeks or even months (Boyce, 2009). It has been shown that there is a difference in the decontamination efficiency that can be achieved on porous and non-porous surfaces. Rogers *et al.* (2005) showed that for the same hydrogen peroxide cycle the log reduction achieved can vary from 1.2 for carpet to 7.5 on plastics and glass.

Galvin *et al.* (2012) suggested that some soft furnishings may absorb hydrogen peroxide during the decontamination process which can then leach out in a process known as "off-gassing". In this study long impractical aeration times of up to 12 h are required for all of the hydrogen peroxide gas to dissipate. However, the study does not state whether an activated carbon was catalyst was used to aid the removal of the hydrogen peroxide after the decontamination process as used by some of the commercial decontamination cycles. The absence of any catalyst to remove the vapour after the decontamination cycle could explain how this has not been identified as an issue with the process already being used in the healthcare environment.

2.4.4 Ozone

Ozone has historically been used mainly in the disinfection of water, although it has also been for the preservation of food during bulk storage (Sykes, 1965). It has been shown to be effective against both Gram-positive and Gram-negative bacteria, however, yeasts and moulds have been shown to be more resistant (Sykes, 1965; Davies *et al.*, 2011). The main advantages of ozone disinfection are that it is cheap to produce and dissociates quickly into oxygen; however, the gas is an irritant, is toxic and can cause respiratory symptoms in humans at concentrations as low as 0.1 ppm (Berrington & Pedler, 1998; Sharma & Hudson, 2008).

Ozone is already used throughout Europe in the washing of highly contaminated hospital linen as a number of microorganisms have become resistant to chlorine (Cardoso *et al.*, 2000). A small number of studies have been carried out investigating decontamination using gaseous ozone, with one study observing the decontamination of a nurses' home persistently contaminated with MRSA. Berrington & Pedler (1998) reported the decontamination of a hospital side room using a very low concentration of ozone (0.13 ppm) over 2 h. Spore samples were placed in the room and exposed for 4 and 7 h, controls were carried out in an adjacent room where samples were exposed to air for identical times. The results obtained showed that methicillin-sensitive *S. aureus* (MSSA) is potentially more resistant to ozone than MRSA. MSSA showed a reduction of 83 % after 4 h exposure,

increasing to 97 % after 7 h when the samples were placed in close proximity to the ozone generator. This reduced to 79 % after 4 h when placed at a significant distance away from the generator with the reduction not significantly increasing after a further 3 h of exposure. The MRSA results show that after 4 h all the MRSA in locations close to the generator was killed, however, when placed further away from the generator only a 43 % reduction was observed after 4 h, increasing 72 % after 7 h. This indicates that the MRSA is more susceptible than MSSA to high concentration of ozone, whereas it could actually be less susceptible if the ozone concentration is decreased. However, no information is given regarding the initial concentration of MRSA or MSSA, also most of the results demonstrated less than one log reduction in the number of viable organisms. The paper also states there is a significant decrease in both MSSA and MRSA when exposed to air alone at flows from 40.2 to 17.2 cfu (42.8 %) between 4 and 7 h, and a reduction from 50.0 to 26.8 cfu (53.6 %) for MRSA over the same period. This shows that not all of the reduction is due to the ozone exposure; this is increasingly likely as the exposure to the ozone only lasts for 2 h and no samples were enumerated immediately following exposure.

de Boer *et al.* (2006) studied the decontamination of a house contaminated with MRSA using ozone. In the investigation, the house was clear of MRSA after exposure to 12 ppm over 10 h. However, the decontamination in this study is over considerably longer time and at a much higher concentration than used by Berrington & Pedler (1998); therefore a much higher reduction would be expected. Decontamination with ozone has also been demonstrated by Sharma & Hudson (2008) to be an effective disinfectant when the bacteria are exposed on a variety of different surfaces, such as plastic, cotton, fabric, filter paper and cardboard, with a greater than 4-$\log_{10}$ reduction being reported on all surfaces after exposure to 25 ppm for 20 min. However, in this study a relative humidity of greater than 80 % was recorded; at a humidity of this order ozone is capable of degrading rubber (Davies *et al.*, 2011).

There are two main concerns with the use of ozone as a vapour disinfectant: firstly ozone is a potent oxidiser and is therefore likely to corrode any metals within the room (Davies *et al.*, 2011); the second is that decontamination with ozone requires the humidity to be maintained between 45

to 80 % as the biocidal activity increases humidity (Davies *et al.*, 2011). The need to maintain the required humidity and the fact the ozone can degrade materials used within the hospital environment makes it unsuitable for use in this manner.

2.4.5 Comparison of Vapour Phase Disinfection Methods

When deciding on the type of gaseous disinfection to be used a number of factors need to be considered, including: any health issue associated with the chemicals being used, the biological efficacy of the system, the downtime of the ward, and the cost of the process. Therefore, although formaldehyde has a long history of being used as a decontamination agent, since being classified as a carcinogen, it is no longer approved for use in the healthcare environment (Grare *et al.*, 2008).

Comparing hydrogen peroxide vapour, ozone and chlorine dioxide disinfection methods there are many similarities between them; all processes required that the area to be treated is completely sealed and a pre-clean is always required to remove any visible contamination and organic material (Davies *et al.*, 2011). All processes are much more expensive than the standard cleaning, with hydrogen peroxide and ozone both costing around £110 per cycle compared to around £15 for a standard clean (wiping all surfaces with a liquid disinfectant and mopping of the floors). These costs are based on the cost of the staffing requirements as the chemicals and any equipment requirements (Doan *et al.*, 2012). These decontamination processes also all take around three times longer than a standard clean (Otter *et al.*, 2009; Doan *et al.*, 2012). Chlorine dioxide is more labour intensive than hydrogen peroxide and ozone as it is requires a high humidity; it can also discolour some materials and becomes explosive at concentrations above 10 %; although this is much higher than the concentrations used it does mean the process require monitoring closely.

Commercially available dry ozone (Meditrox 100) and hydrogen peroxide (Bioquell Q10) decontamination processes were compared by Doan *et al.* (2012). This comparison revealed there is a significant difference in the log reduction of *C. difficile* that can be achieved; a 2.3-$\log_{10}$ reduction was obtained

with the hydrogen peroxide exposure compared to a 1.4-$\log_{10}$ reduction by ozone. This was confirmed with a study by Sharma & Hudson (2008). Ozone also requires the humidity within the room to be monitored to keep the activity high enough to allow for the decontamination, but not too high that it starts degrading other materials within the ward. In conclusion, hydrogen peroxide has been presented as the most feasible chemical for gaseous decontamination as it can be used at room conditions and does not discolour or degrade any materials or electronic equipment used within the healthcare environment. However, as with all of the gaseous decontamination systems it should only be used as a supplement to standard cleaning and not as a replacement.

2.5 Hydrogen Peroxide Disinfection

In this section the different hydrogen peroxide decontamination systems in use are described along with its disinfection properties in laboratory experiments and studies carried out in the hospital setting (Table 2.2). Further to this recontamination after exposure is considered, and finally, the disinfection mechanism employed by hydrogen peroxide during the decontamination process.

Table 2.2 Effectiveness of Hydrogen peroxide vapour decontamination cycles against bacteria from laboratory studies and contaminated hospital surfaces. Summarised from literature.

Proportion of HAIs before decontmination	Decontamination site	Environmental sites sampled	Biolindicator/sampling method	Hydrogen peroxide decontamination method used	Proportion of HAIs after decontamination	Study
6-$\log_{10}$ *C. botulinum* dried on stainless steel discs	Glove box	***	Spore suspensions dried onto stainless steel tabs	Bioquell HPV decontamination, peak concentration 355 ppm	3 to 4-$\log_{10}$ reduction after 12 min	Johnston, Lawson & Otter 2005
B. anthracis, B. subtilis and *G. stearothermophilus* BI of 7-$\log_{10}$	Glove box	***	Spores dried onto different materials including: carpet, wood, glass and metal	Bioquell HPV cabinet decontamination cycle	Lower inactivation on porous materials; log reductions of 3-7 (*B. anthracis*), 1-7 (*B. subtilis*) and 1-4 (*G. stearothermophilus*)	Rogers *et al*. 2005
3-$\log_{10}$ *M. tuberculosis* on stainless steel and 6-$\log_{10}$ *G. stearothermophilus* BI	37 m^3 biological safety level 3 laboratory	Indicators on the floor and surfaces within the room	Spore suspensions dried onto stainless steel tabs	Bioquell HPV decontamination	All indicators negative after HPV cycle	Hall *et al*. 2007
6-, 7- and 8-$\log_{10}$ BI of *B. anthracis*	0.2 m^3 stainless steel fumigation (or test) chamber	***	Spores dried onto different material including: carpet, wood and steel	Steris VHP hydrogen peroxide system	6-$\log_{10}$ reduction on all surfaces other than wood (3-$\log_{10}$) for the 6 and 7-$\log_{10}$ BI. Only 1-4-$\log_{10}$ reduction for the 8-$\log_{10}$ BI	Rastogi *et al*. 2009

Proportion of HAIs before decontmination	Decontamination site	Environmental sites sampled	Biolindicator/sampling method	Hydrogen peroxide decontamination method used	Proportion of HAIs after decontamination	Study
38/40 and 30/40 *M. tuberculosis*-positive BI for 3 and 6 aHP cycles respectively	Biosafety, level 3 tuberculosis laboratory	***	100 µl of 3×10^5 cfu/ml dried in 20 plastic wells	3 or 6 aHP cycles; respective peak concentrations of 88 and 113 ppm	36/40 and 20/40 *M. tuberculosis*-positive BI after 3 and 6 aHP cycles respectively	Andersen *et al*. 2010
MS2 bacteriophage BI of 8-$\log_{10}$	Class III safety cabinet	***	Stainless steel tabs inoculated with MS2 bacteriophage	Steris VHP hydrogen peroxide system	6-$\log_{10}$ reduction achieved within 10 min	Pottage *et al*. 2010
9.7-, 8.2- and 7.5-$\log_{10}$ indicators of adenovirus used in triplicate	Microbiological safety cabinet	***	Adenovirus dried onto disk and grown in 20 ml Trypticase Soy Broth for 7 days	Bioquell hydrogen peroxide cabinet decontamination cycle	8.6-$\log_{10}$ reduction of adenoviruses	Berrie *et al*. 2011
5-$\log_{10}$ feline calicivirus used to inoculate various materials	Class III cabinet	***	1 cm^2 piece of stainless steel, glass, vinyl flooring, ceramic tile and PVC inoculated with feline calicivirus	Bioquell hydrogen peroxide cabinet decontamination cycle	5-$\log_{10}$ reduction show from all indicators after 20 min exposure	Bentley *et al*. 2012
Stainless steel discs containing 7.2-$\log_{10}$ (MRSA), 6.0-$\log_{10}$ (*A. baumanni*)and 5.2-$\log_{10}$ (*C. difficile*)	Test room approximate a large hospital room (50.1 m^3) with an *en suite* (13.2 m^3)	All room corners and bench tops in both the main and side room	Stainless steel indicators inoculated with MRSA, *A. baumanni* and *C. difficile*	Bioquell HPV decontamination system with a peak concentration of 125 ppm	Complete inactivation of all BI	Fu, Gent & Kumar, 2012
Stainless steel discs containing 7.2-$\log_{10}$ (MRSA), 6.0-$\log_{10}$ (*A. baumanni*)and 5.2-$\log_{10}$ (*C. difficile*)	Test room approximate a large hospital room (50.1 m^3) with an *en suite* (13.2 m^3)	All room corners and bench tops in both the main and side room	Stainless steel indicators inoculated with MRSA, *A. baumanni* and *C. difficile*	aHP disinfection system with a peak concentration of 30 ppm	1.7-7.3 (MRSA), 0.5-6.1 (*A. baumanni)* and 0.6-5.6 (*C. difficile)* log recovery from indicators.	Fu, Gent & Kumar, 2012

Proportion of HAIs before decontmination	Decontamination site	Environmental sites sampled	Biolindicator/sampling method	Hydrogen peroxide decontamination method used	Proportion of HAIs after decontamination	Study
G. stearothermophilus and MRSA BI of 6-$\log_{10}$	20.7 m^3 exposure chamber	***	Commercially available *G. stearothermophilus* indicators and MRSA-inoculated stainless steel tabs	Steris VHP hydrogen peroxide system designed to exposure the spores to 750 ppm	5-$\log_{10}$ (*G. stearothermophilus)* and 3-$\log_{10}$ (MRSA) reductions after 30 min exposure	Pottage *et al*. 2012
poliovirus BI of 6-$\log_{10}$	87.5 m^3 room	In corners of the room, on table, on closet and in closed closet	Stainless steel carriers inoculated with poliovirus	Bioquell HPV disinfection with a peak concentration of 127 ppm for 1 hr	6-$\log_{10}$ reduction for all sites, except closed closet (1-$\log_{10}$)	Tuladhar *et al*. 2012
B. subtilis BIof 8-$\log_{10}$	Specially designed environmental exposure chamber	***	Millipore 0.2 µm membranes with spores uniformly filtered on the surface	Hydrogen peroxide solution vaporized on a hotplate to achieve and maintain concentrations of 10, 50, 75 and 90 ppm	6-, 4- and 1-$\log_{10}$ reduction shown for 6 h exposure to 90, 75 and 50 ppm hydrogen peroxide respectively. 2-$\log_{10}$ reduction after 36 h exposure to 10 ppm hydrogen peroxide	Malik *et al*. 2013
61/85 (72 %) MRSA-positive	Four single side rooms (35 m^3) in a 1200-bed London teaching hospital	Floor, over-bed tables, bed frames and raising panels, chairs and lockers, door handles, light switches, sink taps, televisions and remote controls	Sterile cotton-tipped swabs moistened in sterile brain heart infusion (BHI) broth	Bioquell HPV decontamination	1/85 (1.2 %) MRSA-positive	French *et al*. 2004

Proportion of HAIs before decontmination	Decontamination site	Environmental sites sampled	Biolindicator/sampling method	Hydrogen peroxide decontamination method used	Proportion of HAIs after decontamination	Study
8/50 (16 %) *C. difficile*-positive after deep clean	20-bed Nightingale-design surgical ward	Reception telephone, suction pump, bedside chair, TV remotes, window sill and floor samples	Sterile swabs moistened in nutrient broth	Deep clean followed by a Bioquell hydrogen peroxide decontamination cycle	0/50 (0 %) *C. difficile-positive*	Jeanes *et al*. 2005
8/27 (27.6 %) MRSA-positive	28-bed surgical ward with four six-bed bays and four isolation rooms	Bedrail, bedframe, locker and bedside telephone in each six-bed bay	Sterile composite swabs plated on chromogenic MRSA selective media	Hydrogen peroxide vapour decontamination	1/27 (3.4 %) MRSA-positive	Dryden *et al*. 2007
18/30 (60 %) (MRSA) and 9/30 (30 %) (GNR (gram-negative rods)) sites positive	35 m^3 single occupancy room	Floor, bed-frame, control panel, bedside furniture, TV remote and stand, door handles	Sterile cotton-tipped swabs moistened in nutrient broth	Bioquell Hydrogen peroxide vapour decontamination	0/50 (0 %) GNR, 1/30 (3.3 %) MRSA-positive	Otter *et al*. 2007
48/203 positive for *C. difficile* (24 %) 100 % (10/10) of the room sampled had at least one positive sample	11 rooms within three high risk elderly care wards	Bedside table, armchair, mattress, bed frame, bin, floor, toilet, toilet roll holder, towel dispenser, call button, radiator, bedside cabinet, lamp, door handles	Sterile cotton-tipped swabs moistened with Ringer's solution	ASP dry mist hydrogen peroxide cycle	7/203 (3.4 %) with 50 % (5/10) of the rooms sampled had at least one positive sample	Shapey *et al*. 2008

Proportion of HAIs before decontmination	Decontamination site	Environmental sites sampled	Biolindicator/sampling method	Hydrogen peroxide decontamination method used	Proportion of HAIs after decontamination	Study
11/43 (26 %) *C. difficile*-positive	Five wards with the high number of incidents of *C. difficile* infection	Bedrail, bed-raising buttons, nurse call button, intravenous pumps, chair arm, dresser, over-bed table, sink and toilet	Pre-moistened cellulose sponges	Wards were cleaned of any visible dirt before Bioquell HPV decontamination	0/37 (0 %) *C. difficile*-positive	Boyce *et al*. 2008
34/180 (19 %) *C. difficile*-positive	2 French university hospitals	Toilet seat, sinks, bedside and care tables, phone, door handle, chair, remote controls, bedrail, hand gel dispensers	Swabs pre-moistened with Schaedler broth	Conventional cleaning followed by aHP disinfection	4/180 (2 %) *C. difficile*-positive	Barbut *et al*. 2009
10/21 (46.6 %) sites GNR-positive after cleaning	1389 m^3, 12-bed intensive care unit	Mattress, bedside table, door knobs and medical equipment	100 cm^2 sterile cotton gauzes moistened in sterile water used to sample 1 m^2 areas	Bioquell HPV decontamination system	0/63 (0%) sites GNR-positive	Otter *et al*. 2010
Samples ranging from 22-187 cfu/plate (no specific bacteria)	1-2 bedrooms with *en suite* in a 300-bed Melbourne teaching hospital	Toilet flush, bedside table, call button, bed rail and keyboard	55 mm contact plates containing neutralising agents	Dry mist hydrogen peroxide vapour cycle (Nocospray)	Samples ranging from 0-8 cfu/plate (no specific bacteria)	Chan *et al*. 2011

Proportion of HAIs before decontmination	Decontamination site	Environmental sites sampled	Biolindicator/sampling method	Hydrogen peroxide decontamination method used	Proportion of HAIs after decontamination	Study
MRSA and *A. baumannii* BIof 4-$\log_{10}$	Single isolation rooms in intensive care unit	On top of the wardrobe, bedside table, inside a drawer, on the ground floor	Stainless steel tab inoculated with MRSA and *A. baumannii*	aHP peroxide cycle	4-$\log_{10}$ reduction for all sites, except closed drawer (1-$\log_{10}$)	Piskin *et al*. 2011
C. difficile BI of 5.8-$\log_{10}$	Furnished single occupancy (33-45 m^3) hospital room	Near the sink	Spores dried on 2 cm^2 square pieces of polyvinyl chloride (PVC) or laminate	Bioquell HPV decontamination cycle	All indicators showed complete inactivation	Barbut, Yezli & Otter 2012
0.88/1000 patient days lost to *C. difficile*	Hospital-wide decontamination including: 1 burn unit, 5 adult intensive care units, and 2 intermediate care	***	*C. difficile*-positive faecal specimens after 72 h and up to 7 days after discharge	Daily bleach cleaning while occupied; bleach cleaning followed by Bioquell HPV decontamination following discharge	0.55/1000 patient days lost to *C. difficile*	Manian *et al*. 2012

2.5.1 Hydrogen Peroxide Decontamination Systems

There are three main types of hydrogen peroxide decontamination devices on the market, inactivation studies using each of these are summarised in Table 2.2. Two of these (Bioquell Clarus and Steris) are very similar with both using an initial hydrogen peroxide solution of around 30 % (w/v) which is vaporised and produces a peak hydrogen peroxide concentration within an enclosed space of 130 to 150 ppm. The difference between the two systems being that the Bioquell Clarus is a 'wet vapour' system, where the hydrogen peroxide condenses on the surfaces and is otherwise known as HPV (hydrogen peroxide vapour). In contrast there is no micro-condensation with the Steris system which is known as VHP (vaporised hydrogen peroxide). With both of these decontamination cycles an activated carbon catalyst is used to absorb the hydrogen peroxide so that the treated area can be repopulated (Boyce, 2009; Pottage *et al.*, 2010; Chan *et al.*, 2011; Barbut, Yezli & Otter, 2012; Fu, Gent & Kumar, 2012). A third method is aHP (aerosolized hydrogen peroxide) (Advanced Sterilization Products, ASP - previously Sterinis) device where a 5 % (w/w) hydrogen peroxide solution (Sterusil) is sprayed into the room as micro-droplets (small than 10 μm) which then evaporate in the environment. These droplets contain a small amount of silver ions (less than 50 ppm) which, according to the manufacture charge the droplets to help them be attracted to the surfaces. Silver ions (Ag+) are added to the hydrogen peroxide solution for their ability to kill microorganisms by blocking the respiratory enzyme system as well as altering microbe DNA and the cell wall (Bartels *et al.*, 2008). Using this method produces a much lower peak hydrogen peroxide concentration of around 30 ppm to 45 ppm, meaning that no catalyst is required to breakdown the hydrogen peroxide after exposure obviating the need for an active removal stage (Barbut *et al.*, 2009; Fu, Gent & Kumar, 2012).

The efficiency of the three decontamination systems has been evaluated in a number of studies (Table 2.2). In these studies 6-$\log_{10}$ bioindicators (BI) of *Bacillus atrophaeus* (ASP) and *Geobacillus stearothermophilus* (Steris and Bioquell) were used to identify the spore inactivation level that could be achieved (Kahnert *et al.*, 2005; Andersen *et al.*, 2006; Hall *et al.*, 2007; Boyce *et al.*, 2008). Andersen *et al.* (2006) used one, two and three disinfection cycles

of the Advance Sterilization Products system. The study found that within the room a peak hydrogen peroxide concentration of around 45 ppm was reached. It was observed that after exposure to one or two cycles there was no inactivation detected in *B. atrophaeus* bioindicators. However, complete inactivation (24/24 indicators) was observed after 3 decontamination cycles. In comparison to this both the Steris and Bioquell systems were found to inactivate 5-log_{10} to 6-log_{10} *G. stearothermophilus* bioindicators after a single cycle. Hall *et al.* (2007) and Boyce *et al.* (2008) show complete inactivation and inactivation of 87/95 *G. stearothermophilus* indicators respectively. Often incomplete information is given regarding the hydrogen peroxide concentration during a decontamination cycle; rather than the complete concentration-time profile being provided which could then be used to compare the different methods. For example, Hall *et al.* (2007) state that they achieve a peak concentration of greater than 2000 ppm, whereas Boyce *et al.* (2008) do not provide any details on the hydrogen peroxide concentrations. Likewise the study by Kahnert *et al.* (2005), which shows complete inactivation of 5-log_{10} *G. stearothermophilus*, gives no information about the hydrogen peroxide concentrations achieved.

More recently Fu, Gent & Kumar (2012) compared the Advance Sterilization Products system to that of the Bioquell HPV, demonstrating only 9.1% of the 6-log_{10} *G. stearothermophilus* indicators exposed showed growth after the Bioquell cycle, all of which were in an adjoining room, whereas, 63.6 % and 93.2 % of the 4-log_{10} and 6-log_{10} bioindicators, respectively, were positive when exposed to the Advance Sterilization Products decontamination methodology. However, the bioindicators using in this study have only incubated for 24 h and no quenching agent was used to scavenge any residual hydrogen peroxide. Malik (2012) compared the incubation time used by Fu to that used in other studies and found it to be much shorter: 24 h compared to 72 h. This could mean that some of the bacteria which have been sub-lethally damaged do not have sufficient time to grow, as they require longer to germinate than their healthy counterparts.

2.5.2 *Hydrogen Peroxide Disinfection Efficiency in Fume Cupboards and other Controlled Environments*

Hydrogen peroxide vapour has been found to be effective against many different types of bacteria as shown in Table 2.3. The most commonly used test organism is *G. stearothermophilus* as it has been shown in a number of studies to be one of the most resistant organisms to hydrogen peroxide decontamination and as has already been discussed is often used in commercial indicators (Meszaros *et al.*, 2005; Rogers *et al.*, 2005; International Organization for Standardization, 2009). Although Pottage *et al.* (2012) recently suggested that MRSA may be potentially more resistant to hydrogen peroxide than commercial *G. stearothermophilus* bioindicators, which the authors attributes to MRSA producing catalase which breaks down hydrogen peroxide. Despite this Otter & Yezli (2012) defend the use of *G. stearothermophilus* indicators to measure the efficiency of the Bioquell vaporised hydrogen peroxide cycle in the healthcare environment. The basis of their claim is that the indicators having a significantly higher spore concentration than is found for bacteria such as MRSA in the healthcare environment. The efficacy of the Bioquell inactivation cycle against MRSA on a hospital ward was investigated by Jeanes *et al.* (2005) who show a reduction from 8/50 surface swabs after a deep clean to no positive swabs after hydrogen peroxide decontamination.

The main failing of the current literature concerning the inactivation of bacteria using hydrogen peroxide is that most studies do not report the concentration of hydrogen peroxide vapour used and the fact that the hydrogen peroxide concentration is not constant over the duration of the experiment. For example, Bentley *et al.* (2012) reported that a 5-$\log_{10}$ reduction of feline calicivirus can be achieved on a variety of surfaces within 20 min, however, they claimed that a Bioquell Clarus L HPV generator was used. This precludes any comparative studies to investigate the data futile as the hydrogen peroxide concentration is unknown. The same issue has been identified with Pottage *et al.* (2010), in which the Bioquell Clarus L HPV generator is compared with the Steris VHP 1001 generator. It was shown in this study that both systems were

effective against MS2 bacteriophage; it was also suggested that longer decontamination times were required when biological soiling was present.

Table 2.3 Inactivation of bacteria by vaporised hydrogen peroxide.

Organism	Log_{10} reduction achieved	Hydrogen peroxide concentration	Exposure time	Hydrogen peroxide system	Study
Escherichia coli	7.0	NR (not recorded)	4 hr	Steris	Galvin *et al.* 2012
Staphylococcus aureus	5.0	NR	300 s	Steris	Meszaros *et al.* 2005
Enterococcus faecalis	7.0	NR	4 hr	Steris	Galvin *et al.* 2012
Pseudomonas aeruginosa	7.0	NR	4 hr	Steris	Galvin *et al.* 2012
Aspergillus fumigatus	6.0	NR	4 hr	Steris	Galvin *et al.* 2012
Acinetobacter baumannii	7.0	NR	4 hr	Steris	Galvin *et al.* 2012
Clostridium difficile	7.0	NR	4 hr	Steris	Galvin *et al.* 2012
Geobacillus stearothermophilus	2.0	≥1000 ppm	40 min +aeration	Bioquell	Rogers *et al.* 2005
Bacillus anthracis	7.5	≥1000 ppm	40 min +aeration	Bioquell	Rogers *et al.* 2005
Bacillus subtilis	6.4	≥1000 ppm	40 min +aeration	Bioquell	Rogers *et al.* 2005
Clostridium botulinum	3.0	355 ppm (peak)	12 min	Bioquell	Johnston, Lawson & Otter 2005

Even when details of the hydrogen peroxide cycle are provided the bacteria are not exposed to a uniform concentration. Johnston, Lawson & Otter (2005) investigated the effect of hydrogen peroxide on *Clostridium botulinum*. The experiment required all of the samples being placed into a glove box attached to a Bioquell Clarus L system to ensure they could be transferred into a catalase quench system after the required exposure time. The results from this study showed approximately a 3-log_{10} reduction in the number of viable spores after 5 min. These authors attempted to fit their data linearly but this was not entirely satisfactory as there was a lag of about 2 to 3 min before any inactivation was observed This could be attributed to the hydrogen peroxide cycle used which took 2 min to reach >100 ppm, a further 2 min to reach >300 ppm, and up to 12 min to reach the maximum concentration of 355 ppm.

As an extension to the work discussed in this section, some studies were carried out to investigate the efficiency of hydrogen peroxide in larger enclosed

areas of size comparable to that of a hospital isolation room. Four main studies have been carried out investigating the decontamination effects of hydrogen peroxide in enclosed areas at this scale (detailed in Table 2.2). Both Fu, Gent & Kumar (2012) and Tuladhar *et al.* (2012) report on the effects following exposures in rooms containing a desk, with the former including a side room and the latter having open and closed cabinets. In both studies hydrogen peroxide vapour systems with peak concentrations of around 125 ppm were used. Fu, Gent & Kumar (2012) obtained a 6-$\log_{10}$ reduction of *G. stearothermophilus* within every location in the main room with a 4-$\log_{10}$ reduction achieved in the side room, and just under half of the 6-$\log_{10}$ coupons yielded viable cells from the side room. However, this group only incubated the plates for 24 h which may be insufficient for the resuscitation and germination of sub-lethally damaged spores. Tuladhar *et al.* (2012) showed a 6-$\log_{10}$ reduction in every location, apart from within the closed closet where less than 1-$\log_{10}$ reduction was observed. This was also found by Piskin *et al.* (2011) who showed a significant difference in inactivation efficiency between MRSA (1-$\log_{10}$) and *A. baumannii* (1.7-$\log_{10}$) when they were placed in a drawer throughout a decontamination cycle. In contrast to these studies, Galvin *et al.* (2012) used a portacabin containing soft furnishings likely to be found in a hospital room, such as a mattress, blankets and curtains, as well as floor and table materials. Their experiment showed that although a 7-$\log_{10}$ reduction was observed, when many different organisms are exposed on the surfaces, the soft furnishing can absorb and continue releasing the hydrogen peroxide for up to 3 days after exposure. Unfortunately however, this group did not provide details of the hydrogen peroxide vapour concentration used in the decontamination cycle. The authors used a Steris system in comparison to other studies and therefore may not be comparable. Regardless of this, it is worth noting that the use of hydrogen peroxide systems could cause problems when used with soft furnishings, and so further investigation building on the results shown within this thesis maybe required.

2.5.3 Hydrogen Peroxide Disinfection in the Healthcare Environment

Many studies have been carried out investigating the disinfection properties of hydrogen peroxide in the hospital environment. The majority of these studies are conducted with the focus being on the ability of hydrogen peroxide to inactivate MRSA and *C. difficile*; however, there are some more general reports which summarise the overall practicality of hydrogen peroxide decontamination cycles (Table 2.2).

Studies to investigate the efficacy of the Bioquell decontamination system *in situ* to inactivate MRSA were carried out by French *et al.* (2004) in four separate isolation rooms. The group found that before the decontamination cycle, 72 % of samples were positive for MRSA, whereas after the decontamination cycle only one of the sites (1.2 %) tested was confirmed as positive. This was compared to conventional cleaning in a separate room where a reduction in the positive sites was observed from 89.5 % before cleaning to 66.1 % afterwards. This work is supported by Dryden *et al.* (2007) who used hydrogen peroxide to disinfect a whole ward. In this study, 27.6 % of sites were found to be positive for MRSA before the decontamination, decreasing to 3.4 % (one positive sample) afterwards. Bartels *et al.* (2008) used the ASP decontamination system and found that using three cycles could reduce MRSA to undetectable levels on hard surfaces. However, MRSA was still found on the soft furnishing such as the pillows of patients. Using the decontamination cycles three times rather than once represents a much longer decontamination time and also suggests a lower concentration of hydrogen peroxide is achieved in the cycle. This means that more time is required for the same amount of spore or bacterial inactivation.

C. difficile inactivation was also tested *in situ* using the Bioquell system in two separate studies. Different approaches were taken in each: in a study by Boyce *et al.* (2008), *C. difficile* was isolated from various areas using pre-moistened sponges, whereas, Barbut, Yezli & Otter (2012) placed coupons contaminated with *C. difficile* in a room whilst the decontamination cycles were completed. Both studies showed that the *C. difficile*, either on the contaminated

coupons or collected using the pre-moistened sponges, were reduced to below the detection level. In the case of the coupons, this showed that a greater than 5-$\log_{10}$ reduction could be achieved during a normal decontamination cycle. The experiment by Boyce *et al.* (2008) delved further by investigating the number of *C. difficile*-associated disease (CDAD) cases in the five weeks before and during the decontamination cycle. These results show that the number of people contracting CDAD went down from 2.28 cases per 1,000 patient-days before the hydrogen peroxide intervention to 1.28 cases per 1,000 patient-days afterwards. A weakness with these results however, is that they were not run co-currently, and therefore they cannot be effectively compared, especially since previous work has shown seasonal trends in *C. difficile* cases (Polgreen *et al.*, 2010). In order to represent these results in a more reliable way, two different wards or a number of different isolation rooms in the same hospital should be monitored simultaneously with one undergoing treatment with hydrogen peroxide and the other not. An example of a larger study was conducted by Shapey *et al.* (2008), using a single ASP cycle in an elderly care ward. This group demonstrated that 100 % of the rooms tested were positive before the decontamination cycle, and falling to 50 % afterwards. The reductions achieved were slightly increased for samples taken from specific areas showing a reduction from 24 % before decontamination to 6 % afterwards. This shows that as with the MRSA decontamination, the Advance Sterilization Products system is much less efficient than the Bioquell system, which is likely to be due to the much lower hydrogen peroxide concentration achieved, as reported by Fu, Gent & Kumar (2012).

2.5.4 Feasibility of Hydrogen Peroxide Decontamination

The feasibility of using hydrogen peroxide as a routine decontamination process in hospital wards was assessed by Otter *et al.* (2009). These authors concluded that hydrogen peroxide decontamination is more reliable than bleach cleaning, as it required less intervention from hospital staff as once the cycle is operational, all sites will receive exposure to the hydrogen peroxide. However, hydrogen peroxide decontamination takes three times longer than standard bleach cleaning and requires the room to be emptied of patients and personnel; therefore it can only be used as a supplement and not a replacement. In a

review by Falagas *et al.* (2011), demonstrated that all the commercial hydrogen peroxide decontamination systems show reduction in the number of viable bacteria in the environment. In addition, it was reported that one of the main advantages of the use of hydrogen peroxide is that it is a broad-spectrum disinfectant efficient against a vast number of pathogenic bacteria. In another study by Otter *et al.* (2007), the recontamination of MRSA after hydrogen peroxide decontamination was assessed. The authors found that MRSA was identified on surfaces between 2 to 5 days after the decontamination cycle, with the number of contaminated sites reaching the same level as before the decontamination after just 6 days. These findings suggest that although hydrogen peroxide decontamination is effective it does need to be carried out on a reasonably regular basis.

2.5.5 Disinfection Mechanism

Very little is currently known about the mechanism by which hydrogen peroxide induces death in bacteria (Labas *et al.*, 2008). Linley *et al.* (2012) claimed that there are two possible mechanisms by which this occurs. The first uses the Fenton reaction to break down the hydrogen peroxide and form hydroxyl radicals which are then able to react with the DNA chain and cause damage (Imlay *et al.*, 1988). The second mode of action is the oxidation of proteins and lipids within the bacteria. One of the advantages of hydrogen peroxide is its permeability into bacteria is similar to water and it can therefore easily pass through the cell membrane (Mishra & Imlay, 2012).

More is known about the inactivation of spores using oxidising agents, such as hydrogen peroxide. Oxidising agents attack the inner membrane of the spore causing it to rupture on spore germination (Cortezzo *et al.*, 2004; Setlow, 2006). For most oxidising agents the outer spore membrane and coat provides some protection to the spore, however, because hydrogen peroxide is able to pass easily through the membrane this protection is limited (Setlow, 2006; Mishra & Imlay, 2012). The method by which the spores are produced is also important as those produced a high temperatures have lower core water content making them more resistant to high humidity and therefore they could be more resistant to hydrogen peroxide also (Melly *et al.*, 2002).

2.6 Inactivation Models

Many different inactivation models have been described to fit and predict the inactivation kinetics of microorganisms upon subjection to chemical disinfectants, UV light and temperature among other processes. In this section four different inactivation models are detailed, these are: Chick-Watson, Series-Event, Baranyi and Weibull.

In order to accurately model the inactivation there are three different inactivation stages that need to be considered as detailed in Figure 2.3. A "shoulder" or lag time is often observed in the early stages of treatment (Figure 2.3), which has been shown to be important for inactivation using oxidising agents (Haas & Kaymak, 2002; Cortezzo *et al.*, 2004). The ability of microorganisms to repair damage inflicted upon them The shoulder can be viewed as a manifestation of the microorganism to repair the damage inflicted on them. Similarly there may be a critical amount of damage required for the bacteria to be inactivated, with the lag period being the time for this damage to be accumulated (Xiong *et al.*, 1999; Peleg, 2000; Corradini & Peleg, 2003).

The final is a "tail" phase (Figure 2.3) where the inactivation rate decreases during the exposure (Haas & Kaymak, 2002). Tailing is more prevalent when dealing with fungal spores rather than bacterial spores, as it is much more difficult to completely inactivate fungal spores (Fujikawa & Itoh, 1996). Although tailing can also be observed with bacterial spores, it can be attributed to a subset of more resistant spores or the spores being protected by bacteria which has been inactivated (Xiong *et al.*, 1999; Hijnen, Beerendonk & Medema, 2006).
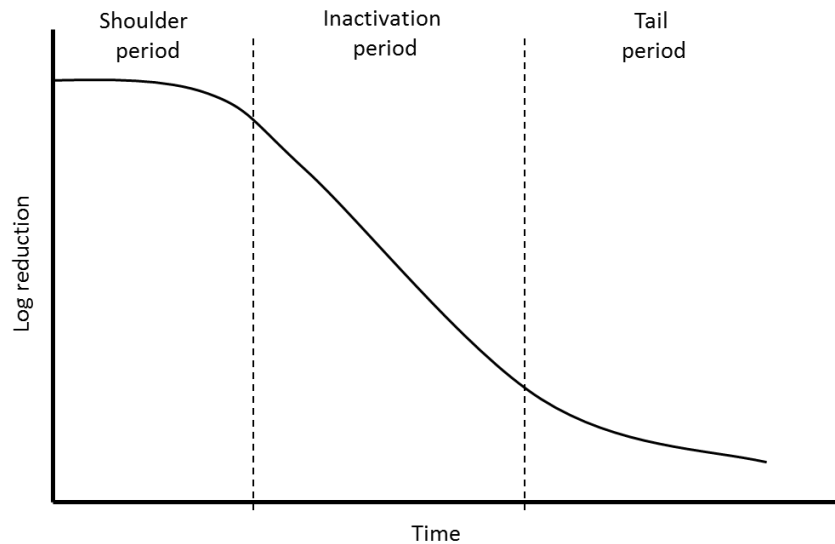
Figure 2.3 A typical graph showing the shoulder, inactivation and tail periods.

2.6.1 Chick-Watson Law

The Chick-Watson Law (Equation 2.1) is the simplest inactivation model and is a log-linear model which was first proposed in 1908 (Chick, 1908; Watson, 1908).

$$N = N_0 \exp\left(-kC_P^b t\right)$$

(2.1)

N and N_0 are the number and initial number of viable organisms (cfu/ml), k is the inactivation constant (ppm^{-n} min^{-1}), C_P is the hydrogen peroxide concentration (ppm), b is the reaction order and t is the time (min).

For the Chick-Watson law to be used the concentration of the disinfectant (in this case hydrogen peroxide) needs to be constant (Haas & Karra, 1984). It is also assumed that there is a linear relationship between the log reduction of the organisms and the exposure time. The main problem with the assumption is that the exponential decay kinetics is typically not encountered.

2.6.2 Series-Event Inactivation Model

The Series-Event inactivation model is based on the Poisson distribution and was first proposed by Severin *et al.* in 1983 for UV disinfection of water. In

this model, an "event" is assumed to be a unit of damage which occurs in a series of discrete steps. An organism can be thought of requiring a discrete number of unit damages before it is inactivated; damage below this level will not inactivate it and it will survive, exposure exceeding this level will lead to death. In the original model, it is assumed that the rate at which this damage is accumulated can be characterised as first order (Severin, Suidan & Engelbrecht, 1983; Dalrymple *et al.*, 2010). Other studies have modified the equation to overcome this problem as it cannot always be assumed that it is a first order reaction (Labas *et al.*, 2008). The modified form of the Series-Event model equation is:

$$\frac{N}{N_0} = \exp\left(-kC_P^b t\right) \sum_{i=0}^{n-1} \frac{\left(kC_P^b t\right)^i}{i!} \qquad (2.2)$$

N and N_0 are the number of microorganisms and the initial number of microorganisms respectively (cfu/ml), C_P is the hydrogen peroxide concentration (ppm), t is the exposure time (min), i and n are the number of hits or event level and the required number of hits, respectively, k is the inactivation kinetic constant (min^{-1}), and b is the reaction order.

As with the Chick-Watson law the Series-Event inactivation model is an integral model and can therefore only be applied where a constant hydrogen peroxide concentration is used. The main limitations with this model are that it is not very flexible as it can only be used to describe a concave curve with no tail, which is only one of many different inactivation kinetics observed. The model makes no assumptions about the disinfection mechanism and can therefore be used to model many different microorganism inactivation techniques. The Series-Event model has historically mainly been used to investigate the disinfection of water and wastewater (Severin *et al.*, 1983; Haas & Karra, 1984; Labas *et al.*, 2008; Labas *et al.*, 2009). Severin *et al.* (1983) and Ye *et al.* (2007) both demonstrate a good correlation between the Series-Event model and experimentally derived UV decontamination data. Both studies showed coefficient of multiple determination, r^2 values of greater than 0.96 for *Candida parapsilosis*, *Escherichia coli*, *Yersinia pseudotuberculosis* and the f2 virus. The model has also been shown to describe liquid phase hydrogen

peroxide disinfection particularly well (Labas *et al.*, 2008; Labas *et al.*, 2009). Different liquid concentrations of hydrogen peroxide have been shown to follow the Series-Event model for the inactivation of *E. coli* (Labas *et al.*, 2008; Labas *et al.*, 2009), although no r^2 values or other measures of correlation between the model and experimental data were given.

2.6.3 Modified Baranyi Inactivation Model

The modified Baranyi or Geeraerd inactivation model is based on an initial model proposed and described by Baranyi *et al.* (1996). The original model, which was used to predict the growth of microbial cultures, was re-parameterised by Geeraerd *et al.* (2000) to give a dynamic model which can show an initial lag or shoulder period; it can also reveal a saturation or tail period. As with the Series-Event model this is an empirical model (Albert & Mafart 2005; Geeraerd *et al.* 2005). The model first defined by Geeraerd is shown in Equation 2.3:

$$N = \left(N_0 - N_{res}\right)\exp\left(-kt\right)\left(\frac{1 + K_C C_P}{1 + K_C C_P \exp\left(-kt\right)}\right) + N_{res} \qquad (2.3)$$

N and N_0 are the number of viable organisms and the initial number of viable organisms, respectively (cfu/ml), *t* is the time (min), *k* is the inactivation kinetic constant which is dependent on the hydrogen peroxide concentration (C_P) (min^{-1}), N_{res} is the residual population density, C_P is the hydrogen peroxide concentration and K_C is a Michaelis constant used to normalise the hydrogen peroxide concentration.

The model can be further simplified by assuming that there is no residual population, such that N_{res} = 0. This removes the tail portion of the model and is therefore only a valid assumption if the residual population is not being investigated. The simplified model can therefore be written as:

$$\frac{N}{N_0} = \exp\left(-kt\right)\left(\frac{1 + K_C C_P}{1 + K_C C_P \exp(-kt)}\right) \qquad (2.4)$$

Greenacre *et al.* (2003) used the simplified Baranyi equation to model the inactivation of *Listeria monocytogenes* by acetic and lactic acids. The model

fitted their data well; however, they did not compare the model to any other inactivation model that could be used. Albert & Mafart (2005) compared the Weibull (Section 2.6.4) and the modified Baranyi model for the thermal inactivation of *Bacillus pumilus*, *Bacillus cereus* and *Listeria innocua*. The findings from Albert & Mafart (2005) imply that both models provided a good fit for the data, however, no coefficient of multiple determination (r^2) values were determined, which made it difficult to identify which model provided the best fit.

The model can also be manipulated to yield an estimate of the decimal reduction value or *D*-value (*D*), which is defined as the time required for a 1-$\log_{10}$ reduction in the initial value of microorganisms (Equation 2.5). By substituting $\dfrac{N}{N_0}$ for 0.1 (a 1-$\log_{10}$ reduction) and the time for *D*, the resulting equation can be rearranged to give a simple equation that can be used to find the *D*-value (mathematical proof shown in Appendix 1):

$$D = \frac{1}{k}\ln\left(10 + 9K_C C_P\right) \tag{2.5}$$

2.6.4 Weibull Inactivation Model

The Weibull inactivation model is based on the Weibull distribution which is used in reliability engineering to estimate and describe electrical and mechanical failures. This model can also be used to describe the inactivation of bacteria as the "time to fail" after the application of stress (van Boekel, 2002). The Weibull inactivation model has been used to describe may different types of inactivation including pH, thermal and hydrogen peroxide (Albert & Mafart, 2005; Raffellini *et al.*, 2011). The cumulative form of the equation used to characterise the survival has been derived by Buzrul & Alpas (2007):

$$\frac{N}{N_0} = 10^{-\left(\frac{t}{\alpha}\right)^{\beta}} \tag{2.6}$$

N and N_0 are the number of viable microorganisms and the initial number of microorganisms, respectively (cfu/ml), *t* is the time (min), *α* is the scale parameter (min), and *β* is a dimensionless shape parameter.

The main limintation of this model is that unlike the other models discussed it is very flexible allowing it to fit most survival curves. This means that the model can be used to fit even small sets of data where there is not necessarily a good correlation (Todinov, 2010).

It is possible to show that the scale parameter (α) is equivalent to the *D*-value (Equation 2.7). This is a well-known biological parameter which represents the time taken for a one log reduction. The value of the shape parameter (β) defines the shape of the curve; three different type of curves can be achieved: upward concavity ($\beta < 1$), linear ($\beta = 1$) and downward concavity ($\beta > 1$). Therefore, knowing the value of the shape parameter can give an indication of the physiological effects of the decontamination. A value of $\beta < 1$ reveals the cells are adapting to the stress and becoming increasingly more difficult to kill, whereas a value of $\beta > 1$ suggests that the cells are becoming increasingly damaged and easier to kill (van Boekel, 2002).

$$\frac{N}{N_0} = 10^{-\left(\frac{t}{D}\right)^{\beta}} \tag{2.7}$$

van Boekel (2002) uses the Weibull inactivation model for data from many different sources using a variety of inactivation techniques. The results suggest the model provides a good fit for a large variety of different applications. It has also been shown to provide a more accurate description of thermal inactivation than a first-order ($\beta = 1$) kinetic model, which is often used. This is because the model allows for curvature, on a semi-log$_{10}$ plot which is more realistic than the commonly held assumption that inactivation curves are linear (Buzrul & Alpas, 2007). Raffellini *et al.* (2011) have reported the use of the Weibull model for inactivation using liquid hydrogen peroxide. This makes the model a good choice for when investigating the inactivation achieved by gaseous hydrogen peroxide as it has already been demonstrated for use in hydrogen peroxide disinfection, irrespective of whether it is a different phase.

2.7 Key Points

- Although the numbers of reported HAIs has shown a downward trend in the last few years, it is still a major concern for the NHS and is generating significant costs every year.

- *C. difficile* is a spore-forming bacterium, making it much more resistant to standard cleaning methods and allowing it to survive in the environment for up to five months.

- Chlorine-based disinfection agents have been introduced for use during standard cleaning practice to help eradicate the spores. However, their efficiency is disputed due to the relatively high concentrations (>1000 ppm) and reasonably long exposure times (≥10 min) required to inactivate the *C. difficile* spores. This is not possible in the healthcare environment due to the time constraints on the hospital cleaning personnel.

- One method being investigated for healthcare decontamination is the use of gaseous decontamination.

- Hydrogen peroxide has been shown to be sporicidal and a good disinfectant when in the vapour phase.

- The efficiency of hydrogen peroxide has only ever been presented during a decontamination cycle and therefore at differing concentrations. With this in mind, more investigation is required into the disinfection properties at constant hydrogen peroxide concentrations to allow for a better understanding of the system.

Chapter 3
Materials and Methods

3.1 Introduction

This chapter provides details of the materials and methods used to carry out the experiments described in this thesis. It provides detailed information of chemicals, bacteria and media utilised. It also outlines the protocols employed, including the production of the spores, preparation of the spores for exposure to hydrogen peroxide, the exposure chamber used to generate inactivation data, recovery and enumeration of spores.

3.2 Calibration of Hydrogen Peroxide Sensors

Electrochemical hydrogen peroxide sensors (ATI GasSens A11/34 hydrogen peroxide sensor/transmitter) (ATI Limited, UK) were used to monitor and record the hydrogen peroxide concentration during experiments. The hydrogen peroxide concentration was recorded from the sensor using an EasyLog USB data logger (Lascar, UK). In order to ensure that the sensor readings were correct the calibration was checked every two weeks, with recalibration being required every 3 to 4 months depending on usage. The calibration checks were carried out at a constant temperature of 25 °C in a sealed 1 l quickfit vessel (Figure 3.1 and Figure 3.2) containing 500 ml of hydrogen peroxide solution at a concentration where the vapour-liquid equilibrium had been calculated as shown in Appendix 2 (Table 3.1) (Schumb, Satterfield & Wentworth, 1955). Hydrogen peroxide solutions as supplied by Fisher (30 % (w/v) specified solution (Fisher, UK)) were diluted down as required using deionised water.

Table 3.1 The vapour equilibrium concentration of hydrogen peroxide variation with the liquid concentration at 25 °C.
Data calculated from equations given by Schumb, Satterfield & Wentworth (1955)

Liquid Mole Fraction	Liquid Concentration (%) (w/v)	Vapour Concentration (ppm)
0.00	0.00	0
0.02	3.71	16
0.04	7.30	35
0.06	10.76	55
0.08	14.11	78
0.10	17.35	103

The calibration bath consisted of a cylindrical glass reaction flask and lid with five ports to allow the instrumentation to be attached (Figure 3.1 and 3.2). The temperature in the vessel was controlled by submersion into a water bath and temperature readings were made in the vapour phase using a thermometer. A double stirrer with a blade in both the vapour and liquid phase was operated at 1000 rpm to ensure that both phases were well-mixed. Once sealed, the chamber was left for 2 h to reach equilibrium before the hydrogen peroxide sensor was exposed to the vapour phase for 1 to 1.5 h. After exposure, the equilibrium value was plotted against the hydrogen peroxide liquid concentration.
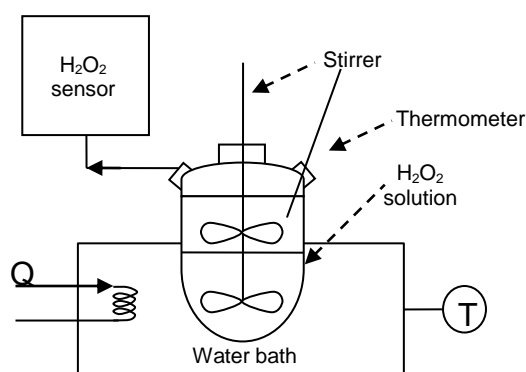
Figure 3.1 Schematic diagram of the hydrogen peroxide calibration bath.
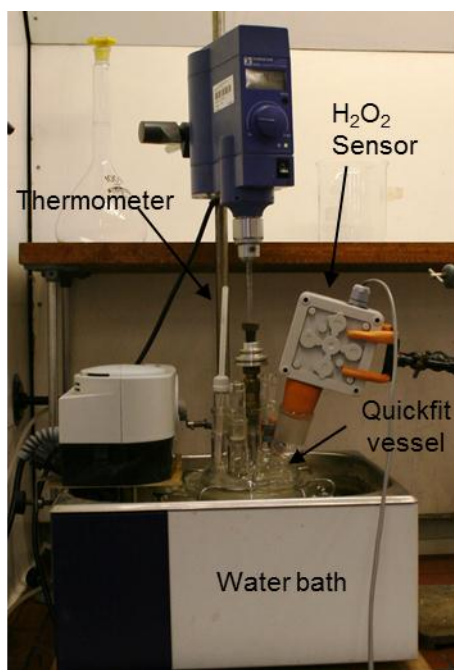
Figure 3.2 Photographic representation of the hydrogen peroxide calibration bath.

3.2.1 Titration Method for Evaluating Liquid Hydrogen Peroxide Concentration using Potassium Permanganate

To find the exact concentration of liquid hydrogen peroxide used in the calibration of the sensor, it was titrated against potassium permanganate (Jeffery *et al.*, 1989). The reaction is as follows:

$$2KMnO_4 + 5H_2O_2 + 3H_2SO_4 \rightarrow K_2SO_4 + 2MnSO_4 + 8H_2O + 5O_2 \qquad (3.1)$$

Using the stoichiometry of the reaction equation, if the sulphuric acid is in excess then two moles of potassium permanganate would be required for every five moles of hydrogen peroxide for a complete reaction. From this, using the molar concentration ($n = CV$ where n is the number of moles, C is the molar concentration and V is the volume of the solution) it is possible to derive the following equation:

$$2C_{H_2O_2}V_{H_2O_2} = 5C_{KMnO_4}V_{KMnO_4} \qquad (3.2)$$

Using the volume and concentration of potassium permanganate required to react with the initial volume of hydrogen peroxide, it is possible to calculate the exact concentration of the hydrogen peroxide.

For the titration, a 0.1 M solution of potassium permanganate was produced from 99+ % specified crystallised form of potassium permanganate (Fisher Scientific, UK) and deionised water. As the concentration of hydrogen peroxide was approximately known, the volume used for the titration was selected accordingly. The required volume of hydrogen peroxide was then added to a conical flask with excess (5 ml) 1 M sulphuric acid. A 25 ml burette was used to slowly add the potassium permanganate solution to the hydrogen peroxide mixture until the solution turned a faint purple colour, indicating that all the hydrogen peroxide has been reacted. If the permanganate was added too quickly or the solution was left too long after the titration, then the liquid became a brown colour, due to the formation of manganese dioxide. If this happened during the titration, the liquid was discarded and the procedure restarted as it could lead to errors within the results (Jeffery *et al.*, 1989). It is also important to note that at the beginning of the titration the liquid could turn the same faint purple colour as at the end. This lag was observed occasionally and gentle mixing was applied until the purple colour disappeared and then the titration was completed as normal. This process was repeated three times and an average of the resulting concentrations used. An overall concentration was calculated through titration of the hydrogen peroxide solution before and after the calibration.

3.2.2 Calibration Procedure for Hydrogen Peroxide Sensors

The vapour-liquid equilibrium curve for hydrogen peroxide at 25 °C was calculated alongside the measurements taken using the sensor is shown in Figure 3.3 (Schumb, Satterfield & Wentworth, 1955). This shows that the data points are all clustered around the equilibrium line indicating that the sensor is well-calibrated. When there was a relative error of more than 10 % between the equilibrium line and the data point, the sensor was recalibrated.

To calibrate the sensor, it was placed in the stirred tank over a titrated 3 % (w/v) solution and the span control was adjusted to give a vapour concentration of 13 ppm. For the calibration the lid of the sensor was removed; a multi-meter was attached to the metal pins and the span switch adjusted until the voltage was the same as the calculated vapour concentration. After the sensor had

been calibrated it was left in the hydrogen peroxide vapour for at least 30 min to ensure that it continued to read the correct value. The sensor was then removed from the calibration bath and exposed to air to zero it overnight. The next day, the calibrated sensor was placed over a liquid hydrogen peroxide concentration of 16 % (w/v) which gave a vapour concentration of 92.4 ppm to ensure it was correctly calibrated over the whole range.

The sensor readings are shown alongside the vapour-liquid equilibrium line in Figure 3.3. This demonstrates that the hydrogen peroxide sensors were kept well calibrated with all of the sensor readings being within 10 % of the expected equilibrium values. Table 3.2 shows the time to reach 95 % and 99 % of the equilibrium value; the data shows that it takes between 5 min and 15 min to reach the value. The time required to reach the equilibrium concentration varied depending on the extent to which the vapour phase was disturbed when the sensor is put in place, with a longer T_{95} or T_{99} time meaning that the vapour phase was more greatly disturbed.
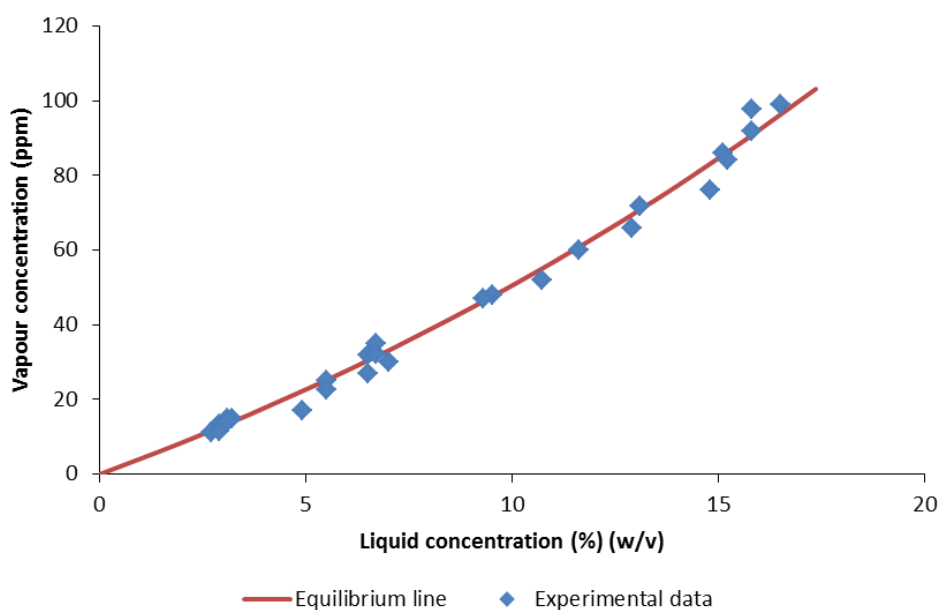
Figure 3.3 Calibration data for the hydrogen peroxide sensor carried out at 25 °C and presented alongside the corresponding calculated equilibrium curve.

Table 3.2 Time required to reach 95 % and 99 % of the vapour-liquid equilibrium value using the calibration bath

H_2O_2 vapour concentration (ppm)	T_{95} (min)	T_{99} (min)
90	7.1	9.7
75	11.2	15.7
50	4.0	4.5
25	7.2	13.8
10	1.5	2.7

3.3 Humidity Sensors and their Calibration

Six combined temperature and humidity sensors (OM-62 Temperature/Humidity data logger) (Omega, UK) were used throughout the project to record the humidity in the chamber when the spores were being exposed to the hydrogen peroxide. In order to verify their calibration was correct, the humidity was measured over different salt solutions, pure water and silica gel.

Table 3.3 Comparison between salts used and expected relative humidities.

Salt	Chemical Formula	Relative Humidity (%)
Water	H_2O	100
Potassium iodide	KI	69
Calcium nitrate	$Ca(NO_3)_2 \cdot H_2O$	57
Sodium iodide	NaI	38
Silica Gel		0

The saturated salt solutions were placed into the bottom of a desiccator and left overnight to reach equilibrium. Once equilibrium was reached, the sensors were all placed into the desiccator and set to a frequency of once every 15 min. The sensors were left in the desiccator recording for at least 12 h before they were removed and the results plotted (Figure 3.4).
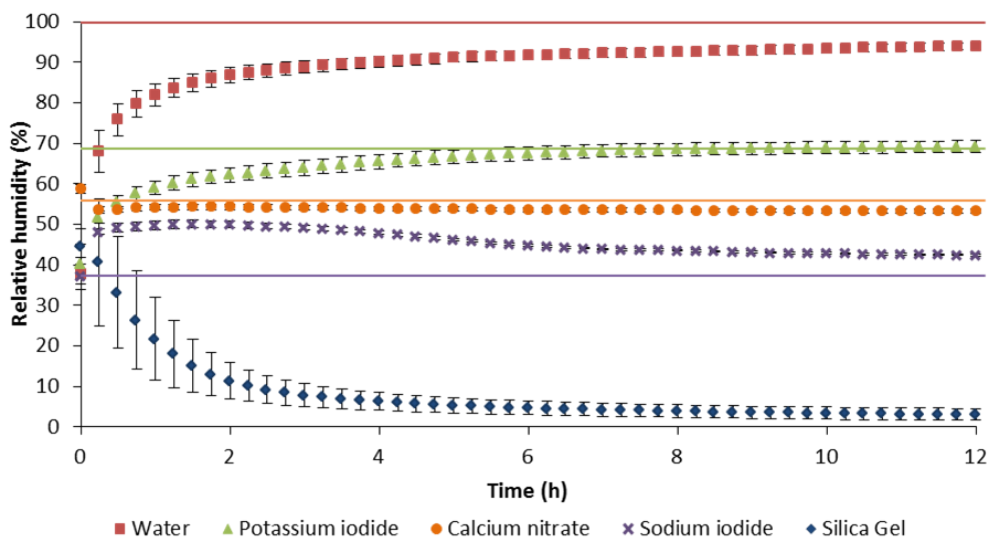
Figure 3.4 The relative humidity recorded by the humidity logger over different salt solutions.

From the data generated, it was concluded that the humidity sensors were accurate around their mid-range and begin to deviate towards the extremes. The data demonstrate the sensors took about 2 h to achieve a steady reading; however, this is most likely due to the environment within the desiccator being disturbed when the lid was opened so it represents the time taken to reach equilibrium. This does nevertheless show that the humidity readings from the sensors are sufficiently accurate for the range used within this study.

3.4 Preparation of *Bacillus subtilis* Spore Stock

B. subtilis spores (from a spore stock produced by Pawel Wolyniec, Loughborough University) were used for preliminary testing of the exposure chamber as the bacterium is non-pathogenic (Class 1 bacteria) and has been used previously to show the efficacy of hydrogen peroxide disinfection. The spores used were *B. subtilis*, ATCC 6633, produced as described by Gardner and Shama (1998). In brief, 100 ml Oxoid Nutrient Broth (Fisher Scientific, UK) was inoculated with a loopful of *B. subtilis* (ATCC 6633, NCIMB 8054) in an Erlenmeyer flask and incubated at 30 °C for 24 h on a rotary incubator at 200 rpm. Sporulation Agar (BD, USA) plates were then inoculated with 0.2 ml of the resulting culture, using a sterile disposable plastic spreader. The plates were then incubated for 14 days at 30 °C before the spores could be harvested. To harvest the spores, 5 ml sterile water was pipetted onto each of the plates and

the surface was scraped with an inoculation loop to detach them from the agar and the liquid collected into centrifuge tubes. The resulting suspension was then washed immediately by centrifuging at 8000 g for 20 min. The supernatant fluid was poured off and replaced with 10 ml PBS and vigorously mixed using a vortex mixer to re-suspend the pellet. This process was then repeated a further two times in order to remove any remaining vegetative cells. The resulting spore suspension was heat treated at 70 °C for 30 min in order to inactivate any remaining vegetative cells. Resulting spore suspensions were serially diluted and grown on TSA (tryptone soya agar) (Oxoid Ltd., UK) plates, which were enumerated after 24 h. The resulting spore stock had a concentration of 10-$\log_{10}$.

3.4.1 Preparation of Bacillus subtilis Spore-Laden Membrane Bioindicators

Spores were filter-deposited onto 13 mm isopore membranes (Millipore, GTTP01300) for exposure in the chamber. Spores with approximately a 2 μm length were captured by the membranes (shown by the SEM images in Section 3.4.2) which have a pore size 0.22 μm. Before the spores could be deposited onto the membranes, it was necessary to sterilise the membranes. For this, the membranes were placed into filter holders (Sartorius 13 mm syringe filter holder) (Sartorius, UK) using 30 μl deionised water to moisten and keep them in place, and the top secured. A small amount of non-absorbent cotton wool was then used to seal the syringe hole in the top. Once assembled each filter holder was loosely wrapped in aluminium foil and placed in a beaker; they were then autoclaved at 121 °C for 15 min before use.

Using stock prepared as previously described (Section 3.4), a 30 ml 8-$\log_{10}$ spore stock was produced; 1 ml of this spore stock was filtered through each membrane. To do this, each filter holder was unwrapped, the cotton wool removed and the bottom half of a disassembled 2 ml DB Plastipak syringe was placed in the top of the filter holder. To remove any remaining water the filter holder was then placed into a rubber bung with a suitable aperture on top of a Büchner flask and a vacuum of 0.5 bar was achieved in the flask using a vacuum pump. Once the spores had been deposited on the membrane, the

filter holder was dismantled and the membrane removed using tweezers, the membranes were then either placed onto sticky tape ready (Figure 3.5), on a acetate sheet inside a Petri dish to stop any contamination, to be exposed to the hydrogen peroxide or placed into 10 ml PBS containing Tween-80 and catalase as a control (Section 3.8.1). Once the membranes had been removed, the used filter holders were autoclaved at 121 °C for 15 min, washed using deionised water and dried ready to be reassembled.
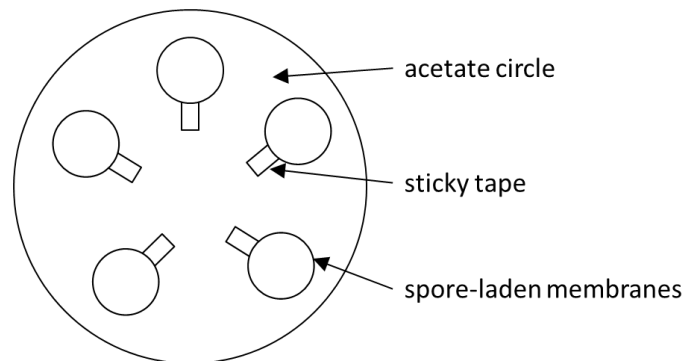
Figure 3.5 Image detailing how the spore-laden membranes were arranged for inactivation in the exposure chamber.

3.4.2 SEM Images *Bacillus subtilis* Spore Membrane Bioindicators

To evaluate whether uniform coverage of the *B. subtilis* was achieved across the whole membrane, SEM images were taken. The membranes were prepared as above, following which the spores were set and dehydrated as described by Perdigao *et al*. (1995). To inactivate and set the spores on the membranes they were placed in a 1 % glutaraldehyde solution for 1 h before being removed and replaced in a 2 % glutaraldehyde solution overnight. Once set, the spores were dehydrated in ascending grades of ethanol (20 %, 40 %, 60 %, 80 %, 100 % and 100 %) for 15 min each. After the ethanol dehydration, the spores were dried using hexamethyldisilazane (HMDS); the membranes were first placed into a 50/50 mixture of HMDS and ethanol followed by 100 % HMDS both for 15 min. The membranes were then removed from the solution and left in a fume hood to evaporate off any HMDS. Once dry, the membranes were mounted onto 13 mm aluminium tabs using carbon sticky tabs and coated with a layer of gold-palladium approximately 20 nm thick using a sputter coater (Quorum Technologies Ltd, UK) at 20 mA for 60 s. After the membranes were coated with gold-palladium, a small amount of silver paste was placed on the

edge of the membrane to ensure it was completely grounded. The samples could then by viewed and imaged using a Stereoscan 360 scanning electron microscope (Cambridge Scientific Instruments Ltd, UK) operated at 15 kV using a tungsten filament at a working distance of 25 mm.
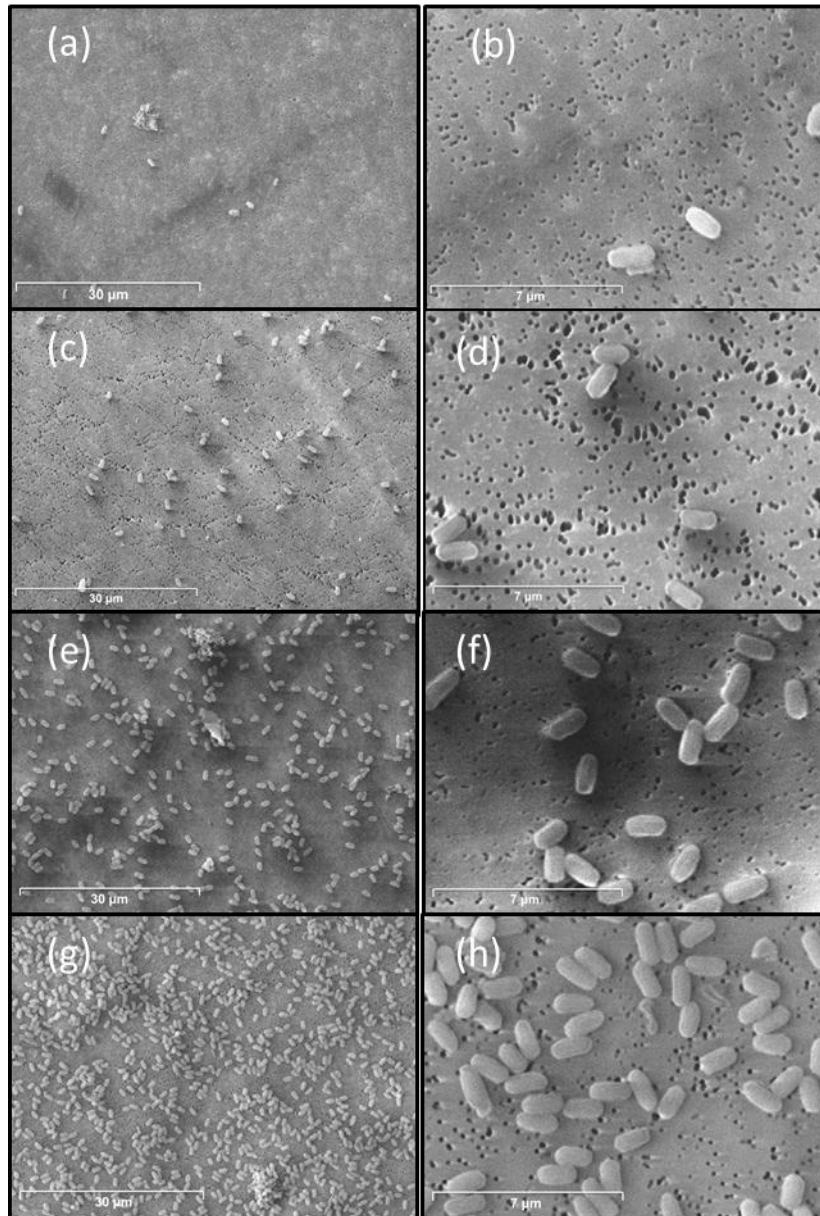
Figure 3.6 SEM images of *B. subtilis* showing membranes laden with 5-$\log_{10}$ (a and b), 6-$\log_{10}$ (c and d) 7-$\log_{10}$ (e and f) 8-$\log_{10}$ (g and h) spores.

Filtering the spores using this method resulted in an even coverage of the *B. subtilis* spores on the membranes allowing for even exposure to the hydrogen peroxide (Figure 3.6). Preparation of spore bioindicators using the filtration method results in more uniform coverage of spores, whereas direct

pipetting of spore solutions onto stainless steel discs followed by air drying can cause stacking of the spores (Bayliss *et al.* 2012). Uniform deposition of the spores is important as any stacking of the spores could result in diffusional resistances which would affect the local concentration of the hydrogen peroxide to which the spores are exposed.

3.5 Production of *Clostridium difficile* Spore Stocks

C. difficile spores were produced using two different methods: firstly, a method where the bacteria were cultured on an agar plate before sporulation and recovery. The second method used liquid media and was able to produce a much higher titre of purified spores.

3.5.1 *Preparation of Clostridium difficile Spore Stock using Agar Plates*

Spore suspensions of *C. difficile* ribotype 027 were prepared in a similar method as described by Shetty *et al.* (1999). To grow the bacterium, an inoculation loop of *C. difficile* from cryopreserved stocks was isolated from a faecal specimen (University Hospital of Leicester, NHS trust) from a *C. difficile*-infected patient and PCR ribotyped by Krusha Patel (PhD student, Loughborough University). This was then struck out onto brain-heart infusion (BHI) agar containing 7 % defibrinated horse blood and incubated in an anaerobic chamber (mini MACS, Don Whitley Scientific Ltd., UK) for 6 days at 37 °C. After incubation, the plates were left in aerobic conditions at room temperature overnight to allow sporulation of the organism. The spore-vegetative cell mixture was collected using an inoculation loop and washed in a 50 % IMS/PBS (industrial methylated sprits/phosphate-buffered saline) solution to kill any vegetative bacteria. Samples were then centrifuged at room temperature for 20 min at 3500 *g* (Beckman-Coulter, Allegra-X-22R) to separate the spores from the IMS/PBS solution. The pellet was then re-suspended in PBS and heat-shocked at 60 °C for 10 min to ensure that only the *C. difficile* spores remained viable. Samples were serially diluted in PBS and plated onto BHI agar supplemented with 0.1 % sodium taurocholate (Sigma-Aldrich, UK). Plates were incubated for 24 h before cfu were enumerated.
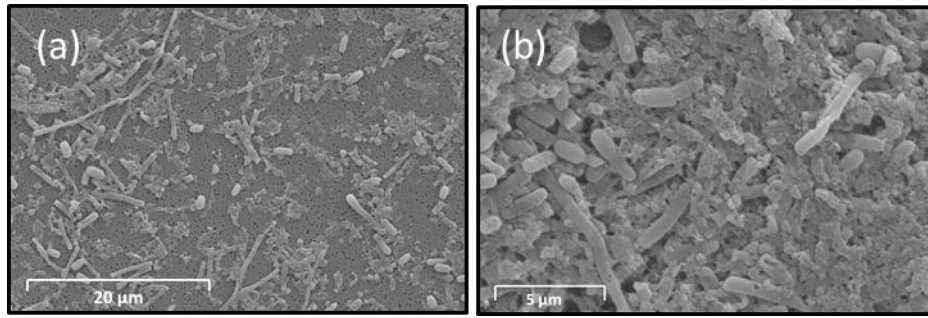
Figure 3.7 SEM images of 6-log$_{10}$ *C. difficile* spore stocks produced using agar plates and deposited on membranes.

SEM images of the spores produced using this method are shown in Figure 3.7. These images reveal much debris including agar left in the spore stock prepared by this method. This could affect the resistance of the spores to the hydrogen peroxide vapour making them either more or less resistant than a uniform layer of purified stock.

Using this method, spore stocks of different ribotypes were produced to identify any differences between the different strains of *C. difficile*. Three other strains were used: (i) strain isolated from a faecal specimen from an infected patient (ribotype 014), (ii) strain isolated from hospital environmental sampling and not a faecal sample, (ribotype 103) both isolated by Krusha Patel, (iii) isolated from an estuary sediment sample (ribotype 220) by Katherine Hargreaves (PhD student, University of Leicester).

3.5.2 Preparation of Clospore Liquid Medium and Clostridium difficile Spore Stock

C. difficile (ribotype 027) spore stocks were produced using Clospore media as described by Perez *et al*. (2011). This method can be divided into three steps: the production of a working suspension, the making of the high titre stock and the purification of the spores.

Step 1 – Making the spore suspension: In this stage, an initial low titre spore stock (4-log$_{10}$) was produced which could be used as an inoculum for step 2. For this a *C. difficile* strain, ribotype 027, was grown for 48 h on fastidious anaerobic agar (fastidious anaerobe broth (Oxoid, UK) containing 1 %

agar bacteriological (Oxoid, UK), FAA) anaerobically. From this plate, a single isolated colony was suspended in 10 ml CB (Columbia broth) (difco, BD, UK) and grown for 24 h in an anaerobic chamber. Using the resulting culture, 50 µl was used to inoculate ten 100 mm Petri dishes, containing CB with 7 % defibrinated horse blood and 1.5 % agar. The plates were then incubated anaerobically, firstly for 7 days at 37 °C, then for a further 15 days at 23 °C. Once the cultures had grown, the plates were scraped to harvest the spores, which were then suspended in 200 ml double-distilled water. This solution was then washed by centrifugation for 10 min at 10,000 g at 4 °C. The pellet left after the supernatant was poured away and were re-suspended in the same 200 ml double-distilled water twice before the final pellet was re-suspended in 2 ml to increase the concentration. This resulting solution was then heat shocked at 70 °C for 10 min to kill any remaining vegetative cells. This spore suspension could then be kept in the fridge at 3 °C and used as the inoculum for the next stage.

Step 2 – Making a high titre spore stock: An inoculation loopful of the stock produced in step 1 was streaked out onto an FAA plate and incubated for 48 h anaerobically at 37 °C. A single isolated colony was then picked from the plate and suspended in 5 ml CB in a 15 ml centrifuge tube; the solution was then placed back into the anaerobic chamber for a further 48 h. An aliquot (50 µl) of the resulting culture was then used to inoculate a further 20 ml of CB in a 50 ml centrifuge tube and incubated for a further 24 h. The entire 20 ml culture was finally equally split between two 500 ml centrifuge bottles containing 250 ml Clospore broth (Table 3.4), and then incubated for a further 72 h at 37 °C before purification.

Table 3.4 Composition of the Clospore media.

Component	Chemical Formula	Composition (g/l)	Supplier
Special peptone	SPM	10	Oxoid, UK
Yeast extract		10	Oxoid, UK
Ammonium sulphate	$(NH_4)_2SO_4$	0.6	Fisher, UK
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	0.12	Fisher, UK
Calcium chloride dehydrate	$CaCl_2 \cdot 2H_2O$	0.08	Acros Organics, UK
Potassium carbonate	K_2CO_3	3.48	Sigma-Aldrich, UK
Potassium phosphate monobasic	KH_2PO_4	2.6	Sigma-Aldrich, UK
pH		7.9 ± 0.1	

Step 3 – Spore purification: The spore suspension produced in step 2 was centrifuged three times at 10,000 g and 20 °C for 10 min. After the first centrifuge step, both pellets were re-suspended in 17.5 ml double-distilled water before being combined to create a single spore suspension. This was then centrifuged as before and re-suspended in 35 ml. The final pellet was weighed and suspended in 10 ml 0.1 M sterile sodium phosphate buffer (pH 7.0) and transferred to a 50 ml centrifuge tube. A further 25 ml of the same buffer this time containing 20 µg lysozyme (from chicken egg white, Sigma) and 15 µg Proteinase K/mg wet pellet (Fisher Bioreagents) was sterilised using a 0.2 µm filter and added to the suspension. The resulting suspension was sonicated at 60 Hz (Grant ultrasonic water (XB2) bath) for 10 min. Once sonicated, the cell suspension was incubated in a water bath at 45 °C for 6 h, during which time it was sonicated every 2 h for 10 min. Once this was complete the cell suspension was left in the fridge at 4 °C overnight. The following day the spore stock was centrifuged and washed 3 times at 10,000 g and 20 °C for 10 min and re-suspended in the same volume of double-distilled water. The final pellet was re-suspended in 10 ml double-distilled water. The resulting stock was serially diluted and grown on BHIYT-Ly agar (3.7 % BHI, 0.5 % yeast extract, 0.1 % L-cysteine, 0.1 % sodium taurocholate and 1.5 % agar (all w/v)) which had previously been prepared by autoclaving for 15 min at 121 °C and left to cool to 55 °C after which 10 mg/l filter sterilised lysozyme was added. The growth on the plates was counted after overnight anaerobic incubation and the titre calculated ($9\text{-}\log_{10}$).

3.5.3 Preparation of Clostridium difficile Spore-Laden Membrane Bioindicators

C. difficile spore-laden membranes were produced in two different ways for exposure. The first of these were prepared using high concentrations of spores exposed to hydrogen peroxide and recovered as with *B. subtilis*. Membranes were also made up with different spore concentrations and after exposure to the hydrogen peroxide vapour these membranes were subsequently placed in broth to see if any of the spores remained viable (growth/no-growth). The membranes were made up in the filter holders and autoclaved as previously described (Section 3.4.1) for both procedures.

Spore recovery membranes were made up as described for *B. subtilis* membranes. In this instance, a 6-$\log_{10}$ spore stock was used which was produced using the agar plate method. A lower concentration was used because the spore stock produced using this method being yielded only 8-$\log_{10}$. A 30 ml volume of the 6-$\log_{10}$ spore stock was produced to make the membranes for exposure. Spore stock (1 ml) was placed in a syringe on top of the filter holder and pushed though. The filters were then placed into a tight fitting bung and placed on top of a Büchner flask, to remove any residual water using a hand pump to achieve a vacuum of 0.4 bar. Once complete and laden with spores, the membranes were removed from the holder using tweezers. These membranes were then stuck on to acetate film using autoclave tape and placed in a sterile Petri dish ready for exposure to hydrogen peroxide. The control membrane bioindicators were placed directly into the recovery liquid as described in Section 3.8.1.

Different titre *C. difficile* membrane bioindicators with different surface loading of spores (1-$\log_{10}$ to 7-$\log_{10}$) were made from stocks produced using the Clospore liquid media production method. The spore stocks were diluted to a concentration of 7-$\log_{10}$ using PBS for the highest spore concentration. Using the spore stock, different concentrations were made up at 10-fold dilutions. Membranes of the required concentrations were then produced using the method described above for the spore recovery of *C. difficile* from membranes. The spore concentration of the membranes produced depended on the

expected log reduction, with the lower concentration membranes being used for shorter exposure times and vice versa. Three membranes of three or four different concentrations were produced for each exposure time and fixed onto the acetate as before (Figure 3.5). Controls were carried out using membranes without spore deposition and ones laden with the lowest concentration of spores (limit of detection is less than $1\text{-}\log_{10}$); these membranes were placed directly into the recovery medium.

3.5.4 SEM Images of Clostridium difficile Spore Membrane Bioindicators

To image the *C. difficile* spore laden membrane bioindicators using SEM for both spore production methods, spores were deposited on the membrane using the method described previously. These membranes were then submerged in 2 % glutaraldehyde (diluted down from a 25 % solution of glutaraldehyde using deionised water) overnight to fix them to the membrane surface. Once fixed, the glutaraldehyde was removed and the membranes washed three times with deionised water (each wash step had a 30 min dwell stage). The membranes were dried in a laminar flow cabinet and stuck onto 13 mm aluminium tabs using carbon sticky pads. Membranes were then coated with gold approximately 30 nm thick in a sputter coater (Polaron SC7640) for 90 sec at 20 mA. After coating the spores were imaged using a Hitachi scanning electron microscope (S3000H) (Hitachi High-Tech, Tokyo, Japan).
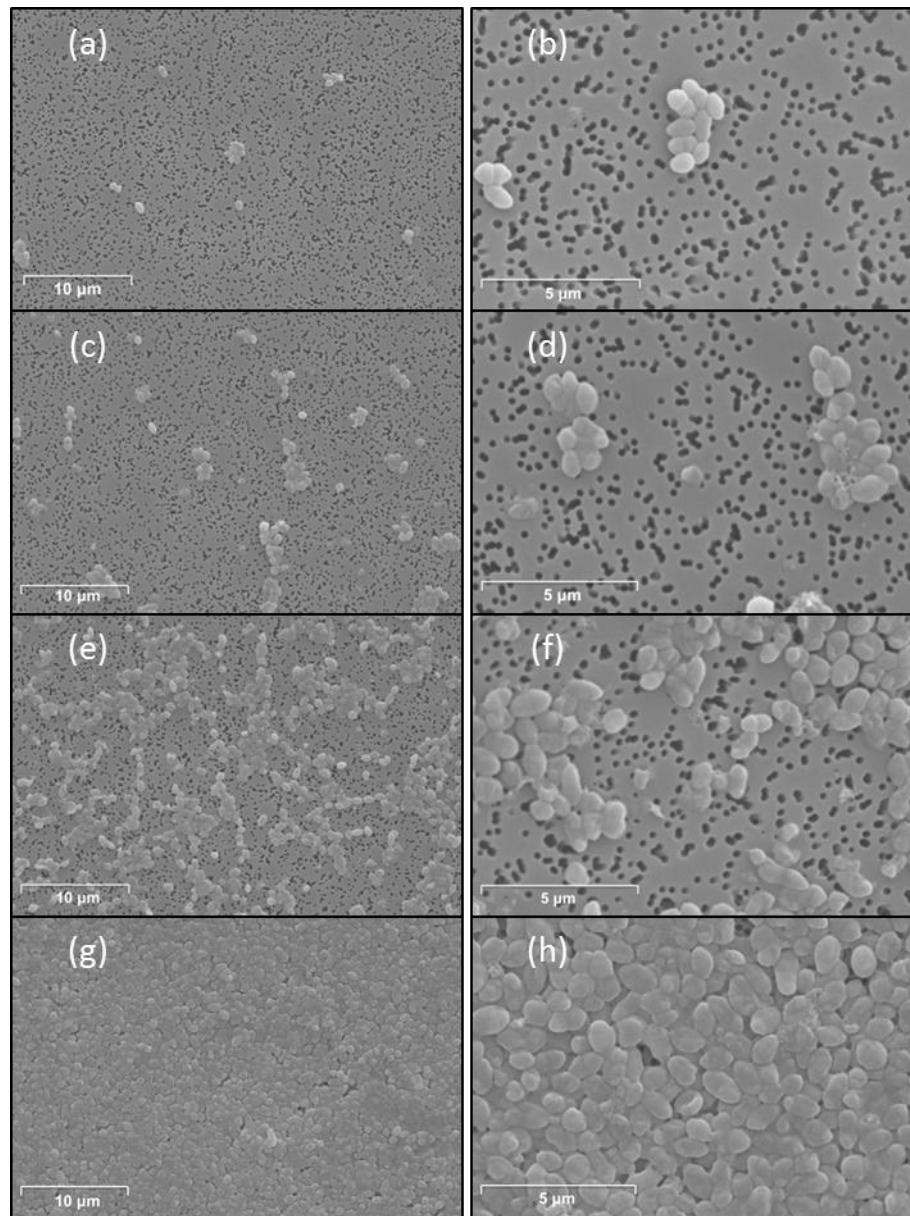
Figure 3.8 SEM images showing membranes laden with *C. difficile* spores produced using the Clospore method: 5-$\log_{10}$ (a and b), 6-$\log_{10}$ (c and d), 7-$\log_{10}$ (e and f) 8-$\log_{10}$ (g and h).

Figure 3.8 shows uniform spore surface coverage ensuring the spores would be exposed to a uniform concentration of vapour hydrogen peroxide in the exposure chamber. The SEM images from the two different methods of production of infer that the Clospore production method produces a much cleaner spore stock, as with the *B. subtilis* membranes, than the agar plate method (Figure 3.7). The main advantage of the Clospore method is that the production of a cleaner spore stock allows for even deposition of the spores and even exposure to hydrogen peroxide.

3.6 Hydrogen Peroxide Exposure Chamber

The hydrogen peroxide exposure chamber was designed to allow spore-laden bioindicators to be exposed to known concentrations of hydrogen peroxide for different exposure times. Hydrogen peroxide inactivation kinetic data were obtained using the bioindicators prepared as described in Section 3.5.3.

3.6.1 Design of Hydrogen Peroxide Vapour Exposure Chamber

Spore laden bioindicator membranes were exposed to hydrogen peroxide vapour in the exposure chamber (Figure 3.9). The chamber was designed to fit into a fume cupboard, with a relatively small footprint of 900 x 670 mm, so it could be operated safely Figure 3.10. It was comprised of three exposure boxes which were identical (dimensions (L x W x D) 665 x 100 x 133 mm) and each had a 30 mm thick sponge diffuser at the air inlet to ensure mixing of hydrogen peroxide within the box thereby resulting in a uniform distribution of hydrogen peroxide vapour within the exposure chamber.

For exposure, a constant air flow through the chamber was required to allow the hydrogen peroxide concentration to be maintained. Air flow was generated using a Vent-Axia mixed flow fan (ACM150) with an extract performance of 460 m^3/h (Vent-Axia Limited, UK), which maintained an air flow of 7 x 10^{-3} m^3/s at the chamber exit, measured using an anemometer (Kestrel 3000 wind meter) (Kestrel, USA). From the fan the air flowed into the mixing chamber where it combined with the hydrogen peroxide vapour as it was produced via flash evaporation. Hydrogen peroxide solution of the required concentration was fed at a pre-determined flow-rate (Table 3.5), using a Cole-Parmer single-syringe infusion pump (WU-74900-05) (Cole-Parmer, UK) fitted with a 60 ml syringe (BD Plastipak, USA), onto a hotplate set to 130 °C, where it flash evaporated and mixed with the air to create the required vapour concentration. The air containing the hydrogen peroxide vapour was then passed through the three boxes which contained racks upon which the membranes could be placed for exposure. The hydrogen peroxide vapour/air mixture passed through the three boxes in series so the membranes could be

removed from the last box without disturbing the others (described in Section 3.7.1). This allowed for exposure of the spores at a number of different time points during the same run. On leaving the third box, the hydrogen peroxide vapour/air mixture was piped into the exhaust unit where the hydrogen peroxide concentration and humidity were measured using an ATI GasSens hydrogen peroxide monitor and an Omega OM-62 Temperature/Humidity data logger.
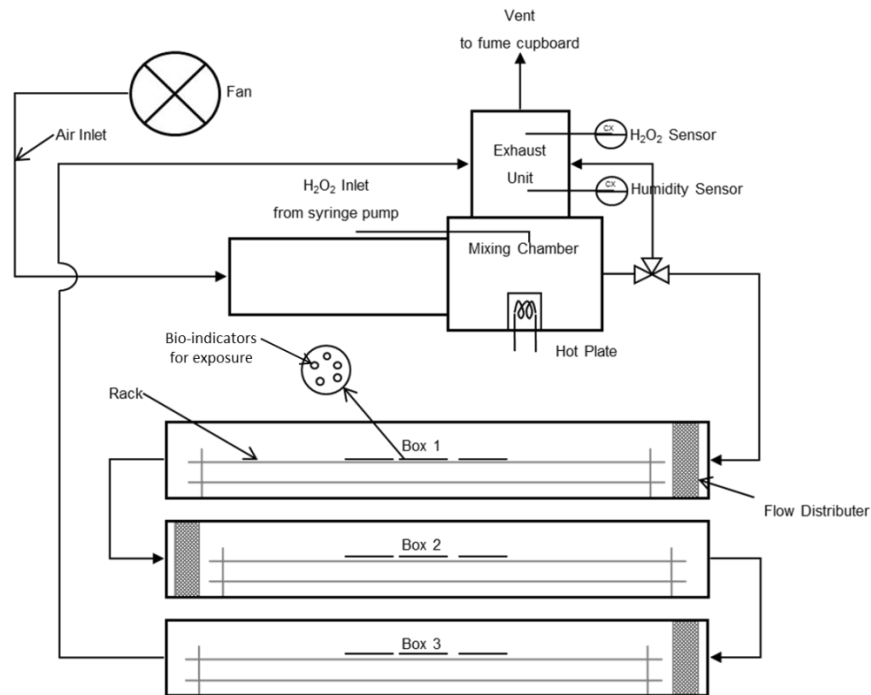
Figure 3.9 A Schematic diagram of the exposure chamber.
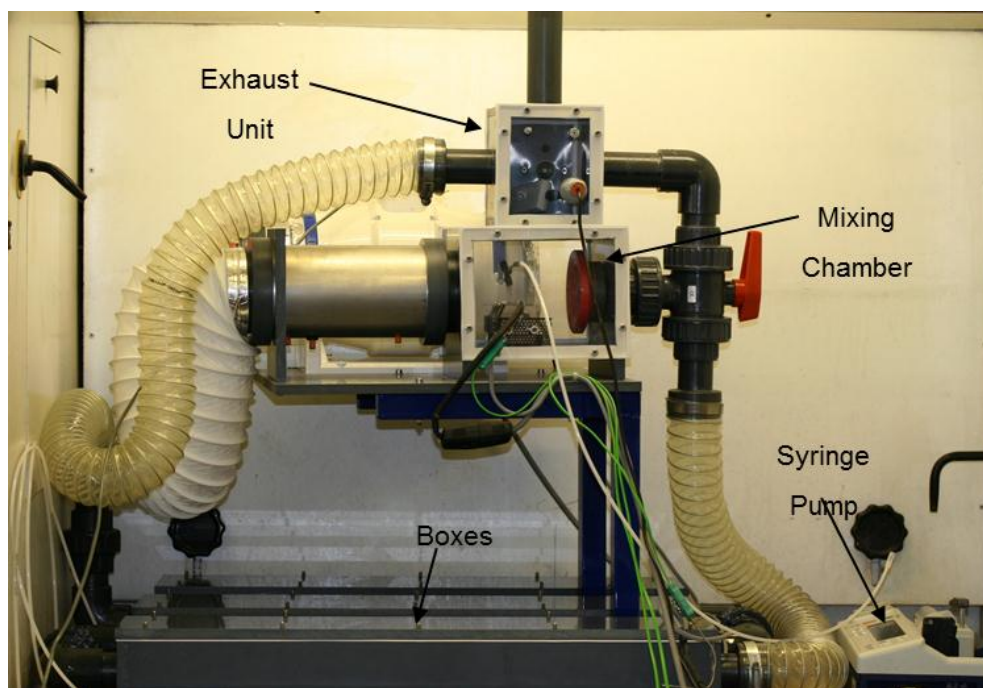
Figure 3.10 Photographic image of the exposure chamber.

Table 3.5 Hydrogen peroxide liquid concentrations and flow-rate used to reach the desired vapour concentrations inside the exposure chamber at an air flow of 7×10^{-3} m^3/s.

H_2O_2 vapour concentration (ppm)	H_2O_2 liquid concentration (%) (w/v)	Liquid flow-rate (ml/h)
90	6	17-13.5
75	4.5	17-13.5
50	3	17-13.5
25	9	4.5-9
10	6	2-4.5

3.6.2 Exposure Chamber Commissioning

Commissioning of the exposure chamber was carried out to ascertain: (i) that it was possible to reach and maintain a required humidity or hydrogen peroxide vapour/air concentration (ii) the uniformity within all three boxes to ensure that the log reductions using the bioindicators achieved could be reproduced in any of the boxes.

Humidity tests were carried out to investigate achieving and maintaining the required absolute humidity of 0.016 kg/kg (relative humidity 80 %) within the boxes. A mass balance (Equation 3.3) of the system was used to estimate the flow-rate of water required to generate the required humidity. For this, the

boxes were sealed and the fan started at the highest setting (8.5 m/s), the syringe pump containing pure water was then started at the calculated flow-rate. Water was dropped onto the hot plate where it evaporated and mixed with the air flow through the system from the fan. Once started, the water flow-rate was then adjusted to achieve the required humidity. This flow-rate was then maintained with the syringe being refilled as required to ensure a stable humidity was maintained, and to investigate what effect refilling the syringe had on the equilibrium.

$$m_W = (m_A)_{wet} (Y_O - Y_I)(1 - Y_I) \qquad (3.3)$$

m_W is the required mass flow-rate of the water (kg/s), $(m_A)_{wet}$ is the mass flow-rate of the air at the inlet (kg/s) and Y_I and Y_O are the absolute humidity of the air at the inlet and outlet respectively (kg water/kg dry air).

As Figure 3.11 shows, the humidity in the exhaust unit could be accurately controlled at both the highest and lowest fan settings. An 80 % relative humidity (corresponding to an absolute humidity of 0.016 kg/kg) could be achieved and maintained over a period of 40 min. Using the mass balance it was calculated that a water flow of 300 ml/h and 113 ml/h were required for the high and low air velocities respectively, whereas the actual flow-rates required were 200 ml/h and 90 ml/h. It took very little time for the chamber to reach steady-state with the sharp drop in the humidity being a result of the syringe pump being refilled after which the humidity recovered in about 1 min. By comparing the two lines it can be seen that at the lower air velocity the syringe only needs refilling half as often to maintain the same humidity. The plot for the lowest fan speed shows a small dip in humidity at the start; this is due to the pump speed being altered to achieve the required humidity.
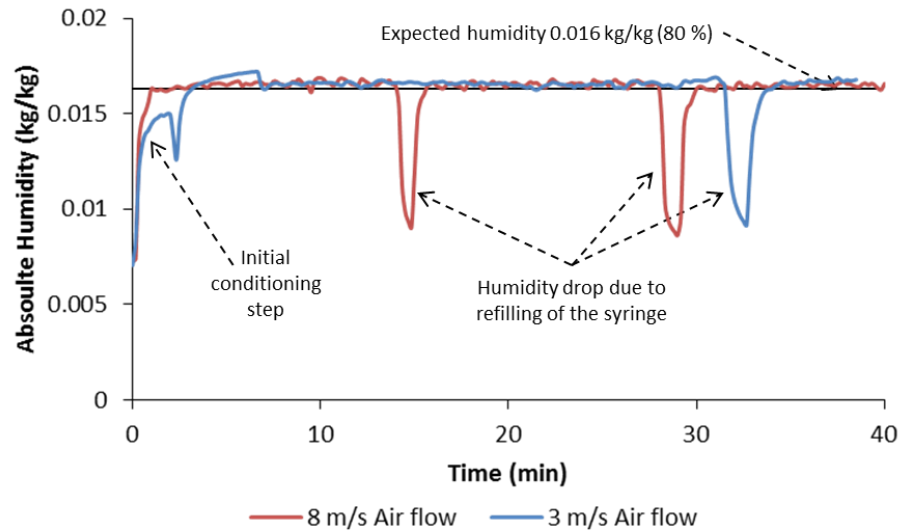
Figure 3.11 The absolute humidity and the effects of different air speeds.

The humidity within the three boxes and the exit box is shown alongside the room humidity in Figure 3.12. Box 1 and 2 show a very similar humidity trace with box 3 having a slightly higher humidity with the exit box being higher still. This is contrary to what was anticipated as it was expected that water within the air would condense on the surfaces within the chamber causing the humidity to decrease. The increase (0.001 kg/kg) in the absolute humidity between boxes 1 and 2 and box 3 could as a result of the error in the sensors as shown in Section 3.3.   Large humidity changes within the exit box are caused by a small amount of extra liquid being deposited onto the hotplate as the syringe pump is refilled. It was also observed that due to the relatively low flow-rate (15 ml/h) of liquid there is only a small increase in the humidity within the exposure chamber.
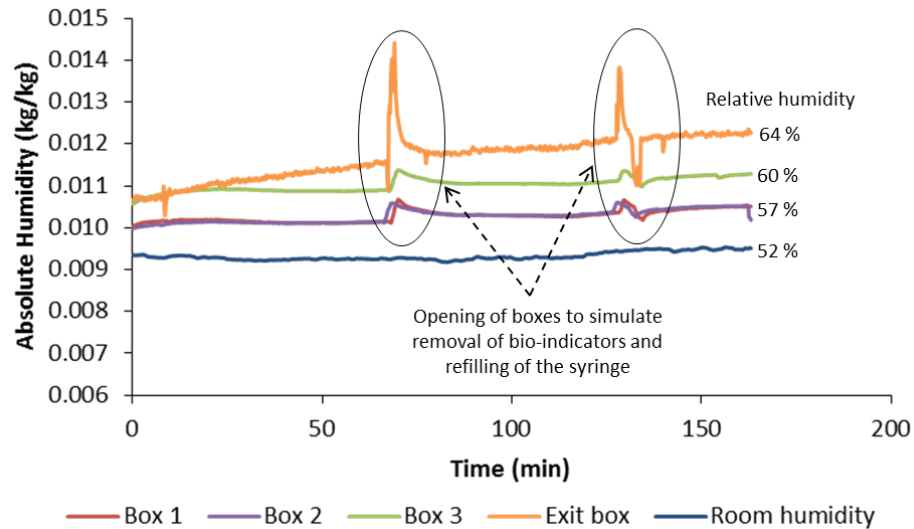
Figure 3.12 Humidity trace within each of the boxes shown against the room humidity at ambient temperature.

The hydrogen peroxide concentration was investigated using a mass balance to calculate how much of a known concentration of liquid hydrogen peroxide was required to generate the expected vapour phase concentration (Equation 3.4). Although this equation does underestimate the required flow of liquid hydrogen peroxide, therefore trial and error is used to find the actual flow-rate required. For example at an air flow of 7 x 10^{-3} m^3/s and a hydrogen peroxide feed of a 6 % (w/v) solution at 15 ml/h the mass balance predicts a hydrogen peroxide vapour concentration of 29 ppm compared to 90 ppm achieved in practice. The chamber was started with the liquid flow-rate being set according to the mass balance calculations as with the humidity tests. Both the hydrogen peroxide concentration and the humidity were recorded in the exhaust unit allowing the time to reach steady-state to be calculated. The test also investigated the stability of the hydrogen peroxide concentration and the effect of refilling the syringe.

$$y_h = \frac{m x_h}{\left(m_A\right)_{wet} + m} \tag{3.4}$$

$(m_A)_{wet}$ and m are the mass flow-rates of the air and liquid respectively (kg/s), x_h is the mass fraction of hydrogen peroxide in the liquid at the inlet and y_h is the mass fraction of the hydrogen peroxide in the air at the outlet.

An example of the ability to reach and maintain a specific hydrogen peroxide concentration is shown in Figure 3.13. In the example, a 6 % or 3 % hydrogen peroxide was fed onto the hotplate at 15 ml/h with an air flow-rate of 7 m/s to achieve a 90 ppm or 50 ppm hydrogen peroxide concentration respectively. Around 90 % of the required concentration was achieved within around 1 h and remained relatively stable, although the chamber is left for 2 h to 2.5 h before exposure of the membrane bioindicators. Drops in concentration occur when the boxes were opened to simulate the samples being removed. As expected when the samples are removed the concentration drops, however, the required concentration recovered within 5 min.
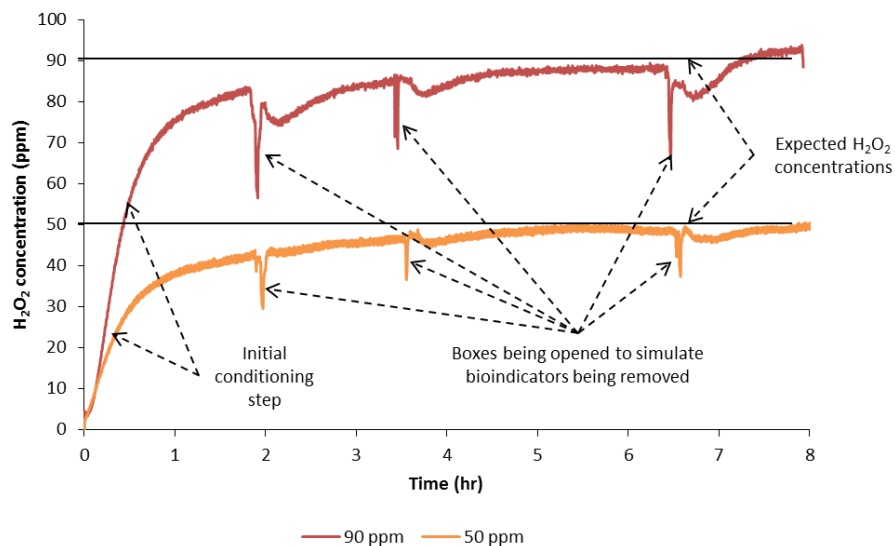
Figure 3.13 Different hydrogen peroxide concentration showing the initial conditioning step and the effect opening the boxes has on the hydrogen peroxide concentration.

It is important to find the time required to reach the hydrogen peroxide steady-state concentration to ensure that the exposure chamber has reached the concentration before the bioindicators are placed inside. Table 3.6 shows the times taken to 95 % and 99 % of the steady-state. In general as expected the time required to reach the higher concentrations were longer, although the time to reach 90 ppm was shorter than that to reach both 50 ppm and 75 ppm (Table 3.6). 95 % of steady-state is reached for all but 75 ppm within 2.5 h increasing to 99 % within 3.5 h. This means that the chamber needs to be set up and left for a minimum of 2.5 h before any bioindicators are added to ensure steady-state has been reached.

Table 3.6 Times to achieve to 95 % and 99 % of the steady-state hydrogen peroxide concentration.

H_2O_2 concentration (ppm)	T_{95} (h)	T_{99} (h)
90	1.83	2.89
75	2.76	3.42
50	2.22	3.24
25	1.19	1.49
10	0.66	0.81

From the commissioning of the exposure chamber, it can be concluded that the exposure chamber allowed control of the desired steady-state concentrations of hydrogen peroxide vapour in order to evaluate inactivation kinetics of bioindicators

3.6.3 Preliminary Testing of the Hydrogen Peroxide Exposure Chamber Using Petri Dishes

Preliminary tests were carried before the development of the membrane bioindicator method out using *B. subtilis* spores produced as described in Section 3.4. For exposure, 1 ml of the spores stock was spread on to the Petri dishes containing 10 ml TSA using a sterile disposable plastic spreader. Different spore concentrations (3-$\log_{10}$ to 8-$\log_{10}$) were used to identify the concentration of hydrogen peroxide vapour at which all of the spores were killed.

In the first test, a hydrogen peroxide vapour concentration of 50 ppm was used and samples were placed in the three exposure boxes for times of 30 min, 1 h and 2 h. The Petri dishes containing the spores were placed onto wire racks and sealed in the chamber; the air flow (maximum) and hydrogen peroxide flow were then started. A hydrogen peroxide concentration of 3 % (w/v) was used with an initial flow-rate of 15 ml/h. During the course of the experiment it became clear that the required 50 ppm concentration was not going to be reached, as the agar was absorbing the hydrogen peroxide. To overcome this, the hydrogen peroxide solution was changed to a 10.8 % (w/v) solution. The higher concentration hydrogen peroxide solution enabled the target vapour phase hydrogen peroxide concentration of 50 ppm to be reached. The flow-rate of the hydrogen peroxide was adjusted manually throughout the experiment to maintain the required concentration. After the first 30 min, box 3

was unsealed and emptied and as soon as the dishes had been removed the chamber was resealed and the experiment continued. Box 3 was emptied first as it is the last box in series; allowing the exposure in the other two boxes to carry on undisrupted. This was repeated for the other two boxes after 1 h (box 2) and 2 h (box 1). Once all the Petri dishes had been removed from the chamber they were incubated overnight at 37 °C to identify any growth.

The second experiment used a lower concentration of 20 ppm and the plates were removed after 15, 30 and 45 min using a similar procedure to the first experiment, but eliminating any weakness that had been identified. The first of these was the chamber reaching steady state at a much lower hydrogen peroxide concentration than expected. To overcome this, a high flow-rate was used at the beginning and reduced to a level where the correct concentration was maintained. Arrows were drawn on the bottom of the plates to show the direction of the flow and the plates were weighed to ascertain whether if they absorbed any hydrogen peroxide. The plates were also placed in the same order along each of the boxes and marked as to whether they were on the top or bottom of the rack.

3.7 Exposure of Spore Membrane Bioindicators to Hydrogen Peroxide Vapour in the Exposure Chamber

3.7.1 *Exposure of Bacillus subtilis Spore Membrane Bioindicators to Hydrogen Peroxide Vapour*

The previous section describes procedures for exposing spore deposited on Petri dished into the chamber; later these were replaced by membrane bioindicators. Once the exposure chamber had been set up (as described in Section 3.6.1) and steady-state reached (approximately 2 h) the membranes were placed into the chambers. The lid of each box was removed in turn, starting with box 3, and the membranes were placed on the shelf (30 cm down the box) before the box was resealed. Once all the boxes had been sealed, the chamber was left for the required exposure time, membranes were then removed from box 3 leaving the other two boxes sealed and undisturbed for longer exposure times. For the experiments carried out over 6 h (50, 75 and 90

ppm) box 3 was emptied after 1.5 h and the membranes replaced. It was then left for a further 3 h before box 2 (4.5 h) and box 3 (3 h) were emptied, and finally box 1 was emptied after a further 1.5 h (6 h time point). This gave exposure times of 1.5, 3, 4.5 and 6 h. A similar method was used for exposure to 10 ppm, however, the exposure times were longer at 6, 12, 24, 36 and 48 h. If required, the syringe pump was also refilled with hydrogen peroxide when the membranes were removed to ensure a constant feed of peroxide.

3.7.2 Clostridium difficile Membrane Exposure

C. difficile was exposed to the hydrogen peroxide using the same methodology as with the *B. subtilis*, and both the high titre (produced using the agar plates) and bioindicators with different spore loading (produced using the Clospore method) were exposed in the same way, too. The exposure chamber was set up and allowed to reach steady state at the required concentration. The membranes were then placed in the boxes for the time required. For *C. difficile*, the exposure time required to deactivate the spores was much lower than with *B. subtilis*. Therefore at 90 ppm, the membranes were removed at intervals of 10, 20, 30 and 40 min to be able to investigate the inactivation kinetics but were also left for up to 80 min to ensure that all the spores had been inactivated. As with *B. subtilis* the membranes were exposed for different times for each of the hydrogen peroxide concentrations. The membranes were exposed to hydrogen peroxide at concentrations of 10 and 50 ppm for up to 420 and 120 minutes, respectively.

Different ribotypes of *C. difficile* were also investigated in the same manner as the 027 strain, used as a model strain throughout the study. All strains were exposed to 90 ppm for up to 40 min to enable comparisons in inactivation of different strains.

The bioindicators with different spore loading were produced according to the inactivation expected and exposed in the same way as both the *B. subtilis* and *C. difficile* recovery method, with maximum exposure times of 10, 15, 45 and 120 min for hydrogen peroxide concentrations of 90, 50, 25 and 10 ppm respectively.

3.8 *S*pore Recovery and Enumeration

3.8.1 *Recovery of Bacillus subtilis spores from Membrane Bioindicators and Subsequent Enumeration*

Following exposure to hydrogen peroxide, the membranes were transferred to sterile vials containing 10 ml PBS (Oxoid Ltd., Basingstoke, UK) containing 0.05 % (w/v) Tween-80 (Fisher Scientific, UK) and 0.2 mg bovine liver catalase (2000-5000 units/mg, Sigma Chemical Co., UK). Catalase was added in order to arrest the action of any hydrogen peroxide that had adsorbed on to the membranes; Johnston, Lawson & Otter (2005) employed a similar approach. Membranes were then vortexed for 1 min and serially diluted as required in PBS before assessment of viability by pour-plating into TSA in triplicate. Agar plates were incubated at 37 ºC overnight and then counted. The agar plates for peroxide exposed samples were incubated for a further 24 h as the samples took longer to grow and the colonies were subsequently counted. The results obtained were then plotted against the time for each of the different hydrogen peroxide concentrations to show the inactivation curves.

3.8.2 *Recovery of Clostridium difficile Spores from Membrane Bioindicators*

After the membranes were exposed to the hydrogen peroxide, spores were recovered in a similar way to the *B. subtilis*. The membranes were placed into a 30 ml vial containing 10 ml PBS, 0.05% (w/v) Tween-80, 0.2 mg catalase and five sterile glass ballotini (2 mm). Glass Ballotini impact beads were added to help the recovery of the spores by striking the membrane as it is vortexed and knocking off the spores. The *C. difficile* spores were found to be harder to recover from the membranes than the *B. subtilis* spores, consequently the membranes were vortexed at 3,000 rpm for 5 min each. Once recovered, the spore solutions were serially diluted and plated out using BHI agar (BHI broth containing 1 % agar) containing 0.1 % sodium taurocholate (both w/v). The plates were then incubated for 48 h anaerobically at 37 °C before being counted.

3.8.3 *Assessment of viability of Clostridium difficile Spore Bioindicators*

This method used a growth/no growth technique to assess the viability of the spore after exposure to the hydrogen peroxide vapour. The exposed membranes and controls were placed directly into 2 ml reduced BHI broth containing 0.2 mg catalase in a 7 ml universal tube. The tubes were then immediately transferred to the anaerobic chamber for 48 h. After the incubation period, growth was determined by visual assessment; if the solution became cloudy, there were still viable spores on the membrane, and if the solution remained translucent then all the spores had been inactivated.

3.8.4 *Clostridium difficile Enumeration*

C. difficile results were enumerated in two ways. Initially, the spore recovery membranes were enumerated in the same way as the *B. subtilis* and plotted against time. With the bioindicators with different spore loading, the log reduction was the highest concentration of spores deposited on the membrane that did not go cloudy. These results could then be plotted alongside the high titre results to identify any differences.

3.9 Data analyses

3.9.1 *Fitting Inactivation Models to the Data and Calculating D-values*

The inactivation models were fitted using Datafit 9.0 software (Oakdale Engineering, USA). Three different models were used as outlined in Chapter 2, these were the Weibull, Baranyi and Series-Event, with the 'goodness of fit' of each model being indicated using the modified coefficient of multiple determination (r^2) (shown in Equation 3.5). The data were weighted using the standard deviation of the experimental data to allow fitting over the entire range without over-biasing at low log reductions. The Decimal reduction or *D*-value was calculated for each model using the equations given in Chapter 2 for the Weibull and Baranyi models; for the Series-Event model the *D*-value was calculated using the Goal Seek function in Microsoft Excel.

$$r^2 = 1 - \left(1 - \left(1 - \frac{\sum_i (y_i - f_i)^2}{\sum_i (y_i - \bar{y})^2}\right)\right) \frac{q-1}{q-p-1} \tag{3.5}$$

y_i and f_i are the experimental results and model value at a single point respectively, $\bar{y}$ is the mean value of the experimental data, q is the sample size and p is the is the number of fitted parameters.

3.9.2 *Hydrogen Peroxide Trace Concentrations Generated Using the Hygienics Biogenie*

Hydrogen peroxide trace concentrations were generated using the Hygienics Biogenie (Hygienics Biosecurity, UK) by Keith Schou (Hygienics Biosecurity) in the Hygienics 50 m^3 test room. Six ATI hydrogen peroxide sensors (ATI GasSens A11/34 hydrogen peroxide sensor/transmitter) (ATI Limited, UK) were calibrated as described in Section 3.2. These were then placed in one of fifteen locations within the room (each corner and the centre of the room at three different heights) (Figure 6.1). The room was sealed and a single hydrogen peroxide cycle was run while the sensors logged the concentration every minute. The data from the six hydrogen peroxide sensors in each of the locations was averaged to give a hydrogen peroxide trace and the cycle repeated for each of the different locations. Six Omega data loggers (OM-62 Temperature/Humidity data logger) (Omega, UK) were placed alongside the hydrogen peroxide sensors to record the humidity and temperature traces throughout the disinfection cycle.

Once the hydrogen peroxide concentration had been found and plotted against time for each location, the positions with the highest and lowest hydrogen peroxide concentrations could be identified. Also using this data the integral $\int C_P dt$ (C_p is the hydrogen peroxide concentration (ppm) and t is the time (min)) was calculated using the Simpson's rule (Equation 3.6) to find the area under the curve.

$$\int_{x_0}^{x_E} ydx \approx \frac{h}{3}\left(y_0 + 4y_1 + 2y_2 + 4y_3 + \ldots + 2y_{e-1} + 4y_e + 2y_{e+1} + \ldots + 4y_{E-1} + y_E\right) \tag{3.6}$$

h is the length of the intervals between the readings which must be even and *E* is the number of intervals.

Chapter 4
Inactivation of *Bacillus subtilis* using Hydrogen Peroxide Vapour

4.1 Introduction

Bacillus subtilis has used as a model spore-forming bacterium as it is has been categorised as a Class 1 microorganism, and is therefore not a human pathogen and consequently safe to utilise in experiments. This makes it a good bacterium for use in the design of experiments before more pathogenic bacteria (such as those causing HAIs) are studied. It has also been used in many other inactivation studies, including UV light irradiation and high pressure temperature treatment (Gardner & Shama, 1998; Gao *et al.*, 2007). *B. subtilis* is highly resistant to hydrogen peroxide and therefore any inactivation results produced are likely to provide an overestimate of the time required for inactivation of the bacteria (Maillard, 2011; Rezaee *et al.*, 2011).

Although hydrogen peroxide can be used for disinfection in both liquid and vapour form, it has been claimed that they both act significantly differently (Finnegan *et al.*, 2010). These researches showed that vapour phase hydrogen peroxide is a more effective disinfectant; they speculated that this was due to it being able to penetrate the macromolecules of the bacteria and thus, break down vulnerable bonds. Fichet *et al.* (2007) suggest that hydrogen peroxide vapour may be more reactive due to its instability, providing more oxidising species such as hydroxyl radicals that damage the bacteria. Hydrogen peroxide has been used as a disinfectant with encouraging results was with the microbial decontamination of fruits including melons, grapes, prunes and apples, nearly twenty years ago (Aharoni, Copel & Falik, 1994; Rij & Forney, 1995; Simmons *et al.*, 1997; Sapers *et al.*, 2003). Vamos-vigyazo (1981) demonstrated that one of the main advantages of using hydrogen peroxide in this way can be attributed to the fruit containing natural catalases and peroxidases that would break down any residual hydrogen peroxide into the harmless products of oxygen and water. More recently there has been interest in the use of hydrogen

peroxide to decontaminate processing equipment, such as aseptic filling machines and in the food packaging industry (Kirchner *et al,*. 2011; Pruss, Stirtzel & Kulozik, 2012).

There have been previous studies investigating vapour phase hydrogen peroxide disinfection, although most were conducted in the region of 1000 ppm (Hall *et al.*, 2007; Pottage *et al.*, 2012). There have also been a few at lower concentrations (Johnston, Lawson & Otter, 2005; Fu, Gent & Kumar, 2012; Tuladhar *et al.*, 2012). Establishing the efficiency of hydrogen peroxide vapour is of great interest because as well as inactivating a wide range of microorganisms, it is also a powerful oxidising agent and hence capable of degrading or corroding many materials through contact at concentrations greater than 1000 ppm (Sk *et al.*, 2011). Therefore, the ability to inactivate bacteria at lower hydrogen peroxide concentrations would minimise the potential damage to the contaminated materials, allowing medical equipment and furniture etc. to be left in the room or area during decontamination.

4.1.1 Aims

The aim of this chapter was to present results for that inactivation *of B. subtilis* spores when exposed to a constant concentration hydrogen peroxide vapour. *B. subtilis* has been used throughout this work as it is not pathogenic and is easier to manipulate than other microorganisms. In this chapter the suitability of the membrane bioindicators was investigated prior to the application of hydrogen peroxide.

Subsequently, the inactivation kinetics of *B. subtilis* was identified at four different concentrations (10, 50, 75 and 90 ppm); three inactivation models, the Series-Event, Weibull and Baranyi were used to model the data. From these, the decimal reduction (*D*) values were determined for the selected hydrogen peroxide concentrations and therefore, the resistance of the *B. subtilis* spores. Through optimising this method using *B. subtilis*, other organisms and the associated inactivation kinetics can be studied and compared; this has been carried out in Chapter 5 with *C. difficile*.

4.2 Results

4.2.1 Preliminary Hydrogen Peroxide Exposure Results

Preliminary tests were carried out using *B. subtilis* spores aseptically spread on Petri dishes containing 10 ml TSA (tryptone soya agar) at concentrations ranging from 3-log$_{10}$ to 8-log$_{10}$. Plates were then placed in the decontamination chamber (described in Section 3.6.3) and exposed to hydrogen peroxide vapour. Tests were conducted at 50 ppm for 30, 60 and 120 min and resulted in complete inactivation of the spores, irrespective of time of exposure. It is possible that the agar could have absorbed moisture from the environment, including some of the hydrogen peroxide vapour. This would increase the hydrogen peroxide concentration in the vicinity of the spores while also affecting the internal hydrogen peroxide concentration within the chamber. Therefore, despite the findings from the chamber commissioning work, the concentrations may not have been maintained at 50 ppm. The liquid hydrogen peroxide concentration was therefore increased during the experiment from 3 % (w/v) to 10 % (w/v) to compensate for this potential loss of hydrogen peroxide vapour. However, prior to this phenomenon being observed, the spores had been exposed to these lower concentrations of hydrogen peroxide for about an hour. In order to calculate the amount of hydrogen peroxide which had been absorbed by the agar, the plates were weighed before and after the next series of exposure experiments. The hydrogen peroxide concentration was reduced to 25 ppm and the times to 15, 30 and 45 min; arrows were also drawn onto the bottom of the Petri dishes to indicate the vapour flow direction so any inactivation patterns caused by the flow of the hydrogen peroxide could be identified.

Spore inactivation, humidity and hydrogen peroxide traces for the tests conducted using the modifications discussed previously, are presented in Figure 4.1. The plot of the hydrogen peroxide vapour concentration demonstrates that for this experiment, only 5 min were required for the chamber to reach 25 ppm. The trace does show that the concentration fluctuated by about 10 ppm. These concentration fluctuations could be explained as a result of the modifications to the liquid hydrogen peroxide flow-rate, which then

maintained the required internal vapour concentration. Therefore, this suggests that manually changing the liquid flow-rate is not necessarily a reliable manner in which to control the vapour phase concentration and a method where the hydrogen peroxide concentration is stable would be required.

Weighing the plates before and after exposure revealed an increase in the weight of plates (from 28.0 g) in all of the boxes: 0.5 g after 15 min, 1.0 g after 30 min and 1.7 g after 45 min. These findings support the theory that the agar was adsorbing some of the hydrogen peroxide and water vapour; furthermore, the spores may still be exposed to hydrogen peroxide after removal from the chamber. Should this be the case, any subsequent inactivation results cannot be directly attributed to the exposure time, as further kill could well be observed after the removal of the samples from the chamber. From Figure 4.1 and Figure 4.2, it can be deduced that there is a difference in the kill achieved between the two shelves, top and bottom. This is most likely due to the hydrogen peroxide vapour entering in line with the top shelf. In order to correct for this, a modification in the chamber design was made and foam flow distributors were placed at the entrance to each of the boxes.
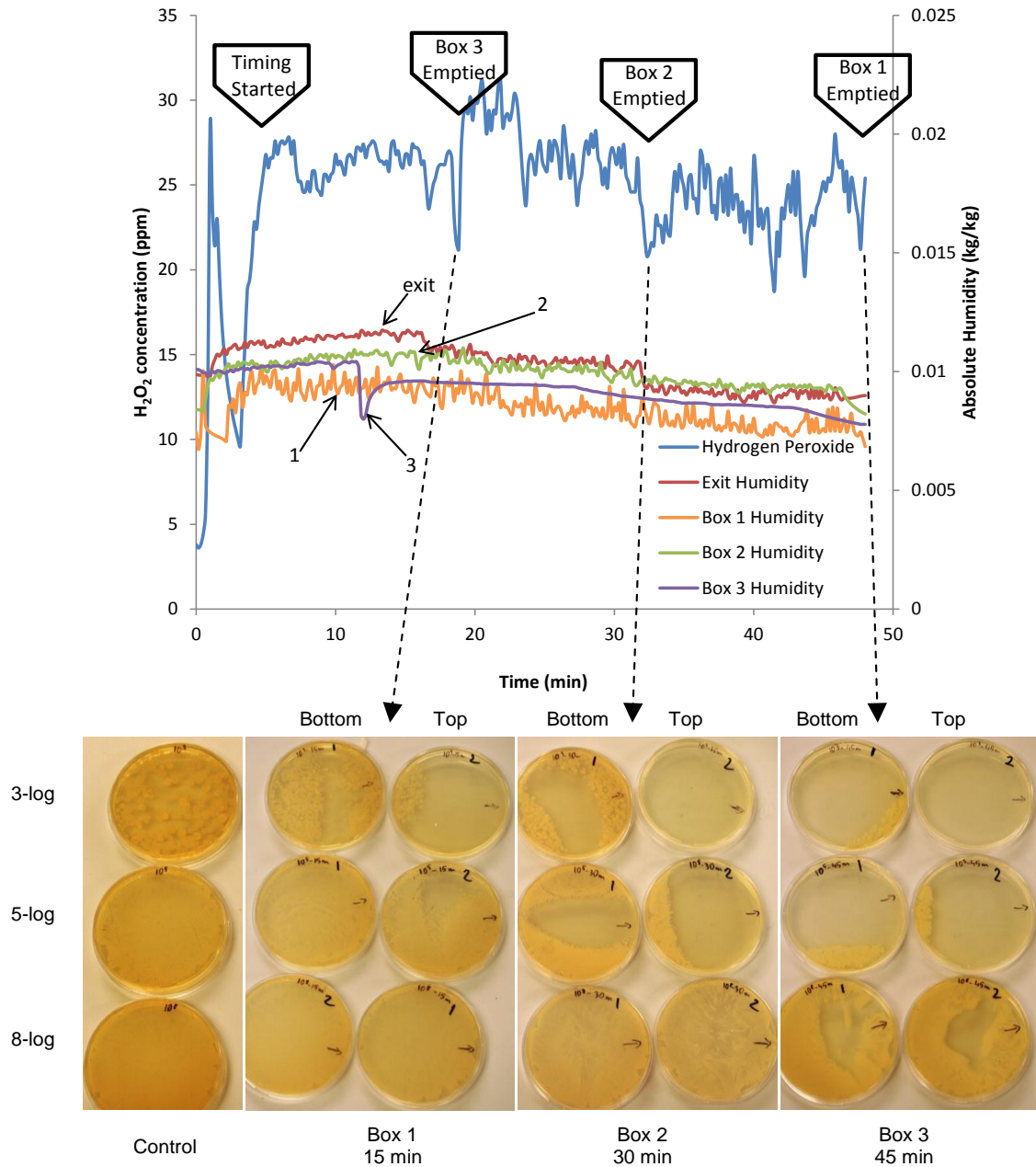
Figure 4.1 Hydrogen peroxide (target 25 ppm) and humidity traces for the preliminary tests. Photographs depict the inactivation of *B. subtilis* achieved over 15, 30 and 45 min.
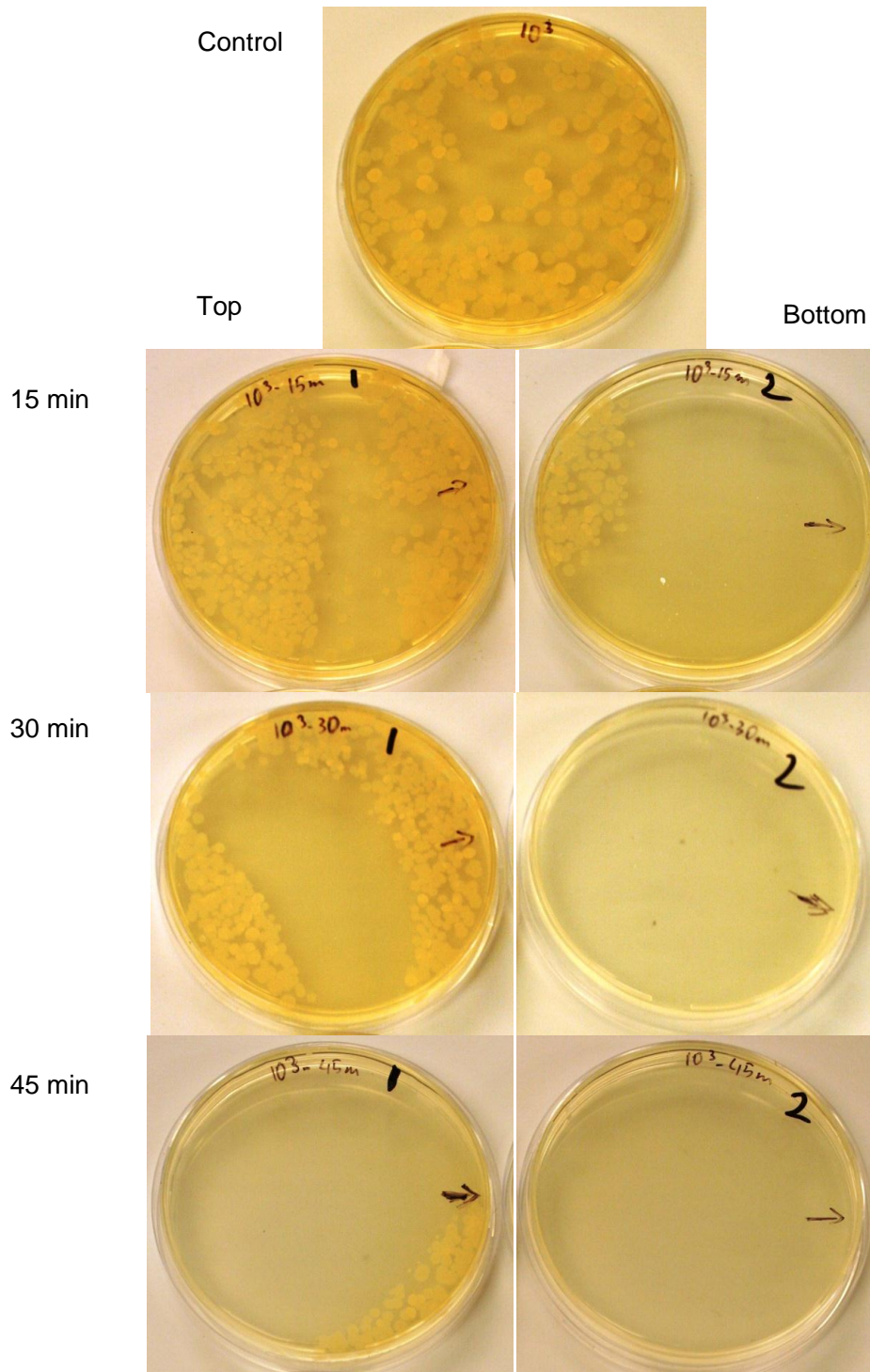
Figure 4.2 Enlarged photographs of *B. subtilis* (3-log$_{10}$) exposed to 25 ppm hydrogen peroxide at 15, 30 and 45 min. Arrows indicate the direction of vaporised hydrogen peroxide flow.

From the preliminary experiments, it was therefore concluded that it was important for the spores to be exposed to hydrogen peroxide using an agar free method to avoid adsorption. In order to do this, the spores were deposited onto membranes (as described in Section 3.4.1) and exposed to hydrogen peroxide

before recovery and enumeration. This ensured the vapour would not be absorbed by the agar, making it easier for the maintenance of the required vapour concentration. It also identified that. Figure 4.2 identified a difference in the inactivation between the top and bottom shelf, with a greater inactivation seen on the bottom shelf which was directly in line with the air inlet to the box. The inactivation is also shown to start in the centre of the Petri dish and spread out to the edge, this implies that the lip around the edge provides some protection to the bacteria around the edge of the Petri dish. A flow distributor was included to ensure an even flow through all of the boxes. A final modification was the addition of catalase in the recovery of spores so any residual hydrogen peroxide would be broken down.

4.2.2 Testing of Bacillus subtilis Spore Bioindicators before Exposure to Hydrogen Peroxide

Testing of *B. subtilis* bioindicators was important to identify weaknesses in the method and address them before exposure to hydrogen peroxide. The following aspects of the bioindicators were investigated:

4.2.2.1 Recovery of *B. subtilis* spores from the membrane bioindicators

A comparative study into recovery times was investigated to establish a replicable protocol for the recovery of spores from the membrane bioindicators. It was important to ensure that the results for the inactivation kinetics were repeatable to identify differences which could result in under- or over-estimation of inactivation. Figure 4.3 illustrates the percentage recovery from the membrane following mixing the samples using a vortex mixer for 1, 2 and 4 min. After 1 min, the recovery was about 60 %; with 2 min vortexing, this increased to 80 %. There was no further increase observed after 4 min. The standard deviations were 5 % (1 min), 7 % (2 min) and 5 % (4 min). Student t-tests identify no statistical significant difference between 2 and 4 min vortexing (P = 0.697), whereas it did highlight a statistically significant difference between a 1 min vortex compared to either a 2 or 4 min vortex (P = 0.000362). As multiple samples had to be processed it was decided to employ vortex mixing for 1 min only. Therefore, despite the lower overall recovery, there was still a significant number of spores recovered.
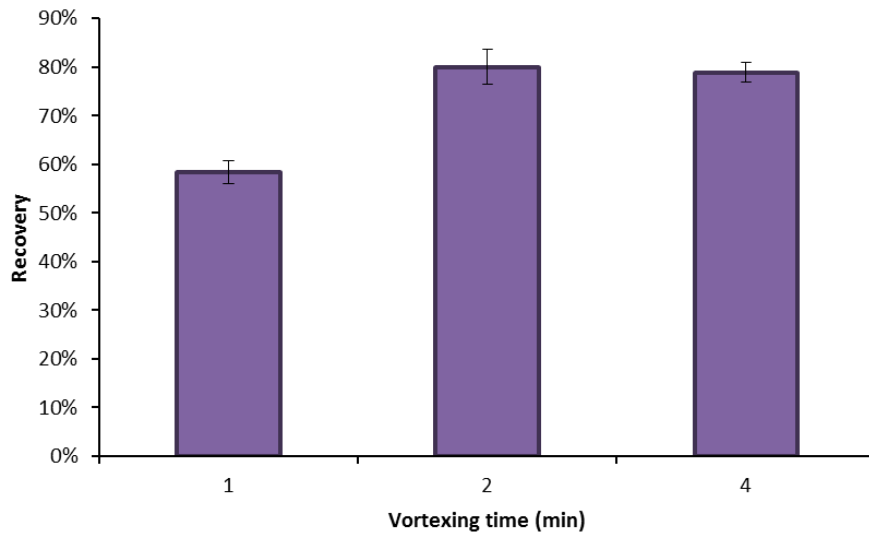
Figure 4.3 *B. subtilis* spore recovery from membranes (5 replicates) at various vortexing times without exposure to hydrogen peroxide vapour.

4.2.2.2 The effect of the addition of catalase on the recovery of *B. subtilis* from the bioindicators

During the spore recovery stage it was important to include the addition of catalase to breakdown any residual hydrogen peroxide present on the membranes. Therefore, it was necessary to identify any reductions in recovery as a result of adding catalase. For this, spores were recovered from bioindicators prior to any exposure to hydrogen peroxide in two different PBS solutions (five replicates for each); one containing 0.2 mg catalase and the other containing no catalase (Figure 4.4). There is a slight reduction in the number of colony forming units of *B. subtilis* when catalase is added; 57 % without catalase and 49 % with catalase. This indicates a statistically significant difference of 8 % (P = 0.0035) in the number of viable spores, however the reduction is small and catalase was added to all of the recovery and control vials, thus the reduction affected all subsequent experiments. The reduction in the number of viable bacteria observed with the addition of catalase is trivial compared to the reduction with is likely to be seen from any residual hydrogen peroxide.
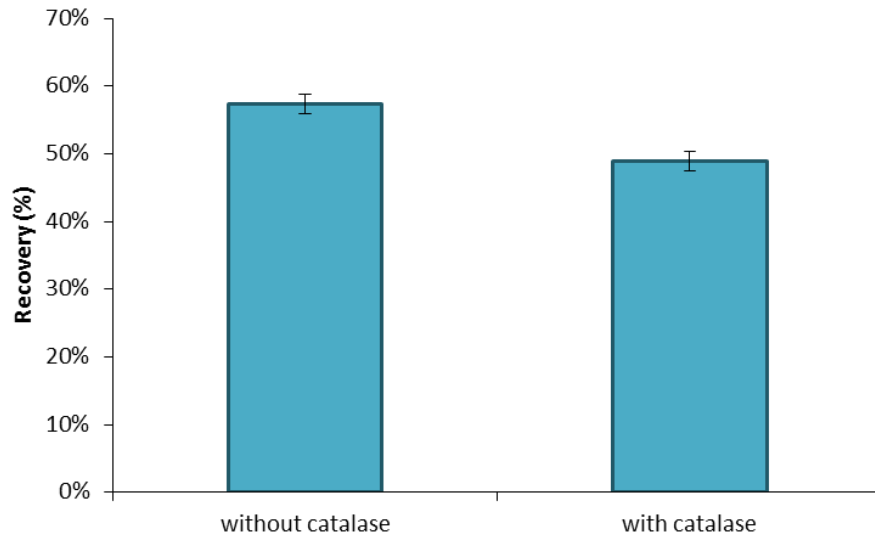
Figure 4.4 *B. subtilis* spore recovery from membranes (5 replicates) following the addition of catalase without exposure to hydrogen peroxide.

4.2.2.3 Investigating the effect of the exposure box used on the log reduction achieved when using *B. subtilis* bioindicators

To ensure that the conditions within each of the three exposure boxes were identical, bioindicators were exposed in each box for the same length of time at a constant hydrogen peroxide concentration (Figure 4.5). A difference of 0.15-log_{10} was observed between the boxes after exposure to 90 ppm for 3 h. This increased to a 0.7-log_{10} difference after 4.5 h. Both time points show a larger log reduction in the first box and a similar log reduction in the second and third boxes. This is most likely caused by some of the hydrogen peroxide breaking down on the hotplate, creating reactive species which damage the spores in the first box. However, analysis of the data with ANOVA tests carried out using Minitab 15 show that the error bars overlap for each of the different boxes at both time points. This clearly demonstrates there is not a significant difference between any of the boxes at the 95 % confidence level.
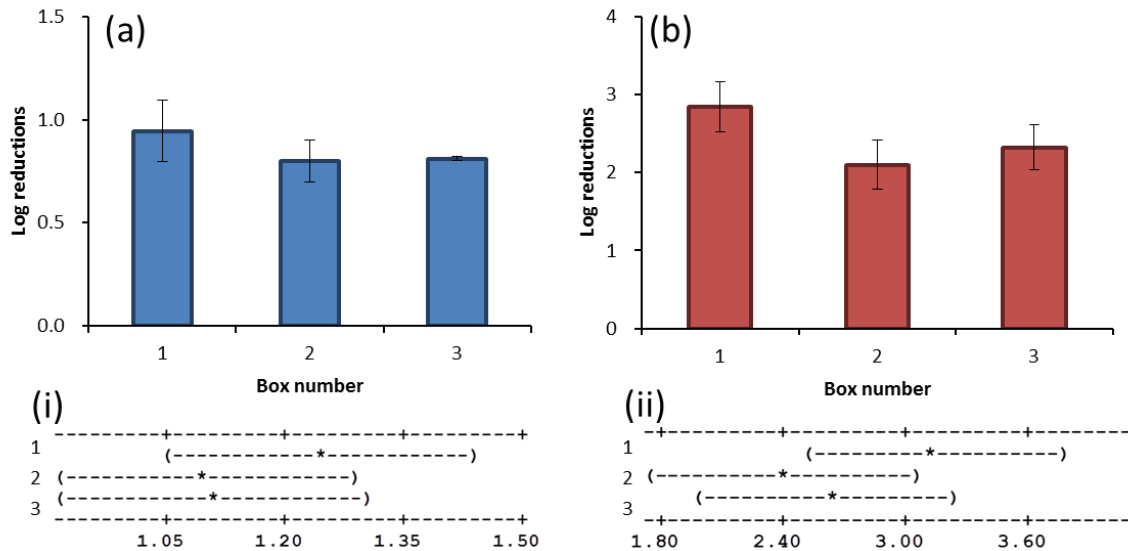
Figure 4.5 Log reductions of *B. subtilis* spores from each of the three boxes following exposure to hydrogen peroxide vapour (5 replicates for each). (a) 90 ppm for 3 hr; (b) 90 ppm for 4.5 h. The corresponding plots underneath (i) and (ii) are the ANOVA test for (a) and (b) respectively.

4.2.2.4 *B. subtilis* spore recovery from bioindicators after time on the membrane without exposure to hydrogen peroxide

The effects of spore recovery from the membranes over time were investigated. This was to identify any differences in exposure for a period of time in the chamber. For this, membrane bioindicators were placed in a desiccator where the desiccant had been replaced with a saturated salt solution of sodium bromide creating a relative humidity of 60 % similar to the relative humidity in the box. The results are shown in Figure 4.6, detailing the percentage recovery of *B. subtilis* after 0, 1.5 and 48 h. Upon recovery directly following deposition onto the membrane (controls), 60 % of the spores can be recovered. However, once the spores have been placed in the humid environment for either 1.5 or 48 h, the number of spores that can be recovered is dramatically reduced to around 30 %. This implies that although the initial recovery of the spores is high, once the spores have adhered to the membrane the strength of the adhesions does not appear to increase over the time intervals examined. With the 48 h recovery not showing statistically significant difference (P = 0.696) compared to that found after 1.5 h. This means that when looking at the inactivation data generated using this method the initial

concentration of the spores need to be corrected to take account of this reduction.
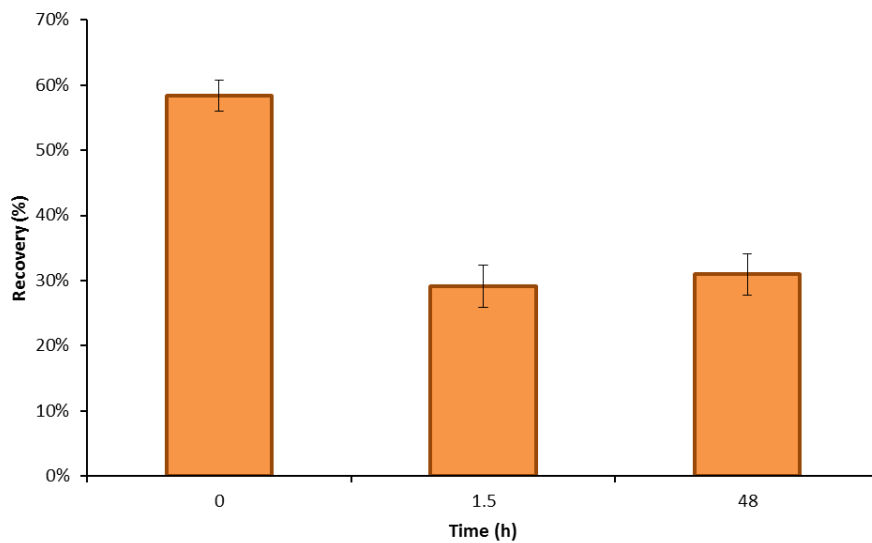
Figure 4.6 *B. subtilis* spore recovery from membranes (5 replicates) exposed to a relative humidity of 60 % for 1.5 and 48 h.

4.2.2.5 Measurement of spore aggregation using light scattering

A spore aggregation check was carried out using a Coulter LS130 particle sizer (Beckman Coulter, UK) which uses light scattering to measure the particle sizes. This was used to establish whether the spores were forming any agglomerates when recovered from the membranes. Again, this was carried out for the controls and membranes left in the humid environment for 1.5 and 48 h. Figure 4.7 shows the percentage differential volumes against the particle size; no measurements are shown below 0.4 µm, as this is the limit of detection for the particle sizer. The spores have an approximate width of 0.5 µm (Figure 3.6), and therefore anything smaller will not be detected. For all three data sets the greatest peak is the first one between 0.4 µm and 2.5 µm, relating to the size of the individual spores. This shows that the majority of the spores are not forming any sort of agglomerates. The control plot also shows that there is another peak around 4 µm suggesting that there is a very small amount of agglomerates containing two or three spores, however, this is negligible compared to the number of single spores. These agglomerates can be seen on the SEM images (Section 3.4.2) where one or more spores are connected by a small amount of

residual agar. With the membranes that were left for 1.5 and 48 h before recovery, the initial peak is significantly smaller and the peak around 4 μm is roughly the same size, there is however an additional peak at 40 μm. This suggests that when the spores are left on the membrane, agglomerates of around twenty spores can form. Even though this accounts for a very small amount of the total spores due to the plot being based on volume, it would make a difference when the plated as a cluster of twenty spores would still only appear as one colony. Therefore, two or three clusters could result in the lower recovery identified when the spores are left on the membranes for 1.5 and 48 h. As this has been identified and proved to be repeatable for both times, and as the reduction is the same for both the shortest and longest exposure time, providing these results are amended by adjusting the control recovery from around 60 % to 30 %, there will be little effect on the inactivation data generated.
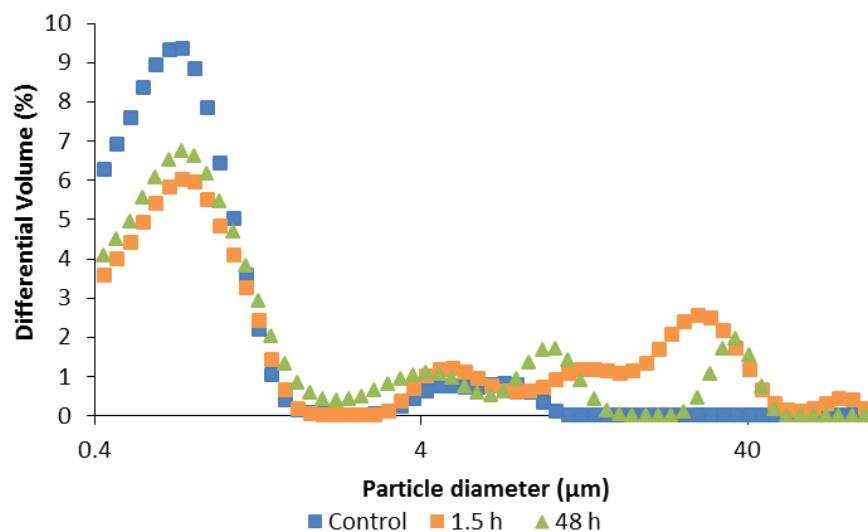
Figure 4.7 Particle size distribution of spores recovered immediately and after 1.5 and 48 h to identify clustering of *B. subtilis* spores on the bioindicator surfaces.

The preliminary testing of the *B. subtilis* bioindicators identified a repeatable recovery of 49 % when using catalase, although this was reduced to 30 % when the spores were not immediately recovered straight away. This reduction in recovery was explained by the increase in the number of aggregates found using the particle sizer. Although the recovery using this

method was found to be low due to the aggregates it was statistically the same after 1.5 and 48 h (the shortest and longest times which would be used for the exposure experiments) which would suggest the recovery would be of an acceptable proportion. Moreover, there was no statistical difference between the log reductions achieved in any of the three boxes when exposed to the same concentration for the same time.

4.2.3 Maintenance of the Physical Conditions within the Exposure Chamber

Hydrogen peroxide traces obtained during exposure of *B. subtilis* to hydrogen peroxide vapour are shown in Figure 4.8. The mean concentrations for the different runs were 49.0 ppm (50.8 ppm over run 1 (R1) and 47.1 ppm over run 2 (R2)), 72.7 ppm (72.9 ppm over R1 and 72.5 ppm over R2) and 92.7 ppm (92.4 ppm over R1 and 92.9 ppm over R2) over 6 h (Figure 4.8a), and 9.5 ppm (9.4 ppm over R1 and 9.6 ppm over R2) over 48 h (Figure 4.8b) experiments. Average concentrations over the two separate runs show very little different between the two runs. The largest difference is seen between the two runs at 50 ppm where a 3.7 ppm is observed, as this is less than the 10 % relative error of the sensor it was seem as an acceptable error. As the relative error of the sensor was 10 % of the nominal value, the concentrations have subsequently been rounded to 10, 50, 75 and 90 ppm throughout the work. The spikes on the plots identify where the spore-laden membranes were removed from the chamber and the pump was refilled with the hydrogen peroxide solution.
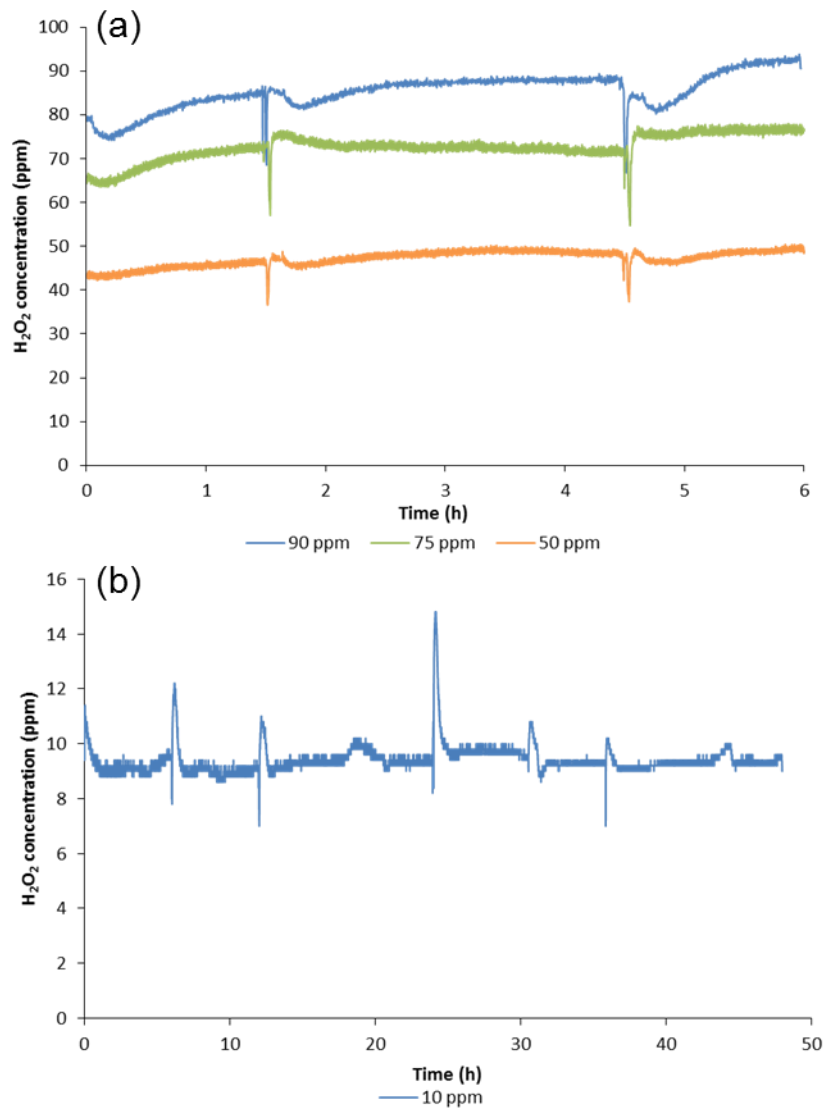
Figure 4.8 Hydrogen peroxide concentration traces for (a) 50, 75 and 90 ppm over 6 h exposure and (b) 10 ppm over 48 h exposure.

The relative humidity and temperature profiles are shown in Figure 4.9. From the relative humidity traces it is observed that the humidity remains between 40 and 50 % during exposure to all four hydrogen peroxide concentrations. There is a peak in humidity, where it increases to 70 %, when the samples are removed and the syringe pump refilled. During all of the 6 h experiments the temperatures remained stable at around 24 °C; however, the 48 h exposure fluctuated slightly more between 20 and 25 °C. This is due to the experiments running over longer time periods and therefore the fluctuations represent the drop in temperature overnight and then increasing again during the day.
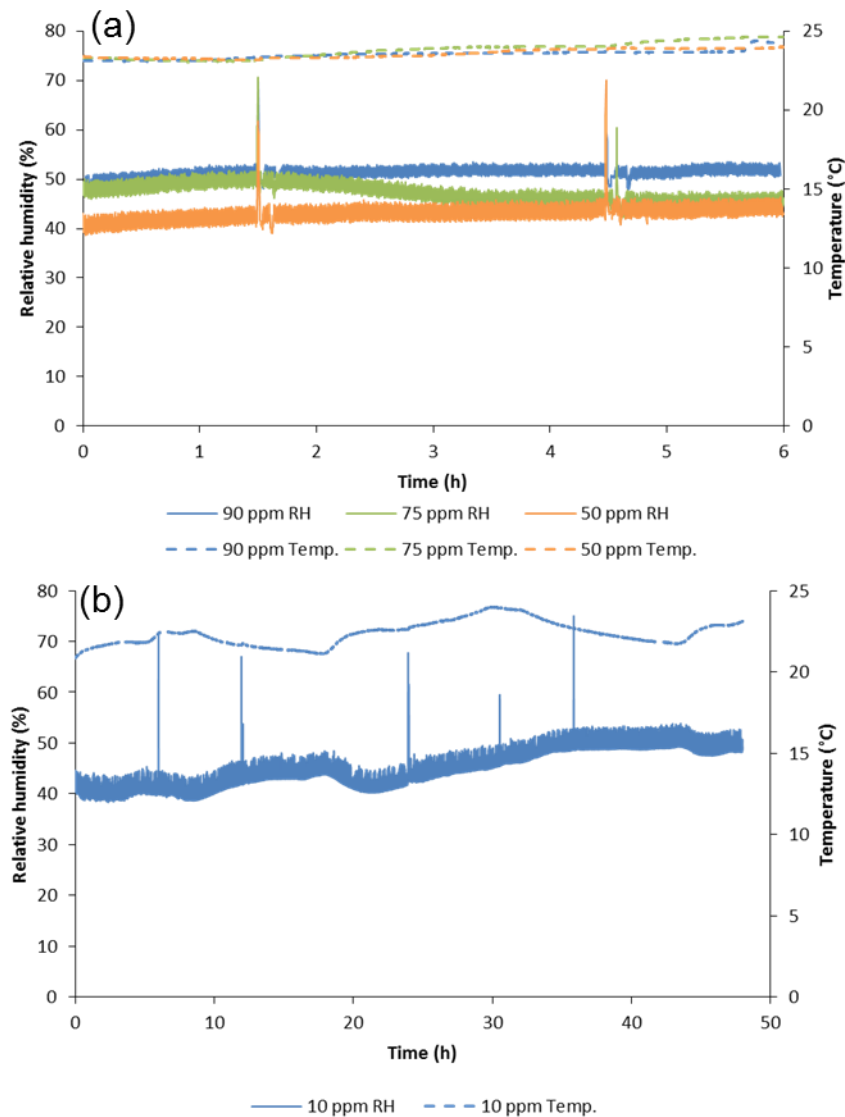
Figure 4.9 Typical relative humidity and temperature profiles for (a) 90, 75 and 50 ppm over 6 h and (b) 10 ppm over 48 h hydrogen peroxide exposures.

4.2.4 Inactivation Results and Fitting the Inactivation Models

The inactivation of *B. subtilis* spores shown in Figure 4.10 and Figure 4.11 detail the results obtained at 10, 50, 75 and 90 ppm. For the Series-Event model the number of hits (*i*) was solved iteratively, over all the hydrogen peroxide concentrations, and found to give a best fit with seven hits. The number of hits is defined as the number of interactions required with the hydrogen peroxide for the spores to become unviable. All three models show a good fit for 50 ppm, however, there is very little inactivation realised over the 6 h exposure making it easier to fit a model as it is close to a linear trend. This is

compared to the 6-log$_{10}$ inactivation observed after exposure to 90 ppm for the same time, which shows very different inactivation curves for the same data. Although all the modelled provide a reasonable fit for the data, the Weibull model yields the best fit over all the hydrogen peroxide concentrations. The error bars shown indicate the standard error from which it can be assumed that there is very little difference between the two runs for each concentration carried out over 6 h. The standard error is increased for the 10 ppm exposure indicating that the longer inactivation times required could make a difference to the reproducibility.
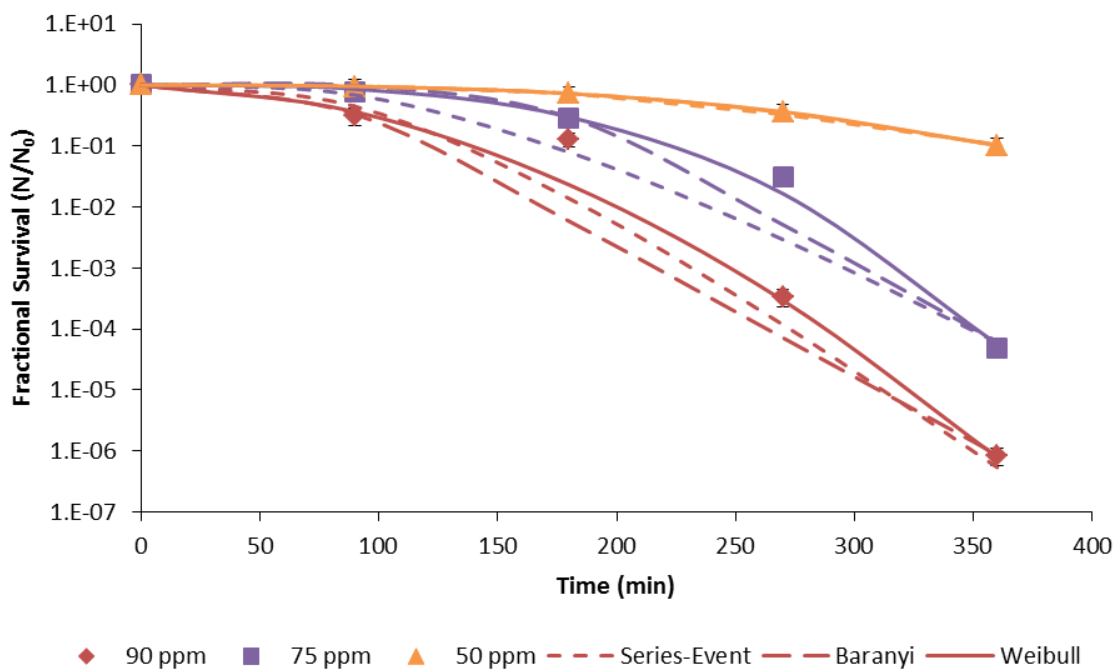
Figure 4.10 Inactivation of *B. subtilis* spore bioindicators (5 replicates) exposed to 50, 75 and 90 ppm (each repeated in duplicate) over 6 h modelled using the Series-Event (*i*=7), Baranyi and Weibull models. Error bars represent the standard error.
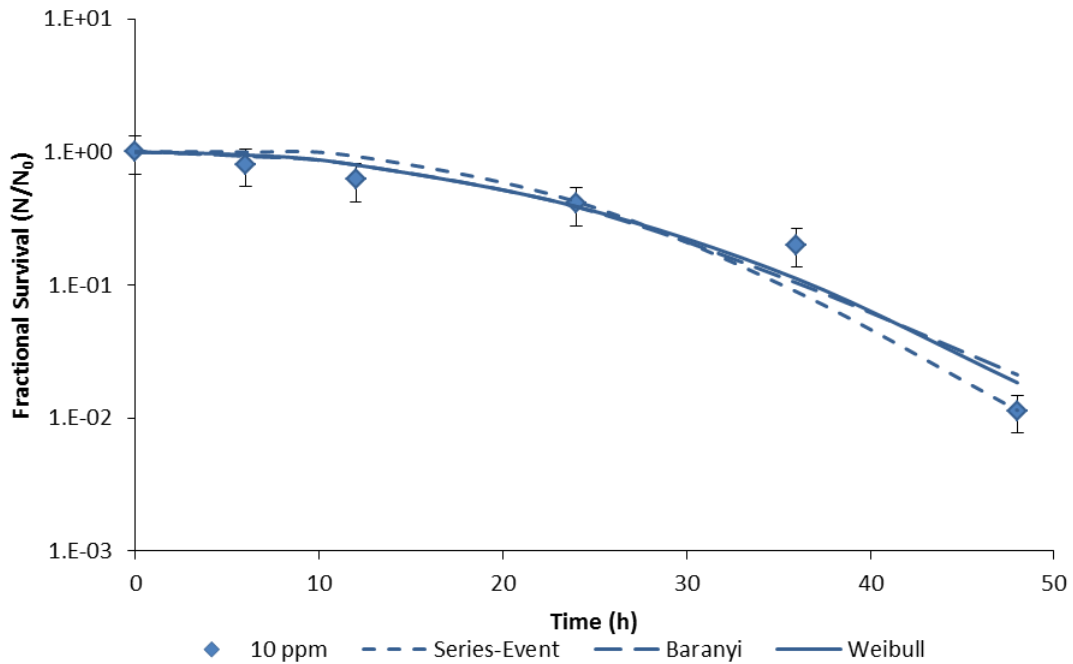
Figure 4.11 Inactivation of *B. subtilis* spore bioindicators (5 replicates) exposed to 10 ppm (repeated in duplicate) hydrogen peroxide over 48 h modelled using the Series-Event (*i*=7), Baranyi and Weibull models. Error bars depict the standard error.

The r^2 values for all 3 models are shown alongside the model parameters in Table 4.1. The *D*-value (expressed in minutes) for each model was predicted using each of the models and show a reasonable agreement between the three models. As the *D*-values would differ depending on what stage of the inactivation they were calculated, all *D*-values shown in this work will be shown for the first log reduction to allow comparison.

Table 4.1 Inactivation model parameters and *D*-values for *B. subtilis* exposed to 10-90 ppm hydrogen peroxide.

ppm	Baranyi				Weibull			Series-Event (*i*=7)			
	k	K_C	*D*	r^2	*β*	*D*	r^2	*k*	*b*	*D*	r^2
10	2.3×10^{-3}	1.6	2176	0.748	2.1	2210	0.731	9.5×10^{-4}	0.72	2111	0.377
50	1.7×10^{-2}	1.2	362	0.999	2.8	362	0.999	9.9×10^{-4}	0.86	364	0.978
75	0.049	38.1	207	0.882	3.0	223	0.972	1.2×10^{-3}	0.91	213	0.758
90	0.049	0.43	120	0.957	1.9	139	0.961	1.3×10^{-3}	0.91	131	0.827

Wang & Toledo (1986) investigated the inactivation of *B. subtilis* spores by hydrogen peroxide at concentrations ranging between 275 and 3879 ppm. However, they neither modelled their data nor attempted prediction of *D*-values calculated. Table 4.2 shows the calculated model parameters for their data

alongside the r^2 and *D*-values. A comparison of r^2 values shows that the Weibull model provides the best fit for the data, as demonstrated with the 10 ppm to 90 ppm data. Therefore, Figure 4.12 shows the data plotted with the Weibull inactivation model alone.

Table 4.2 Inactivation model parameters and *D*-values for *B. subtilis* exposed to 275-3879 ppm hydrogen peroxide.
Data obtained from Wang & Toledo (1986).

ppm	Baranyi				Weibull			Series-Event (*i*=7)			
	k	K_C	*D*	r^2	*β*	*D*	r^2	*k*	*b*	*D*	r^2
275	1.1	99.6	11.5	0.992	3.4	12.2	0.976	3.2×10^{-3}	1.05	9.0	0.739
558	0.9	3.1×10^{-2}	5.9	0.956	2.0	6.8	0.939	2.3×10^{-3}	1.03	6.8	0.728
1131	5.0	26.2	2.5	0.969	2.6	2.3	0.881	2.8×10^{-3}	1.08	1.9	0.559
1859	10.9	7.0	1.1	0.713	3.3	1.2	0.860	4.7×10^{-3}	1.05	0.83	0.638
3879	15.4	12.3	0.84	0.383	3.6	0.91	0.503	2.8×10^{-3}	1.05	0.64	0.674
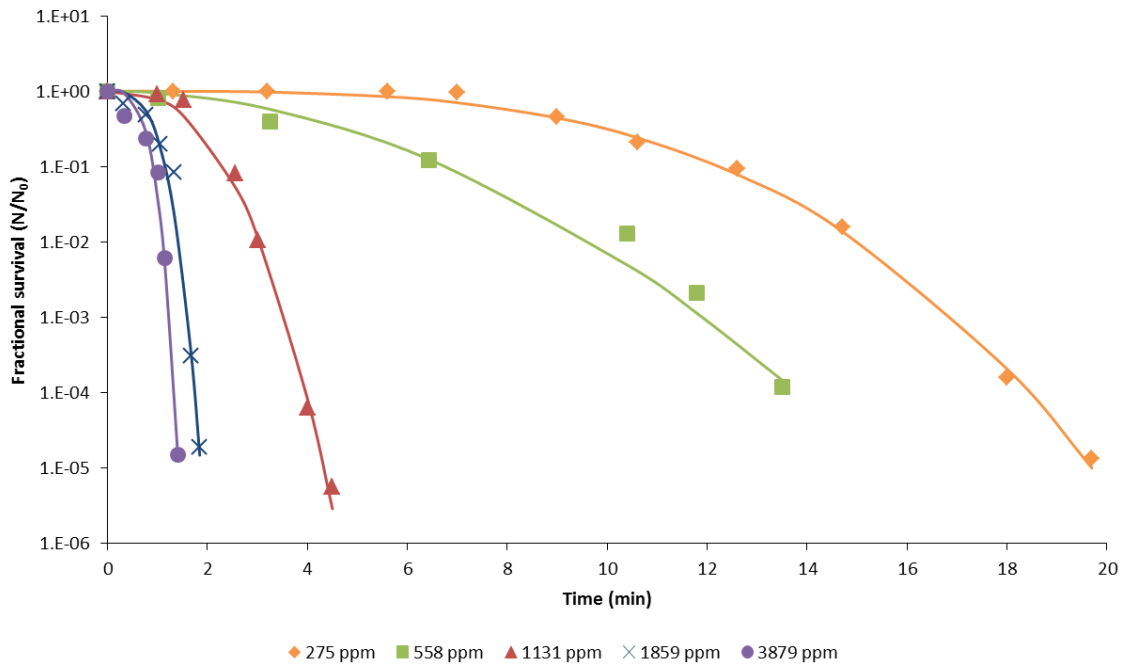
Figure 4.12 Inactivation of *B. subtilis* spores exposed to 275-3879 ppm hydrogen peroxide.
Data obtained from Wang & Toledo (1986).

Figure 4.13 shows the *D*-values for both data sets calculated using the Weibull model show a good fit (r^2 = 0.981) when plotted against the hydrogen peroxide concentration (10 ppm to 4000 ppm). Since both sets of data can be modelled by the same power-law regression model, this suggests that the two methods used to generate the data are compatible.
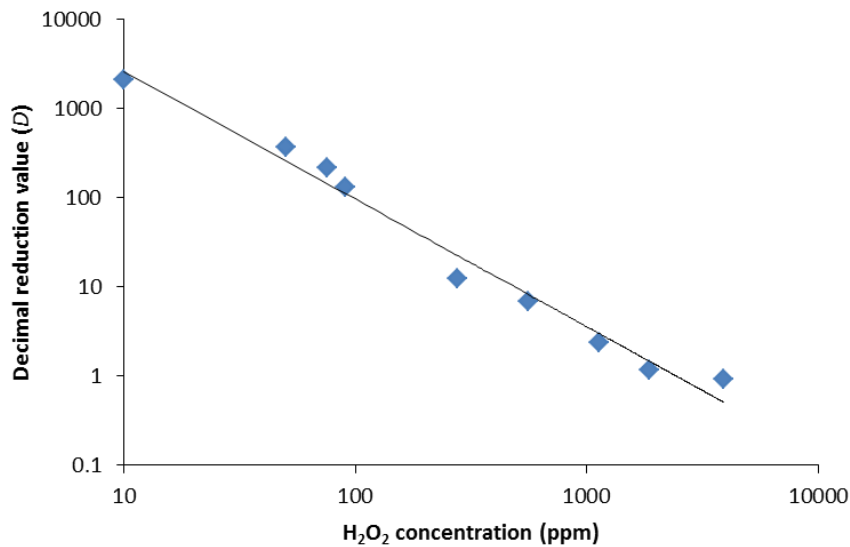
Figure 4.13 *D*-values as calculated from the Weibull inactivation model against hydrogen peroxide concentrations.
Data obtained from this chapter (10-90 ppm) and data produced by Wang & Toledo (1986) (275-3879 ppm).

4.3 Discussion

B. subtilis was used as a model organism as it is not a human pathogen and has been shown to be very resistant to hydrogen peroxide (Klapes & Vesley, 1990). Using this bacterium allowed a method to be developed by which the spores could be exposed to a constant hydrogen peroxide concentration over required times. Methods were developed to produce a uniform distribution of spores for exposure, so that there was no effect of spore shrouding or stacking which could protect some of the spores and the inactivation and recovery results were unaffected. This is important as most studies into the inactivation bacteria exposed to hydrogen peroxide vapour are carried out using commercially available bioindicators. To produce these bioindicators a droplet of the spore suspension is deposited on a metal or paper tab and is left it to dry (e.g. Hall, Otter, *et al.*, 2007; Pottage *et al.*, 2012). Even in studies where the researchers prepare their own bioindicators they follow the same method (e.g. Johnston, Lawson & Otter, 2005; Rastogi *et al.*, 2009;

Bentley *et al.*, 2012). The problem with bioindicators produced in this way is that the spores form non-uniform layers which can include spore stacking at the periphery (Bayliss *et al.*, 2012).

As demonstrated in this chapter, it is possible to compare the data generated in this study at low hydrogen peroxide concentrations (10 ppm to 90 ppm), to that generated by Wang & Toledo (1986) at high hydrogen peroxide concentrations (275 ppm to 3879 ppm). As with this study, Wang & Toledo (1986) also did not employ the commercial available spore strips, instead opting to deposit *B. subtilis* spores on different types of packing material. The compatibility between the two different sets of data suggests that the spores used by Wang & Toledo (1986) were evenly deposited on the different surfaces, as achieved with the spore filtering method used throughout this study (Chapter 3).

The results presented here show that the Weibull inactivation model shows a good fit for inactivation of *B. subtilis* spores using hydrogen peroxide. This contrasts with Johnston, Lawson & Otter (2005) who used first-order inactivation kinetics (log survivors vs. time) for *C. botulinum*. Much higher concentrations (average 355 ppm) were used and as a result exposure times of less than 10 min gave reductions of 4-$\log_{10}$ to 5-$\log_{10}$. However, the use of an inactivation model that includes a shoulder period may provide a better fit for some of the data generated. The importance of the shoulder when modelling hydrogen peroxide inactivation was also shown by Otter & French (2009) and Pottage *et al.* (2010), where the inactivation against a variety of nosocomial bacteria (such as MRSA, *C. difficile*, *K. pneumoniae* and *A. baumannii*) and MS2 bacteriophage was shown respectively. Otter & French (2009) suggested that *C. difficile* was one of the most susceptible organisms to vapour phase hydrogen peroxide showing a 6-$\log_{10}$ reduction after 20 min and reporting 75 min to 90 min were required for the other bacteria to show the same reduction, however, no information was given regarding the hydrogen peroxide concentration, therefore, it is very hard to draw conclusions. The results presented by Pottage *et al.* (2010) suggest that the initial concentration of bacterium on the carrier results in a significant difference in the degree of inactivation achieved; total inactivation of 7.5-$\log_{10}$ after 10 min compared to 60

min for 9-log$_{10}$. This suggests that the samples used in this study may have been stacked at the higher concentrations therefore providing extra protection in the inactivation procedure. As the data demonstrates a shoulder period before inactivation and as different initial concentrations can affect the inactivation achieved care must be taken when comparing the *D*-values as they may depend on the stage of inactivation from which they are calculated and the initial spore concentration. Therefore, in this study all the *D*-values presented represent the time taken for the first log reduction to be achieved to ensure they are comparable.

The survival curves for both data generated in this work and results obtained by Wang & Toledo (1986) depict an initial shoulder inactivation period which are commonly associated with oxidising agents. This represents a period where the spore is accumulating damage but it is not sufficient to inactivate the spore (Cortezzo *et al.*, 2004). Initial resistance of the spores to oxidising agents is due to their structure: low water content, an outer spore coat and exosporium and which protects the inner membrane and core (Maillard, 2011; Leggett *et al.*, 2012). This is important as previous studies have shown that oxidising agents such as hydrogen peroxide damage the spores' inner membrane so that when the damaged spores germinate they rupture resulting in death (Cortezzo *et al.*, 2004; Setlow, 2006).

Rather surprisingly, many of the inactivation studies employing hydrogen peroxide do not state the concentration being used, or if an inactivation cycle has been used, only the maximum concentration is provided. Pottage *et al.* (2010) provided no information about the hydrogen peroxide concentration when reporting on the inactivation of MS2 bacteriophage. However, in a more recent publication they do provide a maximum hydrogen peroxide concentration of 750 ppm used to provide a 3-log$_{10}$ inactivation of MRSA (NCTC 13142) (Pottage *et al.*, 2012). Other studies by Johnston, Lawson & Otter (2005) and Hall *et al.* (2007) also only state the maximum concentration with the latter merely stating that the concentration is greater than 1000 ppm. The whole hydrogen peroxide trace is presented by Fu, Gent & Kumar (2012) and Chung *et al.* (2008), which provides a clearer understanding of the process.

Importantly, however, none of the studies described above report on the inactivation achieved under constant concentrations of hydrogen peroxide over the entire exposure period. Johnston, Lawson & Otter (2005) and Pottage *et al.* (2012) reported their systems introduced hydrogen peroxide at a high flow-rate until the required concentration was reached and then at a lower flow-rate to maintain the required concentration; this method prevents the straightforward calculation of *D*-values and subsequently extrapolation and comparison of data to other findings. Therefore, the hydrogen peroxide inactivation of other bacteria from these studies cannot be compared to the *B. subtilis* inactivation data presented in this chapter.

One of the main advantages in the use of vapour disinfectants over liquid disinfectants is that the latter have been found to be inadequate in removing pathogenic bacteria, in both food processing and healthcare environments (Chambel *et al.*, 2007; Dancer *et al.*, 2009). One of the reasons for this is that the bacteria are able to survive in areas where the liquid detergents cannot access such as medical equipment and has to reach areas behind beds and cupboards. Unger-Bimczok *et al.* (2011) recently showed that hydrogen peroxide can penetrate long narrow cavities that would not be assessed by manual cleaning and application of liquid disinfectant would prove challenging. Therefore, the use of hydrogen peroxide as a disinfectant could be a viable alternative to liquid disinfection in the food processing environment. However, in the healthcare environment it should only be used periodically to supplement manual cleaning, as it is much more expensive than a bleach clean and requires all the patients to be relocated (Otter & French, 2009).

4.4 Chapter Summary

- *B. subtilis* was a good model organism as it is a spore-forming bacterium that has previously been shown to be very resistant to hydrogen peroxide.
- The environmental chamber designed and used in this study provided a controlled environment in which *B. subtilis* spores could be exposed to constant hydrogen peroxide concentrations. The results in this chapter have shown that during exposure, the

conditions inside the chamber were maintained constant and not significantly different from standard room conditions.

- The production of spore-laden membranes and the spore recovery described in Chapter 3 provides a robust method for investigating the inactivation of bacteria using hydrogen peroxide.

- Three different inactivation models were used: Series-Event, Weibull and Baranyi. Of the models, the Weibull gave the best overall fit to the data.

- This approach permitted the calculation of *D*-values of *B. subtilis* over a wide range of hydrogen peroxide concentrations. This should allow for confident predictions of the inactivation times and decontamination process required when using hydrogen peroxide. Although, it was demonstrated that at the lowest concentration (10 ppm) the inactivation times required are very long and therefore inhibitive (>48 h).

- The inactivation models demonstrated the same inactivation kinetics for previously published data at higher hydrogen peroxide concentrations. Allowing the *D*-values to be estimated and compared to those found for exposure to low hydrogen peroxide concentrations in this study.

Chapter 5
Inactivation of *Clostridium difficile* using Hydrogen Peroxide Vapour

5.1 Introduction

Clostridium difficile was identified as a common cause of community-acquired infection in 1982 and is currently the main cause of healthcare-acquired diarrhoea (Poxton, 2013). *C. difficile* costs the NHS in excess of £100 million per year and is responsible for over eight hundred deaths per annum (Health Protection Agency, 2012b; Robotham & Wilcox, 2012). A characteristic of *C. difficile* is that it is a spore-forming bacterium making it more resistant to the standard disinfectants and alcohol-based hand rubs (ABHR) than non-spore-forming bacteria (Wilcox & Fawley 2000; Jabbar *et al*. 2010). Resistance of *C. difficile* to the ABHR can increase the spread of healthcare-acquired infections (HAIs) through hand transfer (Curtis & Cairncross, 2003). However *C. difficile* spores can also be spread by explosive diarrhoea, caused by the infection, making it imperative that the wards are regularly disinfected (Gerding *et al.*, 1995).

He *et al.* (2013) investigated the global spread of a fluoroquinolone-resistant variant of *C. difficile* (027/BI/NAP1). The study concludes that the ability of a single strain of *C. difficile* to spread across the globe is due to ease of world travel resulting in 'interconnectedness of the global healthcare system'.

There has been a substantial reduction in the number of cases of *C. difficile*-associated infection over the last five years, however it still warrants investigation (Health Protection Agency, 2012a). A significant number of cases still being reported worldwide; with 2,000 (Belgium), 23,200 (England and Wales) and 336,600 (USA) cases per annum being seen (Viseur *et al.*, 2011; Lucado, Gould & Elixhauser, 2012; Health Protection Agency, 2012a). The main reason for the fall in the number of cases is reported to be due to an increased awareness in the issues associated with over-prescription of antibiotics (Carling *et al.*, 2003; Davey *et al.*, 2009). There has also a drive

towards increased cleaning and surveillance in hospital wards (Doan *et al.*, 2012). One of the main areas of recent interest is the use of gaseous decontaminant for terminal disinfection once a patient with a HAI is discharged (Davies *et al.*, 2011). Although formaldehyde fumigation has been used for over a hundred years its use in hospitals has been discontinued following its classification as a carcinogen hydrogen peroxide vapour decontamination has become its main replacement (Watling *et al.*, 2002; Grare *et al.*, 2008).

An advantage of hydrogen peroxide breaks down into oxygen and water making it environmentally friendly. It has also been shown to be effective against many different pathogenic bacteria including MRSA and *C. difficile* (French *et al.*, 2004; Boyce *et al.*, 2008). Although there is general agreement that hydrogen peroxide is an effective method for bacterial inactivation the required concentrations are widely disputed (Grare *et al.*, 2008; Davies *et al.*, 2011; Maillard, 2011; Doan *et al.*, 2012). Hydrogen peroxide inactivation systems use a fully automated unit for the release of the hydrogen peroxide vapour; the amount of hydrogen peroxide released is monitored to achieve a pre-determined hydrogen peroxide vapour concentration within the room. For these systems the required peak concentration is reached before the hydrogen peroxide vapour is allowed to decompose naturally or by the use of an activated carbon catalyst. The concentration must drop below a safe level (1 ppm) before staff and patients are allowed to repopulate the ward or room that has been disinfected (Boyce, 2009; Pottage *et al.*, 2010; Barbut, Yezli & Otter, 2012; Fu, Gent & Kumar, 2012). Issues with hydrogen peroxide vapour disinfection include: (i) the requirement for the room to be empty of all patients and staff before commencing the cycle, (ii) the need for trained personnel to operate the system, (iii) the hydrogen peroxide level needs to be monitored to ensure it has dropped below 1 ppm before the ward/room can be repopulated, (iv) the relatively high cost associated with hydrogen peroxide disinfection and the need to retain manual cleaning (Malik, 2013).

There are many different strains of *C. difficile* found in the hospital environment, with 027 historically being the most prevalent; however, there has been an increase in disease outbreaks caused by other ribotypes in recent years (Health Protection Agency, 2012b). Most studies investigating the

efficacy of hydrogen peroxide against *C. difficile* have used a single ribotype (001 or 027) (Boyce *et al.*, 2008; Alfa *et al.*, 2010). Shapey *et al.* (2008) investigated different ribotypes found in a hospital ward before the application of hydrogen peroxide; they found three epidemic strains 001, 027 and 106. However, upon analysis of the decontamination data by hydrogen peroxide, the overall reduction in *C. difficile* is given so no difference between the inactivation of strains could be identified. Barbut, Yezli & Otter (2012) investigated the difference between historical and epidemic strains of ribotype 027 and toxinotype 0. However, no difference between the strains could be identified as they were all reduced below the detection level after the Bioquell decontamination cycle.

5.1.1 Aims

In this chapter, results of the inactivation of *C. difficile* spores exposed to hydrogen peroxide vapour are presented. The aim was to find the inactivation kinetics of *C. difficile* when exposed to a constant concentration of hydrogen peroxide, using a similar approach to that applied in Chapter 4 for *B. subtilis*.

For the experimental work, four different hydrogen peroxide concentrations (10, 25, 50 and 90 ppm) were used with a large proportion of the inactivation studies being conducted using a clinical strain (ribotype 027). Further studies were conducted using three other ribotypes (014, 103 and 220). Using the experimental inactivation results, inactivation models were used to fit the data; these are the Chick-Watson Series-Event, Baranyi and Weibull inactivation models. These models were used to find the *D*-values and the concentration-time integral ($\int C_P dt$) were plotted against the log reduction in order that the decontamination achieved during a hydrogen peroxide cycle can be estimated.

5.2 Results

The results of the two different methods are presented in this section: Method 1 where a high concentration bioindicators using spores grown using the agar plate method are exposed and recovered before enumeration and Method 2 where different concentrations of spores grown using the Clospore

method are deposited on the membrane bioindicators and then a growth/no growth method is used for enumeration.

5.2.1 Method 1 Results

5.2.1.1 Recovery of *C. difficile* spores from the membrane bioindicators

The influence of vortexing time on spore recovery from bioindicators was investigated (Figure 5.1). Spore loadings of 6-log$_{10}$/membrane and vortexing times of 1 min and 5 min were examined using a 5 ml recovery solution of PBS, 0.2 mg catalase and 0.05 % (w/v) tween 80. A 1 min vortexing time resulted in a low (26 %) recovery of the *C. difficile* spores, compared to a 61 % recovery achieved for a 5 min vortex, which shows a statistical difference between the two recovery times (p=0.0004). Increasing the vortexing time from 1 min to 5 min led to an increase in the standard deviation from 6 % to 9 %, therefore, given the increased reliability and repeatability that comes with the higher recovery it was concluded that a 5 min vortex would be used throughout the experiments. The 5 min vortex also resulted in the same recovery (60 %) as was seen with the 1 min vortex used to recover the B. subtilis spores from the membrane bioindicators.
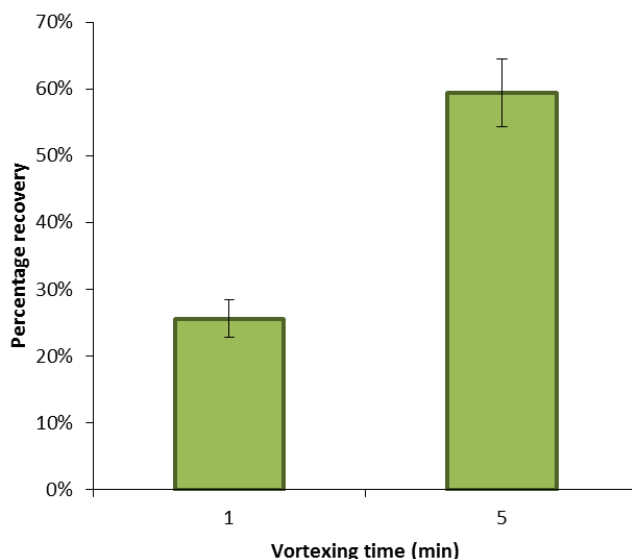
Figure 5.1 *C. difficile* spore recovery from bioindicators (5 replicates) after vortexing with the addition of catalase at 1 and 5 min.

5.2.1.2 The effect of the addition of catalase on the recovery of *C. difficile* from the bioindicators

As with the *B. subtilis* 0.2 mg catalase was incorporated into the recovery media to quench any residual hydrogen peroxide (Johnston, Lawson & Otter, 2005). Figure 5.2 details the recovery of *C. difficile* both with and without catalase. Addition of catalase resulted in a modest increase in the recovery ($p = 0.006$) from around 40 % (without catalase) to around 60 % (with catalase). Five membrane bioindicators were used to reduce the error and provide a clear picture of the inactivation achieved.
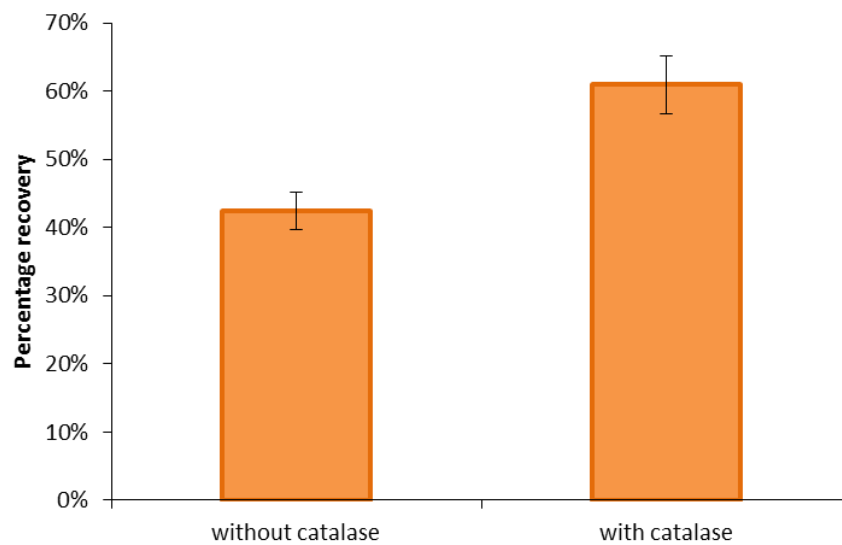
Figure 5.2 *C. difficile* spore recovery from bioindicators (5 replicates) with and without the addition of catalase in the recovery medium.

5.2.1.3 Investigating the effect of the exposure box used on the log reduction achieved when using *C. difficile* bioindicators

The log reduction achieved for samples placed in the various three boxes after 10 min at 50 ppm is shown in Figure 5.3. The difference between the three boxes is minimal, with the inactivation in the first being only slightly higher than the other two. However, based on the findings from an ANOVA test (Figure 5.3i), it can be concluded that there is no significant difference between the reductions achieved in each box at the 95 % confidence level, as all of the bars showing the 95 % confidence level overlap.
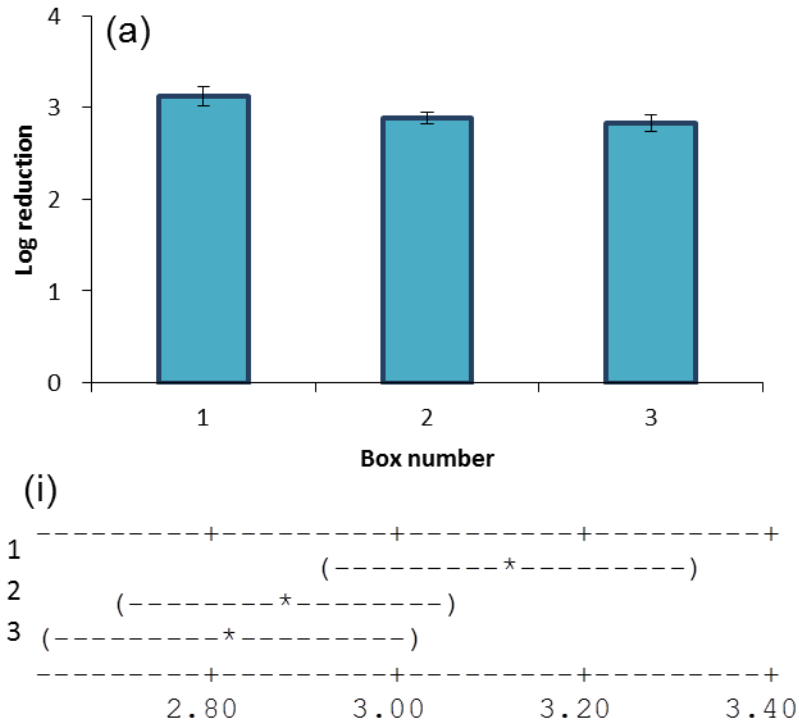
Figure 5.3 Inactivation of *C. difficile* spores achieved in each of the 3 boxes (5 replicates) after 10 min at 50 ppm.

5.2.1.4 *C. difficile* spore recovery from membranes without exposure to hydrogen peroxide

The spore membrane bio-indicators were exposed to a humid environment (60% relative humidity) to identify if any reduction in recovery was likely after exposure within the chamber, as was seen with *B. subtilis*. Spore recoveries from the membranes over time were investigated after 5 min and 420 min at humidity of 60 % before recovery (Figure 5.4). The figure shows that there was a reduction in the recovery from 61 % to 51 % in the first 5 min, with a further decrease to 47 % over the next 415 min. Student t-tests revealed no significant difference between any of the data sets at the 95 % confidence level (P = 0.876 for 5 min and 420 min on membranes; P = 0.079 when comparing the controls to spores left on the membrane for any length of time). Therefore, no corrections were applied to account for decrease in recoveries once the membranes were exposed in the chamber.
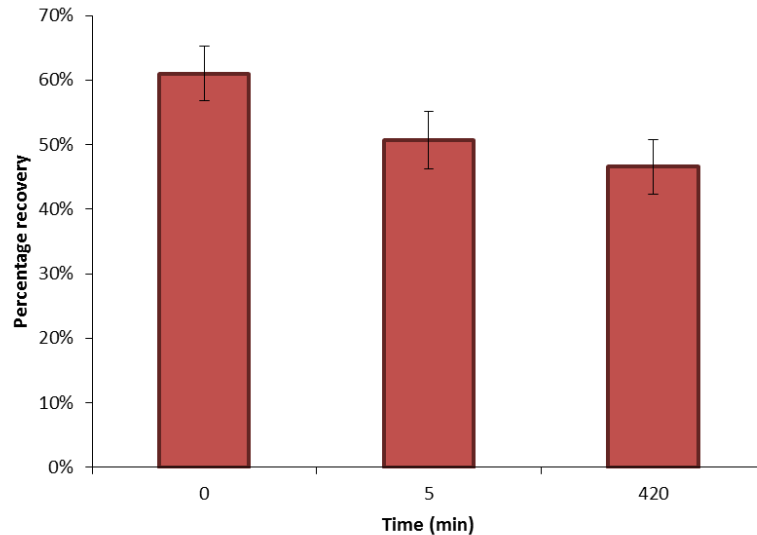
Figure 5.4 *C. difficile* spore recovery from bioindicators (5 replicates) at different time points without exposure to hydrogen peroxide.

5.2.1.5 Maintenance of the physical conditions within the exposure chamber

Typical hydrogen peroxide traces are shown for the inactivation experiments (Figure 5.5) with the averaged concentrations being 11.1 ppm (11.6 ppm over R1 and 10.6 ppm over R2), 51.2 ppm (54.5 ppm over R1 and 48.7 ppm over R2), and 91.2 ppm (94.1 ppm over R1 and 88.3 ppm over R2). As the hydrogen peroxide sensors have a relative error of 10 % of the nominal value these values were rounded to 10, 50 and 90 ppm for ease of reference throughout the chapter. The hydrogen peroxide concentrations remained constant over the exposure times, with the only falls in the concentration observed when the membranes are removed from the chamber after the required time of exposure. It can be inferred that the response characteristics are much quicker than those shown in Section 3.2.2, where it takes the hydrogen peroxide sensors 5 min to 15 min to reach the vapour-liquid equilibrium value inside the calibration bath. This is confirmed by the results shown in Figure 5.5 which demonstrates that when the boxes in the exposure chamber are opened and the hydrogen peroxide drops, less than a minute is required for the steady-state concentration to be re-achieved.
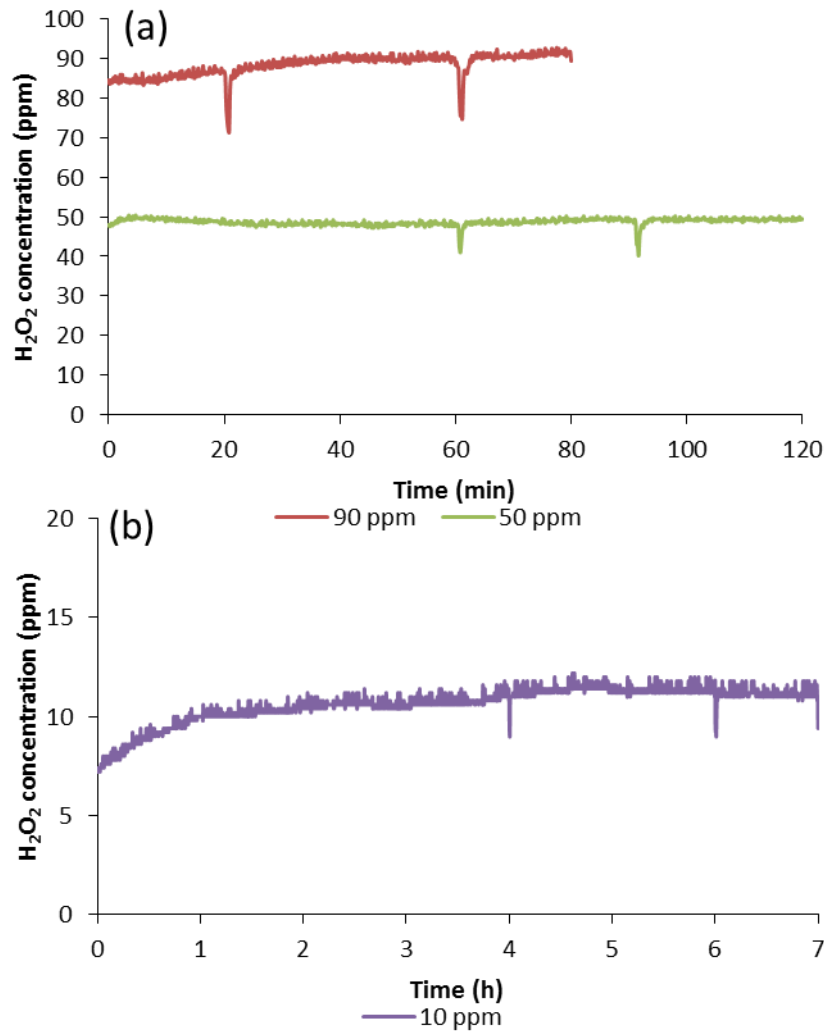
Figure 5.5 Hydrogen peroxide concentration traces over time: (a) 90 and 50 ppm, (b) 10 ppm.

The relative humidity and temperature profiles (Figure 5.6) demonstrate that the relative humidity for both 50 and 90 ppm exposures were stable at around 40 to 45 %, whereas, the humidity during the 10 ppm exposure remains lower at around 30 % relative humidity. Exposing the spore membranes to a relative humidity of 60 % for up to 7 h (Section 5.2.1.4) revealed a slight decrease in the recovery achieved after exposure to the humid environment but for 5 min but no further increase after 7 h, therefore, reduction seen are due to the hydrogen peroxide exposure not the humidity within the chamber. There are no peaks in the humidity as seen with the *B. subtilis* exposure (Section 4.2.3) as the syringe pump did not require refilling during the experiments due

to the shorter exposure times. The temperature during the hydrogen peroxide exposure experiments remained relatively constant at ~ 25 °C.
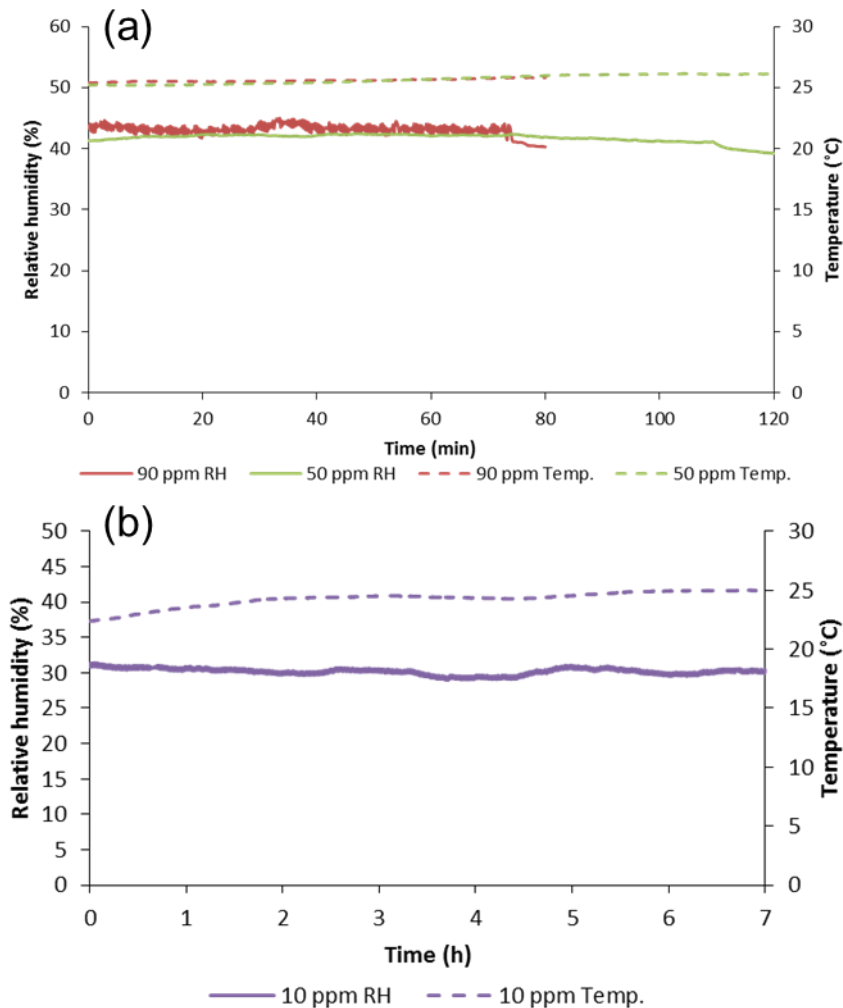
Figure 5.6 Temperature and humidity traces over time for (a) 90 and 50 ppm and (b) 10 ppm hydrogen peroxide vapour concentrations.

5.2.1.6 Inactivation results

Inactivation plots for *C. difficile* exposed to hydrogen peroxide using method 1 are shown in Figure 5.7. All three models demonstrate a good fit for the 50 ppm and 90 ppm data; the inactivation constants (*D*-values shown in minutes) and r^2 values have been compared (Table 5.1). In the Series-Event model two hits (or interactions with hydrogen peroxide) were used for *C. difficile* compared to seven for the inactivation of *B. subtilis* shown in Chapter 4. This is due to the shorter times required for the inactivation and less pronounced shoulder for the *C. difficile* in comparison with data for the *B. subtilis*. For

exposure at 10 ppm, the Weibull and Baranyi models show that the curve has inverted resulting in a much smaller *D*-value than is seen with the Series-Event model (Table 5.1). This implies that the spores are getting more resistant to the hydrogen peroxide. However, based on the SEM images of the spore stock produced from agar plates (Section 3.5.1) it could be suggested that this resistance is caused by the residual dead cells and agar in the stock (Figure 3.7), which may be protecting the spores from the hydrogen peroxide, particularly at 10 ppm.
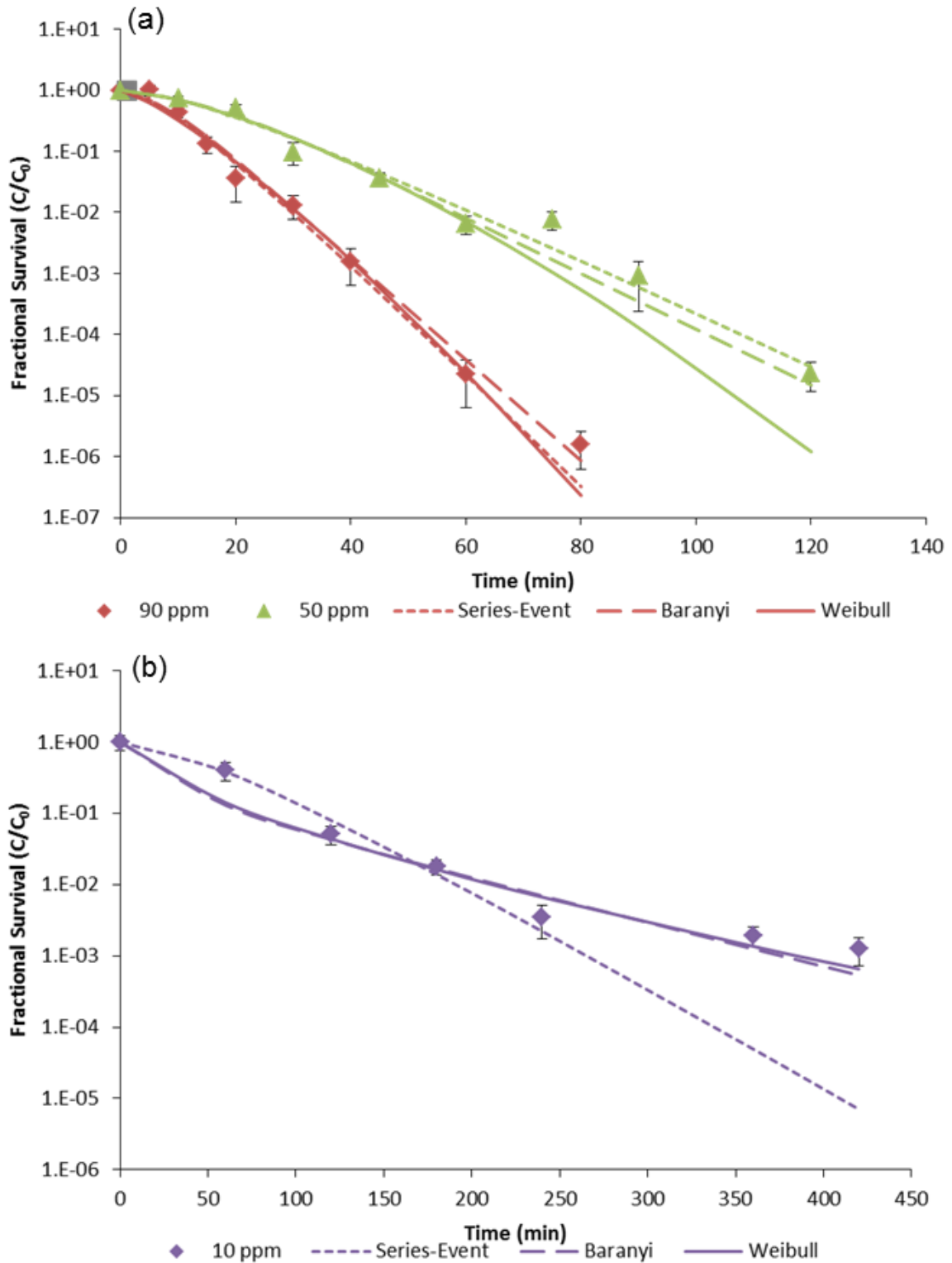
Figure 5.7 *C. difficile* spore inactivation upon exposure to (a) 50 and 90 ppm and (b) 10 ppm hydrogen peroxide vapour (each repeated in duplicate) using the spore recovery method (5 replicates). Data modelled using the Series-Event, Baranyi and Weibull inactivation models.

Table 5.1 Inactivation constants *D*-values and r^2 values for *C. difficile* exposed to hydrogen peroxide using the spore recovery method.

ppm	Baranyi				Weibull			Series-Event (*i*=2)			
	k	K_C	*D*	r^2	*β*	*D*	r^2	*k*	*b*	*D*	r^2
10	1.1×10^{-2}	-8.0×10^{-2}	73.0	0.828	0.68	76.6	0.882	2.7×10^{-3}	1.11	112	0.629
50	1.1×10^{-1}	6.8×10^{-2}	35.5	0.973	1.46	35.5	0935	2.2×10^{-3}	1.00	35.7	0.965
90	1.9×10^{-1}	2.7×10^{-2}	18.2	0.878	1.25	17.6	0.849	2.3×10^{-3}	1.02	17.4	0.881

Using the same spore recovery method, three further *C. difficile* strains were exposed to hydrogen peroxide to identify any differences in their inactivation responses. The strains used for the purpose of this comparative study were: ribotypes 014 and 027 (isolated from faecal samples), ribotype 103 (isolated from a patient's surrounding environment in a hospital), and ribotype 220 (isolated from estuarine sediment). The results suggest that both of the strains isolated from faecal samples show a very similar reduction and showing no statistical difference (P = 0.993) after 40 min. Both the 103 and 220 strains show a statistical difference from 027 (P = 0.022 and P = 0.040, respectively) as well as being statistically different from each other (P = 0.018). It can be inferred from the results that the strains isolated from the faecal samples are the most resistant to the hydrogen peroxide confirming that the use of the 027 strain is a good model as it is the most prevalent and one of the most resistant strains. These findings have been demonstrated in Figure 5.8 and Table 5.2.

Table 5.2 Inactivation model parameters, *D*-values and r^2 values for the different strains (027, 014, 103 and 220) of *C. difficile* exposed to 90 ppm hydrogen peroxide.

strain	Baranyi				Weibull			Series-Event (*i*=2)			
	k	K_C	*D*	r^2	*β*	*D*	r^2	*k*	*b*	*D*	r^2
027	0.16	1.4×10^{-2}	18.9	0.996	1.22	18.9	0.997	2.2×10^{-3}	1.01	19.1	0.975
014	0.19	6.5×10^{-2}	21.4	0.999	1.51	21.6	0.996	2.1×10^{-3}	1.00	20.1	0.941
103	0.26	8.9×10^{-3}	11.1	0.939	1.07	10.4	0.921	2.7×10^{-3}	1.06	12.2	0.938
220	0.17	-7.4×10^{-3}	8.4	0.902	0.80	9.0	0.911	2.7×10^{-3}	1.06	12.2	0.965
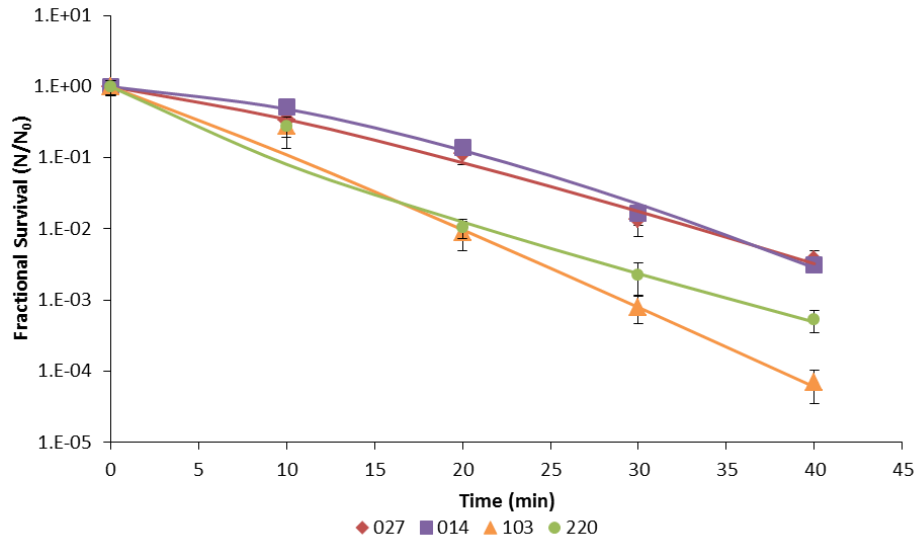
Figure 5.8 Different strains of *C. difficile* (027, 014, 103 and 220) exposed to 90 ppm hydrogen peroxide for 10, 20, 30 and 40 min (each repeated in duplicate) using the spore recovery method (5 replicates), shown with the Weibull inactivation model.

5.2.2 Method 2 Results

The bioindicators with different spore loading were used to expose *C. difficile* spores to hydrogen peroxide without having to recover the spores afterwards (Section 3.8.3). Spores used in this section were produced using the Clospore method as it was shown to produce a purified spore stock with a higher titre (Section 3.5.2). Different concentrations of spores were deposited on the membranes and were then placed in universals containing BHI (blood heart infusion) broth and catalase. If the spores were viable, the broth became turbid, whereas if the hydrogen peroxide had inactivated the spores then the broth remained clear. Controls used blank membranes to ensure that there was no contamination in the broth or on the membrane and for the lowest concentration. With the lowest concentration controls the membranes were placed in an environment at 60 % relative humidity for the longest exposure time to ensure that even the lowest concentration of spores remained viable. All controls used showed positive results for the lowest concentration used on the membranes (generally 1-$\log_{10}$) and negative results for the blank controls.

5.2.2.1 Maintenance of the physical conditions within the exposure chamber

As previous shown with the *B. subtilis* results (Chapter 4) and the spore recovery method for *C. difficile* (Section 5.2.1.5) the hydrogen peroxide concentration is maintained constant throughout the experiment (Figure 5.9). The average concentration over the two exposure experiments for each concentration were 91.2 ppm (91.2 ppm over R1 and 87.7 ppm over R2), 48.9 ppm (50.4 ppm over R1 and 47.5 ppm over R2), 23.5 ppm (24.1 ppm over R1 and 23.0 ppm over R2) and 11.2 ppm (11.3 ppm over R1 and 11.1 over R2). As with the *B. subtilis* exposure (Section 4.2.3) the hydrogen peroxide concentrations over the two runs were within the 10 % relative error of the sensors. This demonstrates that the required concentrations were easily reproducible and that there was clear separation between each of the exposure concentrations. In this case the samples were removed every 2.5 min during the 90 ppm exposure, to achieve this spores were exposed in two stages, 2.5 min and 7.5 min and then 5 and 10 min.
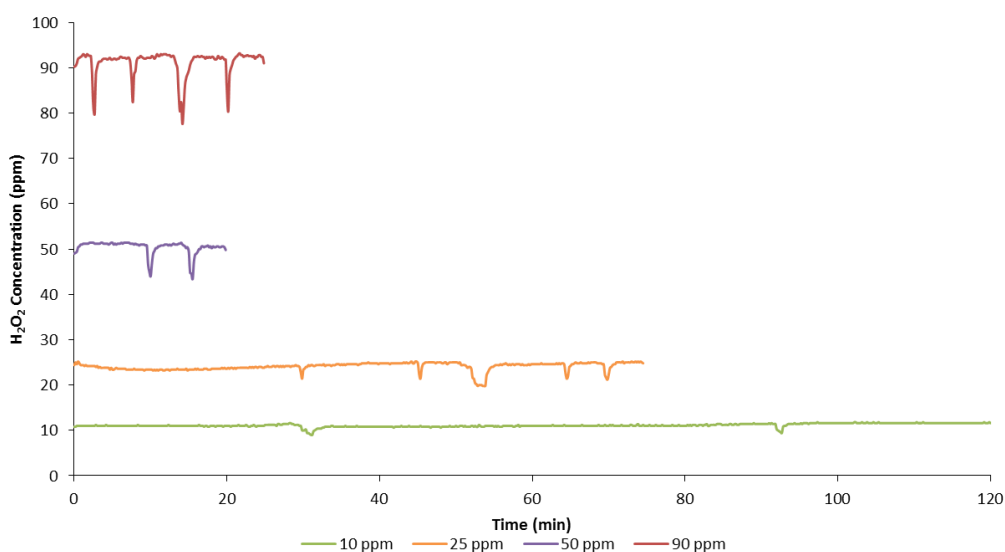
Figure 5.9 Typical hydrogen peroxide concentration traces for *C. difficile* spores on membranes exposed to 10, 25, 50 and 90 ppm over time.

The temperature traces shown in Figure 5.10 demonstrate that the temperature within the exposure chamber was stable at around 25 °C to 30 °C. It is slightly higher for the 25 ppm and 90 ppm traces; however, this was due to

the temperature within the laboratory being higher when the experiments were carried out. Figure 5.10 shows that the relative humidity for the 10 ppm and 25 ppm exposures remained relatively stable at 50 %. Traces for the 50 ppm and 90 ppm are slightly higher at 60 % to 70 %; this is due to the higher liquid hydrogen peroxide flow-rate need to achieve the required hydrogen peroxide vapour concentration. A 4 ml/h liquid hydrogen peroxide flow is used to achieve 10 ppm and 25 ppm, compared to a 15 ml/h flow required to reach 50 ppm and 90 ppm.
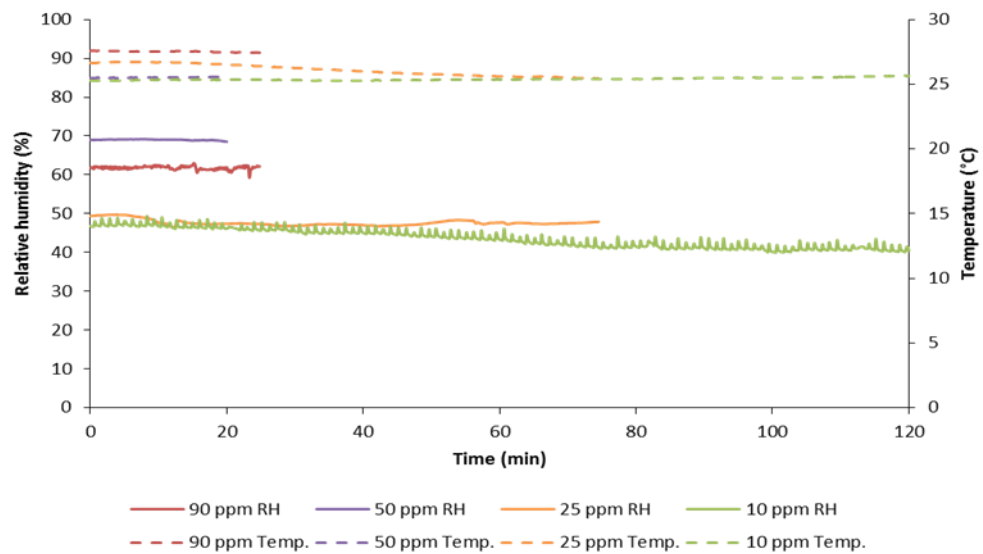
Figure 5.10 Typical relative humidity and temperature traces for *C. difficile* bioindicators exposed to 10, 25, 50 and 90 ppm hydrogen peroxide vapour concentration over time.

5.2.2.2 Inactivation results

The inactivation results for the bioindicators with different spore loading exposed to 10, 25, 50 and 90 ppm hydrogen peroxide were compared (Figure 5.11). A linear trend is indicated by the data; therefore the Chick-Watson inactivation model is fitted alongside the Baranyi, Weibull and Series-Event models. Although the Weibull model gives the best fit (r^2 values shown in Table 5.3) the *D*-values are relatively low compared to the other three models (Figure 5.12) and a linear plot appears to provide a more sensible fit for the data. *D*-values found for the bioindicators with different spore loading (Table 5.3) are far smaller than those for the spore recovery results (Table 5.1). This indicates that there is an apparent difference in the spore susceptibility to

hydrogen peroxide vapour; which could be caused by the spore production method which results in a spore solution containing a lot of debris (Figure 3.7). The nature of the experimental method used to generate these data means that each data point is subject to an error of $\pm$ 1-log$_{10.}$
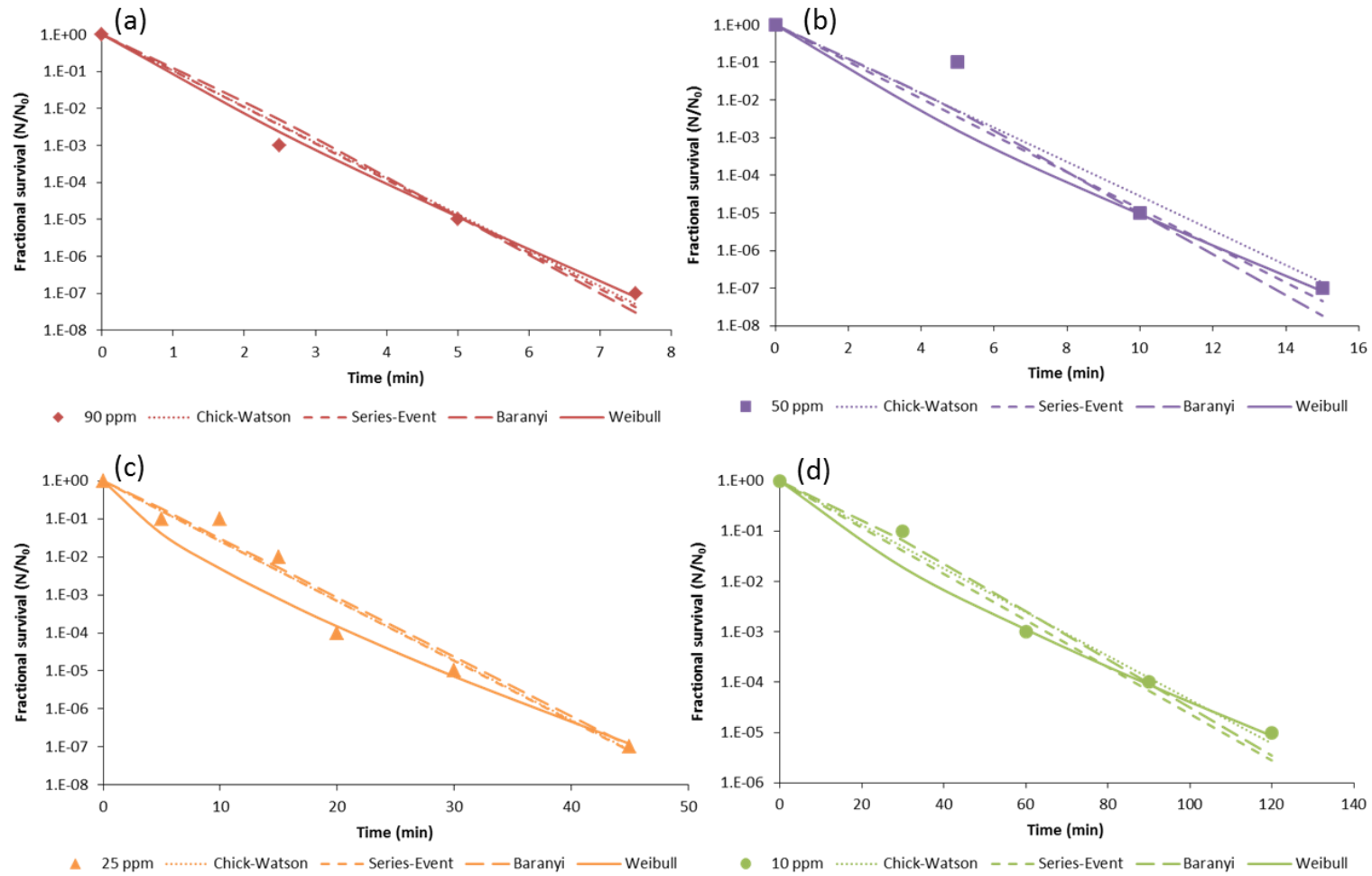
Figure 5.11 Inactivation data for *C. difficile* exposed to different hydrogen peroxide concentrations (a) 90 ppm, (b) 50 ppm, (c) 25 ppm and (d) 10 ppm (two repeateds for each) using the different titre exposure method (3 replicates).
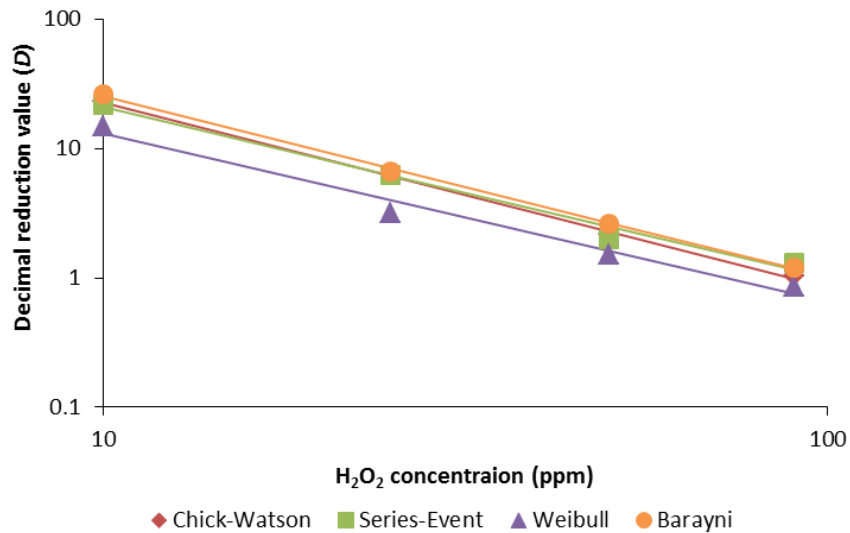
Figure 5.12 *D*-values for the four inactivation models plotted against the hydrogen peroxide concentration.

Figure 5.12 shows the *D*-values calculated from the results obtained using the Clospore production method against the log of the hydrogen peroxide concentration. As with *B. subtilis* a good correlation can be seen between the log of the D-value and the log of the hydrogen peroxide value.

Table 5.3 Inactivation model parameters, *D*-values and r^2 values for *C. difficile* exposed to 10-90 ppm hydrogen peroxide using the bioindicators with different spore loading.

ppm	Chick-Watson			Series-Event (i=1)				Baranyi				Weibull		
	k	*D*	r^2	*k*	*b*	*D*	r^2	*k*	K_C	*D*	r^2	*β*	*D*	r^2
10	1.0×10^{-2}	23.0	0.991	1.5×10^{-2}	0.86	21.6	0.987	0.1	8.6×10^{-2}	26.1	0.997	0.8	15.0	0.986
25	1.5×10^{-2}	6.3	0.977	1.9×10^{-2}	0.91	6.3	0.977	0.4	5.7×10^{-2}	6.7	0.978	0.7	3.2	0.977
50	2.1×10^{-2}	2.2	0.983	2.2×10^{-2}	1.01	2.0	0.961	1.3	1.7×10^{-3}	2.6	0.962	0.8	1.5	0.959
90	2.5×10^{-2}	1.0	0.973	2.1×10^{-2}	1.04	1.3	0.779	2.4	1.1×10^{-2}	1.2	0.740	0.9	0.3	0.999

5.2.2.3 Development of a regression model to estimate the inactivation of *C. difficile* spores using hydrogen peroxide vapour

A method investigated to address the temporal variation of the hydrogen peroxide concentration was to use the concentration-time integral ($\int C dt$) as an independent variable. This is based on the Chick-Watson inactivation model ($\log\left(\dfrac{N}{N_0}\right) = -k\int C_P dt$), shown in Section 2.6.1, with the inactivation being assumed to be first order with respect to the hydrogen peroxide concentration (Chick 1908; Watson 1908). Data plotted in this way could be used to extract an integrated disinfection effect permitting comparison to be made between the data presented here and that presented in literature where the concentration-time data is made available (Fu, Gent & Kumar, 2012), and determine the $\int C_P dt$ value by calculating the area under the concentration-time plot. A plot of $\int C_P dt$ against log reduction is shown in Figure 5.13 for each of the four hydrogen peroxide concentration (90, 50, 25 and 10 ppm). Each individual hydrogen peroxide concentration demonstrates a strong linear correlation with r^2 values of greater than 0.94 being shown for all concentrations. However, the gradient of the fit appears to decrease with the hydrogen peroxide vapour concentration. The gradients do get closer together as the concentration increases, this implies that the inactivation observed at the lower concentrations may be tending towards zero and therefore do not properly fit the model. Although not a perfect solution an overall model was developed including all of the data and is shown in Figure 5.14.
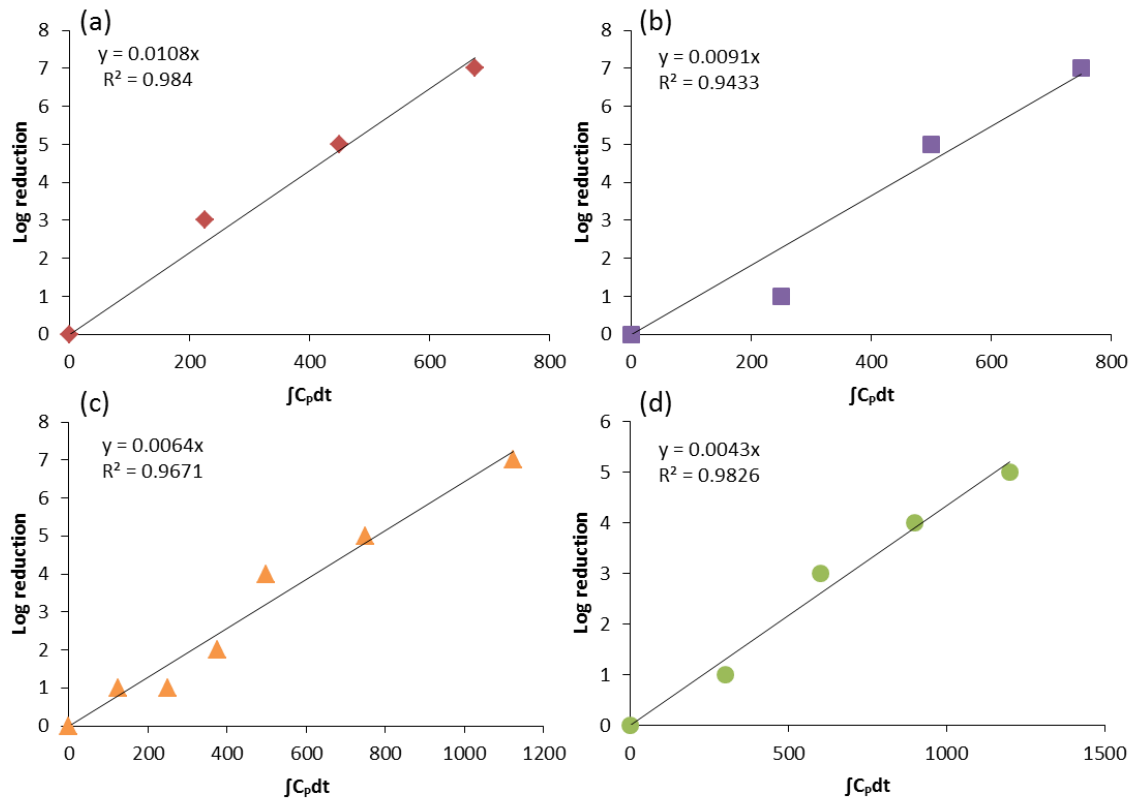
Figure 5.13 $\int C_P dt$ plotted against log reduction for the bioindicators laden with different spore concentrations at: (a) 90 ppm, (b) 50 ppm, (c) 25 ppm and (d) 10 ppm.
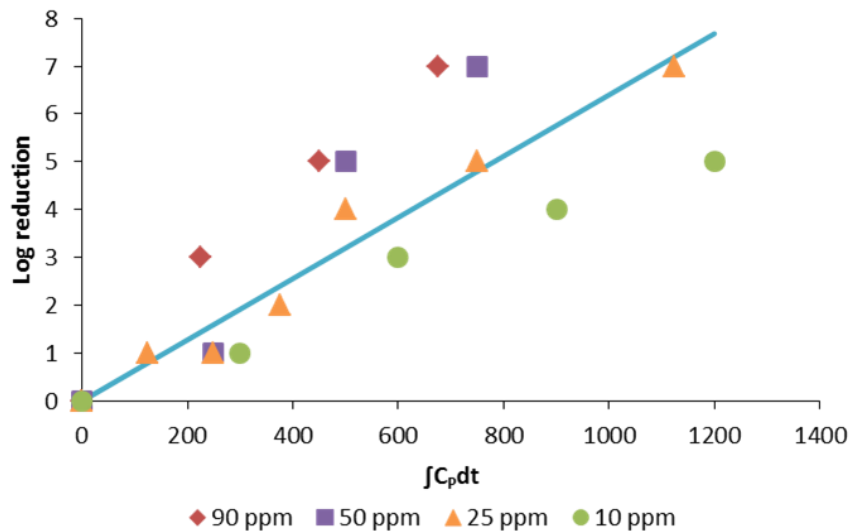
Figure 5.14 Combined plot of $\int C_P dt$ against log reduction for the bioindicators laden with different spore concentrations (regression equation $-\log\left(\dfrac{N}{N_0}\right) = 0.0064 \int C_P dt$, $r^2 = 0.70$).

The combined plot for $\int C_P dt$ against log reduction is shown in Figure 5.14. A linear fit (Equation 5.1) was used to model the data up to 7-$\log_{10}$ and is depicted alongside it. Although the r^2 value is relatively small, the model will still provide an estimation of the log reduction that could be achieved which can then be compared to the log reductions shown in literature (Chapter 6). The model can only be applied up to a 7-$\log_{10}$ reduction as that is the highest concentration of spores that was used in this study.

$$-\log\left(\frac{N}{N_0}\right) = 0.0064\int C_P dt$$

(5.1)

Presenting the data in this way allows for the estimated log reduction to be deduced. It shows that there is a linear relationship up to 7-$\log_{10}$ where all the spores have been inactivated; this happens at a $\int C_P dt$ value of 1093 ppm min. Above this only a 7-$\log_{10}$ reduction can be confirmed, however, a 6-$\log_{10}$ has been used previously to demonstration the efficacy of the hydrogen peroxide inactivation systems (Otter & Yezli, 2011).

5.2.3 Comparison of Spore Laden Membrane Production Methods

Comparing the two different inactivation methods used in this chapter it was deduced that the spore recovery method yielded much longer inactivation times. However, comparison of the SEM images show that spore stock used for the recovery method contained much more debris than that used for the high titre method (Section 3.5), which could provide protection for the spores on the membrane. The spores used in the recovery method were heat shocked at a much higher temperature (60 °C rather than 45 °C) which may result in a more resistant spore (Melly *et al.*, 2002). From the data a 6-$\log_{10}$ reduction is achieved using method 2 after 10 min compared to 80 min for the method 1 at hydrogen peroxide concentration of 90 ppm. For the inactivation at 10 ppm a 5-$\log_{10}$ reduction was achieved after 120 min, however, with method 1 a 2-$\log_{10}$ inactivation was shown after 4 h but the log reduction did not increase any further over the following 3 h. Again this is most likely due to the debris within the spore stock which can protect the spores from the hydrogen peroxide.

5.3 Discussion

In this chapter details were provided on the inactivation of *C. difficile* spores using vapour phase hydrogen peroxide. Further method development was carried out in this chapter and results have been presented for the spore recovery and different titre inactivation methods. As discussed in Chapter 4 with the use of *B. subtilis* the filtering method allowed for the uniform deposition of the spores on the bioindicators ensuring that all the spores were exposed evenly to the hydrogen peroxide vapour.

The results from the two different methods used in this chapter demonstrate that there is a significant difference in the inactivation time required for the same strain of *C. difficile* spores. There are two explanations for this: firstly, the spore stock used for spore recovery experiments was shown to be contaminated with debris of various origins. Otter *et al.* (2012) demonstrated that there was a significant reduction in the inactivation of MRSA achieved upon suspension in 3 % and 10 % BSA (bovine serum albumin) rather than water or 0.3 % BSA. In their study a 6-$\log_{10}$ inactivation was achieved after 30 min when the bacterium is suspended in water compared to 75 min for the same reduction when suspended in 10 % BSA. Therefore, there is difference in the inactivation of bacterium under 'dirty' conditions; however the statistical significance of these results was not established. This suggests that organic material can act to protect the bacteria from exposure to an agent such as hydrogen peroxide (Otter *et al.*, 2012). Nevertheless, there are some discrepancies with this study: predominantly, the lack of information on the concentration of hydrogen peroxide utilised; the group only disclose the type of decontamination cycle employed. There was also no report of a hydrogen peroxide neutraliser used in the recovery process, and therefore it could be argued that the results may be an overestimation of the inactivation achieved. Investigation into bacteria protected by either organic matter, the production of a biofilm or a combination of the two is important as biofilms have recently been shown to be an important aspect of the resistance of HAI-causing bacteria to standard disinfectants (Song, Wu & Xi, 2012; Vickery *et al.*, 2012).

Another explanation for the differences observed in spore inactivation may be attributed to spore production. The spores used for the recovery method were washed in a 50 % IMS/water solution before applying a heat-shock at 60 °C for 10 min; method 1 required heat-shocking the spores at 45 °C for 6 h. Melly *et al.* (2002) demonstrated the effect that a heat-shock temperature can have on spores of *B. subtilis*. There researches reported that spores produced at a higher temperature (48 °C) were more resistant to subsequent thermal inactivation than spores produced at lower temperatures (22 °C); with a 4-$\log_{10}$ reduction being achieved by the thermal inactivation after 30 min and less than 1-$\log_{10}$ reduction after 50 min for the spores produced at low and high temperatures respectively. Although all the results presented throughout this chapter were carried out at room temperature, the difference in inactivation at the same hydrogen peroxide concentration could be due to an intrinsic difference in spore resistance induced by heat shocking at different temperatures.

The data presented supports the use of *B. subtilis* as a model organism (Chapter 4) as it takes significantly longer to be inactivated; *B. subtilis* requires 6 h for a 6-$\log_{10}$ reduction at 90 ppm, compared to 80 min for *C. difficile* using the same method. However, it could be argued that these results are not comparable as the *C. difficile* spore stock possessed cell debris within, which has been shown to increase bacterial resistance to hydrogen peroxide (Otter *et al.*, 2012). When using a purified spore stock, the rate of inactivation of the spores when exposed to hydrogen peroxide significantly increased, showing a 6-$\log_{10}$ reduction after less than 10 min at 90 ppm. *D*-values obtained for the *B. subtilis* were shown to be two orders of magnitude greater than those for *C. difficile*.

Three inactivation models were used to fit the data presented in this chapter, as with Chapter 4: the Weibull, Baranyi and Series-Event models. As demonstrated by Malik *et al.* (2013), the Weibull model provided the best overall fit for the spore recovery results. However, considering the results obtained using method 2 for spore exposure, although the r^2 value for the Weibull model presented the best fit, the Baranyi and Series-Event models produced more realistic linear inactivation models. The inactivation models

used for method 2 results provide similar inactivation kinetics to those reported by Johnston, Lawson & Otter (2005) for the inactivation of *C. botulinum* spores exposed to hydrogen peroxide vapour, with an average concentration of 355 ppm. The data produced by this research would have benefitted from the use of an inactivation model, as shown in this chapter to take account of the initial shoulder. The results comparing the inactivation of four different strains identify both 027 and 014 (both isolated from faecal samples) as the most resistant and 103 which is an environmental strain isolated from a hospital ward as the least resistant. These have been found to be among the most abundant strain found in the healthcare environment (Health Protection Agency, 2012b). As ribotypes 014 and 027 are commonly found within the environment it is implied that they are the most resistant to the cleaning method in place, therefore it is not surprising that they are the most resistant to hydrogen peroxide.

The findings reported in this chapter regarding the resistance of *C. difficile* strains to hydrogen peroxide contrasts with results presented by Patel (2012). This work reported that when exposed to liquid hydrogen peroxide, ribotype 103 was the most resistant strain and 027 was the least. This implies that there may be a difference in inactivation properties of hydrogen peroxide in the vapour and liquid phase which respect to *C. difficile* strains, which has been supported in a study by Finnegan *et al*. (2010).

When the results were presented in the form of a concentration-time integral ($\int C_P dt$) against the log reduction, the results all appear to follow a more or less linear plot. However, at low concentrations (10 ppm and 25 ppm) there were some departures from linearity. Therefore it is important to investigate this further to identify if there is a minimum concentration at which this approach can be used. Plotting results in this manner is important as it will allow for the inactivation achieved in disinfection cycles, where the concentration of hydrogen peroxide varies with time to be predicted.

Inactivation data presented in this study suggested that around a 10 min exposure to a hydrogen peroxide concentration of 90 ppm was required to achieve a 7-$\log_{10}$ reduction in *C. difficile* spores. Otter & French (2009) demonstrated that a 10 min exposure time was required to achieve a 6-$\log_{10}$

reduction in *C. difficile* spores. No details of the hydrogen peroxide concentration used in their study were provided, it is merely stated that the relative humidity and hydrogen peroxide concentration peak at concentration consistent with the onset of microcondensation. Barbut, Yezli & Otter (2012) also showed a 6-log$_{10}$ reduction in *C. difficile*; however, in this study the inactivation is carried out using an automated hydrogen peroxide disinfection cycle in a 33 m^3 to 45 m^3 room and as with the study by Otter no details are provided concerning the hydrogen peroxide concentration used.

The results presented in this chapter show that hydrogen peroxide is a very efficient disinfection method against *C. difficile*. As *C. difficile* is a spore-forming obligately anaerobic bacterium it has not evolved to deal with oxidisation stress, so does not naturally produce catalase, which might offer protection against hydrogen peroxide (Otter & French, 2009). A relationship was obtained between the concentration-time integral and the log reduction that could be used to allow for the estimation of the decontamination level provided from a hydrogen peroxide cycle provided that the concentration-time data were available.

In this study, *C. difficile* was chosen to investigate spore inactivation and kinetics because of its global prevalence as a HAI and as it is one of the most resistant to most common hospital disinfectants. The main cause of its resistance is due to the fact it is a spore-forming bacterium making it very hardy against alcohol rubs and bleach (Wilcox & Fawley, 2000; Jabbar *et al.*, 2010). More recent research has suggested that MRSA, although not a spore-forming bacteria, is more resistant than *C. difficile* to hydrogen peroxide vapour and potentially even *G. stearothermophilus*, which is commonly used in commercial bioindicators to investigate inactivation (Fu, Gent & Kumar, 2012; Pottage *et al.*, 2012). This has been attributed to MRSA being a catalase-positive bacterium and therefore having a natural resistance to hydrogen peroxide (Otter & French, 2009; Otter & Yezli, 2012).

5.4 Chapter Summary

- The method developed for obtaining inactivation data under controlled conditions previously described for *B. subtilis* spores using hydrogen peroxide vapour was extended to *C. difficile* spores.

- A simple growth/no-growth method was shown to remove difficulties with spore recovery and yield data that could be used to estimate the *D*-values.

- *C. difficile* is much more susceptible to hydrogen peroxide vapour than *B. subtilis*. This is potentially due to *C. difficile* being a non-catalase-producing bacterium therefore not having any natural resistance to hydrogen peroxide.

- A model was developed to predict the inactivation based on the hydrogen peroxide integral ($\int C_P dt$) over time. However, this model may not provide a good fit at very low hydrogen peroxide concentrations. Therefore, more research is required to identify a minimum concentration at which the model can be applied.

- The results presented in this chapter showed that there was a difference in resistance between the different strains of *C. difficile* exposed to hydrogen peroxide vapour. This is important as the *C. difficile* strains found within the hospital are becoming more diverse.

- Further work is warranted into the different strains found within the hospital environment to find which are the most resistant and to ensure that inactivation cycles using commercial hydrogen peroxide vapour generators are able to bring about significant reductions in the viability of the strains in question.

Chapter 6
Estimating the Disinfection Performance of Commercial Hydrogen Peroxide Vapour Devices

6.1 Introduction

Hydrogen peroxide decontamination systems are currently used in the healthcare environment for a deep clean after a healthcare-acquired infection (HAI) outbreak. Commercially available devices release a fixed amount of hydrogen peroxide in to a closed space, resulting in a peak concentration which drops rapidly as shown by Chung *et al.* (2008) and Fu, Gent & Kumar (2012). The majority of hydrogen peroxide inactivation studies have employed the same automated equipment as used for hospital disinfection (e.g. Johnston, Lawson & Otter, 2005; Tuladhar *et al.*, 2012). This makes the outcomes of such studies hard to compare as in many of cases the hydrogen peroxide concentration either is not stated or only the peak concentration is provided (Boyce *et al.*, 2008; Otter *et al.*, 2009).

There are a number of hydrogen peroxide vapour disinfection systems currently on the market. The main difference in the systems is the manner in which they generate and distribute the hydrogen peroxide vapour in the space. Some of the systems used forced convection following the release of the spray/vapour into the space; whereas other systems rely solely on diffusion. The different release methods result in significantly different peak hydrogen peroxide concentrations that can be achieved, ranging from 30 ppm to 800 ppm (Johnston, Lawson & Otter, 2005; Andersen *et al.*, 2006; Unger-Bimczok *et al.*, 2008; Fu, Gent & Kumar, 2012). The maximum concentration reached is important as this study has demonstrated that the log of the *D*-value of *C. difficile* and *B. subtilis* is inversely related to the hydrogen peroxide concentration (Chapters 4 and 5). However, the use of higher hydrogen peroxide concentrations increases the risk of the vapour being absorbed by soft furnishings (Galvin *et al.*, 2012). Decontamination cycles which use the higher

hydrogen peroxide concentrations also require additional equipment to aid the removal of hydrogen peroxide to give cycle times that are not excessively long; which increases both the cost and the complexity of the system (Otter & Yezli, 2011). In contrast to this, the systems employing low hydrogen peroxide concentrations allow it to decompose naturally with cycle times of around 2 h (Fu, Gent & Kumar, 2012).

The basis upon which commercial hydrogen peroxide process cycles are designed remains unclear. Commercially available spore bioindicators of *G. stearothermophilus* are normally utilised to demonstrate the efficiency of the cycles (Otter & Yezli, 2012). Only recently has the susceptibility to hydrogen peroxide vapour of common causes of HAIs (MRSA and *C. difficile*) been compared to that of bacteria used in commercial bioindicators (frequently *G. stearothermophilus*). These comparisons have suggested the *C. difficile* is far more susceptible to hydrogen peroxide than the commercial bioindicators, therefore this method results in an overestimate of the inactivation. However, Pottage *et al*. (2012) suggest that MRSA is more resistant to hydrogen peroxide than *G. stearothermophilus* due to its ability to produce catalase. This is of concern for hydrogen peroxide cycles optimised using the *G. stearothermophilus* bioindicators, as it could lead to inadequate decontamination of MRSA.

6.1.1 Aims

In this chapter, the model identified in Chapter 5 was used to estimate the extent of inactivation achieved during typical hydrogen peroxide disinfection cycles. For this aerosolised hydrogen peroxide disinfection cycles generated using a Hygienics Biogenie by Keith Schou (Hygienics Biosecurity, UK) in a 50 m^3 room design were used, to imitate a hospital side room. A typical decontamination cycle involved a 3 % hydrogen peroxide solution being sprayed into the room at a rate of 30 ml/min for around 20 min before the hydrogen peroxide was allowed to degrade naturally. The results generated using this method were then compared to the hydrogen peroxide decontamination cycles presented by Fu, Gent & Kumar (2012).

For the comparison, hydrogen peroxide concentration traces were measured at five different locations around the room (all four corners and the centre of the room) at three different heights. The hydrogen peroxide traces were integrated to find the concentration-time ($\int C_P dt$), this was then compared to the model detailed in Chapter 5 to estimate the inactivation of *C. difficile* in all areas of the room.

6.2 Results

6.2.1 Hydrogen Peroxide and Humidity Results within a Test Room

Hydrogen peroxide concentration traces (Figure 6.3) were recorded during the disinfection cycle of a Hygienics Biogenie in the 50 m^3 Hygienics test room shown in Figure 6.1. An array of six hydrogen peroxide sensors were placed in one of the fifteen positions in the test facility and the standard Biogenie disinfection cycle was run by Keith Schou (Hygienics Biosecurity). The sensors were placed in a group of six so that an average concentration could be calculated.
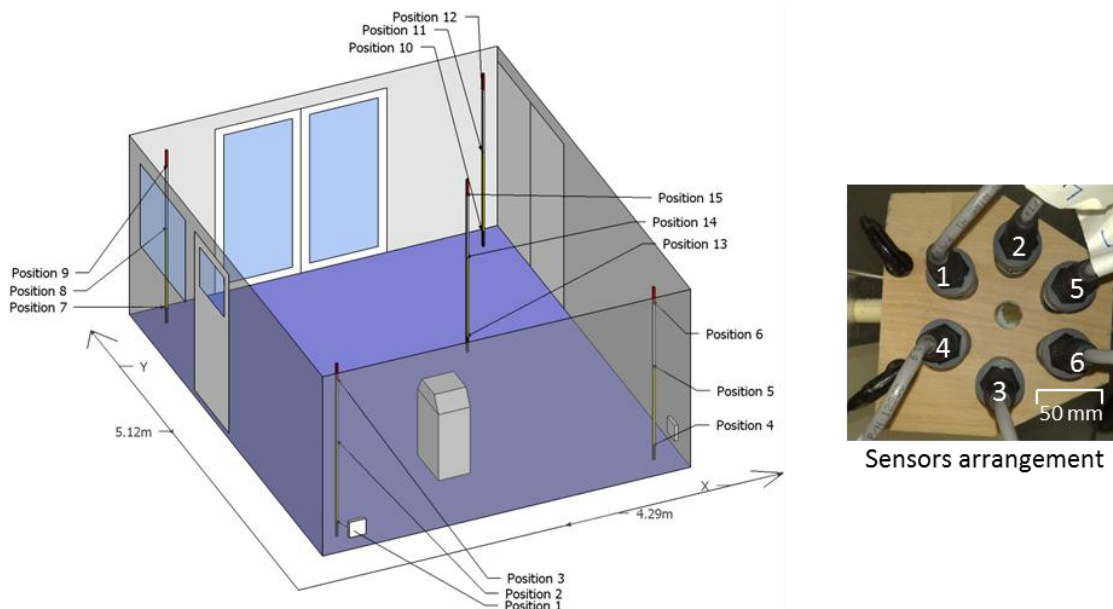
Figure 6.1 Test room showing the hydrogen peroxide sensor locations and arrangement. Image produced by Keith Schou (Hygienics Biosecurity).

Figure 6.2a shows typical discrepancies between the six sensors placed in a single locations; demonstrating that the traces show a range of the peak concentration of between 68 and 86 ppm, which is around the 10 % relative error of the hydrogen peroxide sensors. This suggests that the averaged results of the six sensors shown in Figure 6.3 should provide a reasonably good estimation of the hydrogen peroxide concentration in each location. The sensors have been shown to respond quickly to any changes within the environment in Section 5.2.1 where it was demonstrated that the hydrogen peroxide concentration dropped and recovered within a minute when the chamber was opened to remove the samples.

Humidity sensor traces shown in Figure 6.2b for the six humidity sensors in the same location, demonstrate that there is a reasonable agreement between the six sensors. A 10 % difference in the relative humidity was observed between the sensors; therefore, only locations with greater than 10 % difference in relative humidity can be compared to demonstrate any difference in the spatial and temporal humidity. The response characteristics of the humidity sensors are shown in Section 4.2.3 when the syringe is refilled during the exposure of bioindicators in the exposure chamber. It can be inferred that when the syringe is refilled there is a sharp spike in the humidity due to the extra liquid being added to ensure no air was in the system. The spike was shown to be take around a minute to peak and then fall back to the average humidity within the exposure chamber.
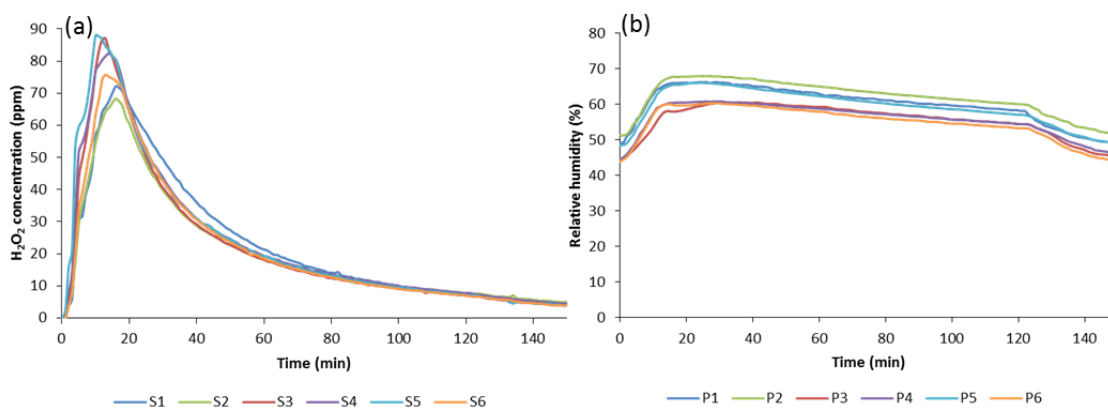
Figure 6.2 Typical (a) hydrogen peroxide traces and (b) relative humidity for the six hydrogen peroxide and humidity sensors placed at position 11.
Data generated by Keith Schou (Hygienics Biosecurity).

The hydrogen peroxide concentration traces for the different positions around the test room are shown in Figure 6.3. Most of the hydrogen peroxide traces show a peak value between 60 ppm and 85 ppm. The peak concentration within the room is reached after the first 15 min; it then dropped rapidly after the hydrogen peroxide stopped being sprayed into the room, to around 30 ppm after a cycle time of 50 min in all cases. A further 100 min is required for the hydrogen peroxide concentration to drop below 1 ppm where the room can be repopulated. The two lowest hydrogen peroxide traces are shown in the centre of the room (P13 and P14), with the floor and mid concentrations reaching peak concentrations of 30 ppm and 40 ppm respectively. These are the sensors in the middle of the room in front of the Hygienics Biogenie, demonstrating that spraying the hydrogen peroxide upwards resulting in the middle of the room is exposed to a much lower concentration. This is supported by the highest hydrogen peroxide concentration being observed at the ceiling in the centre of the room (P15). Unsurprisingly the hydrogen peroxide traces also show that the readings from behind the Biogenie are much lower than those in front of the device. This is due to the hydrogen peroxide being sprayed upwards into the room and then allowed to disperse through the room by convection and diffusion.

To date very few studies have published data presenting the hydrogen peroxide concentration-time data and none in which the spatial and temporal

variations observed during a decontamination process. When the concentration-time data is given, such as by Fu, Gent & Kumar (2012), it is measured by a unit within the hydrogen peroxide vapour generator. This means that the hydrogen peroxide concentration traces provided are likely to presenting the profile with the lowest peak concentration as demonstrated by Figure 6.3. The results presented by Fu, Gent & Kumar (2012) for the aHP disinfection cycle suggest that the inactivation of *G. stearothermophilus* bioindicators is lower at the far end of the room in front of the vapour generator. This is likely to be caused by the non-optimal positioning of the generator, with it being positioned in a corner rather than in the centre of the room.
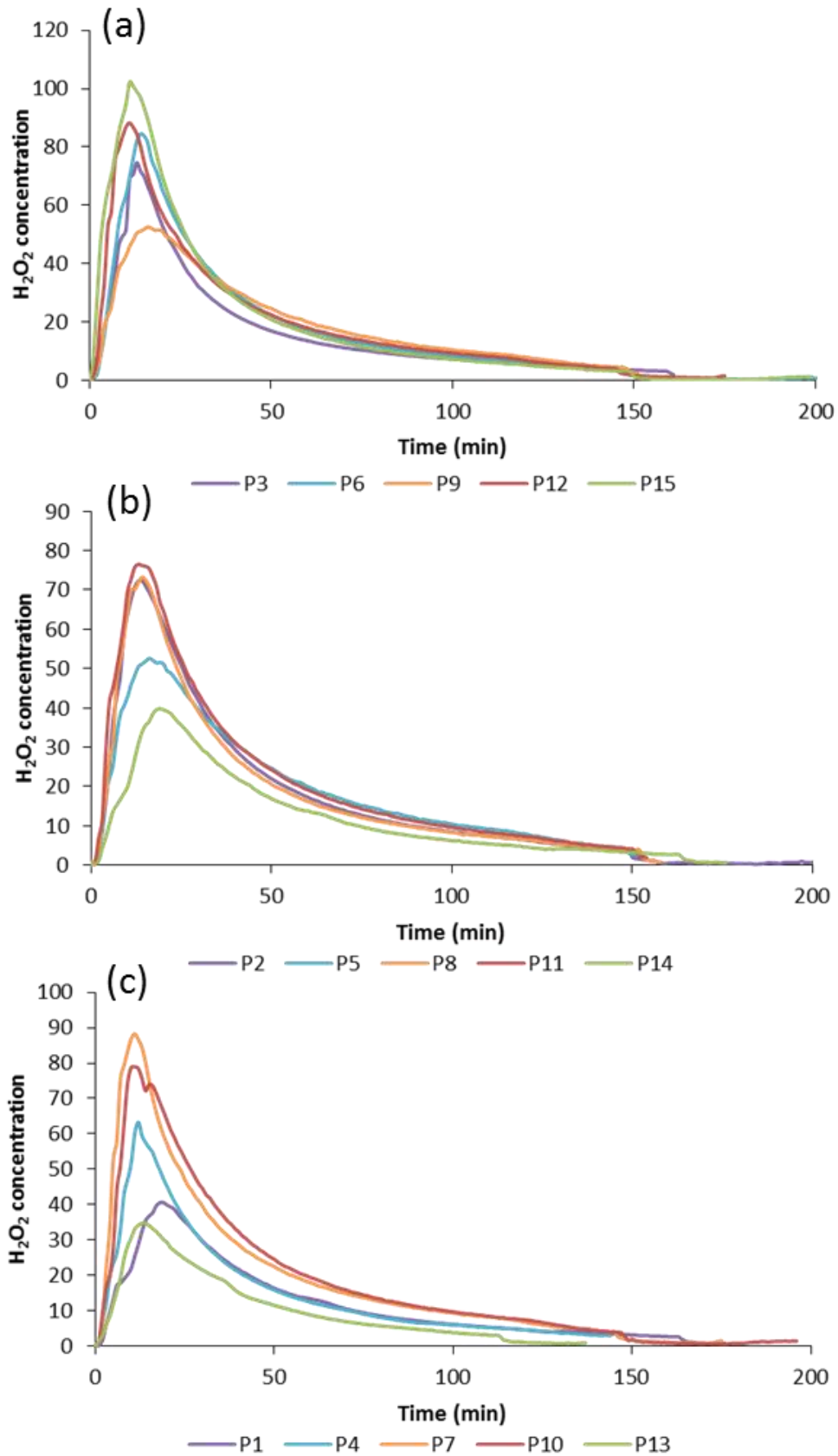
Figure 6.3 Averaged hydrogen peroxide traces (from 6 sensors) for (a) the ceiling (b) mid-way up the test room and (c) the floor.
Data generated by Keith Schou (Hygienics Biosecurity).

The humidity traces (Figure 6.4) within the room reveal an increase during the initial spraying stage of the disinfection process (relative humidity only is presented as the temperature within the room is stable at 26 ±1 °C). This is due the introduction of the dilute hydrogen peroxide solution into the room, after the initial spraying process the relative humidity steadily decreases. There is no apparent connection between the position at which the highest hydrogen peroxide concentration is achieved and that at which the highest humidity is obtained. The highest humidity is identified for position 7, which is in the mid-range for the hydrogen peroxide. Position 3 demonstrates the lowest humidity and the third highest hydrogen peroxide concentration.
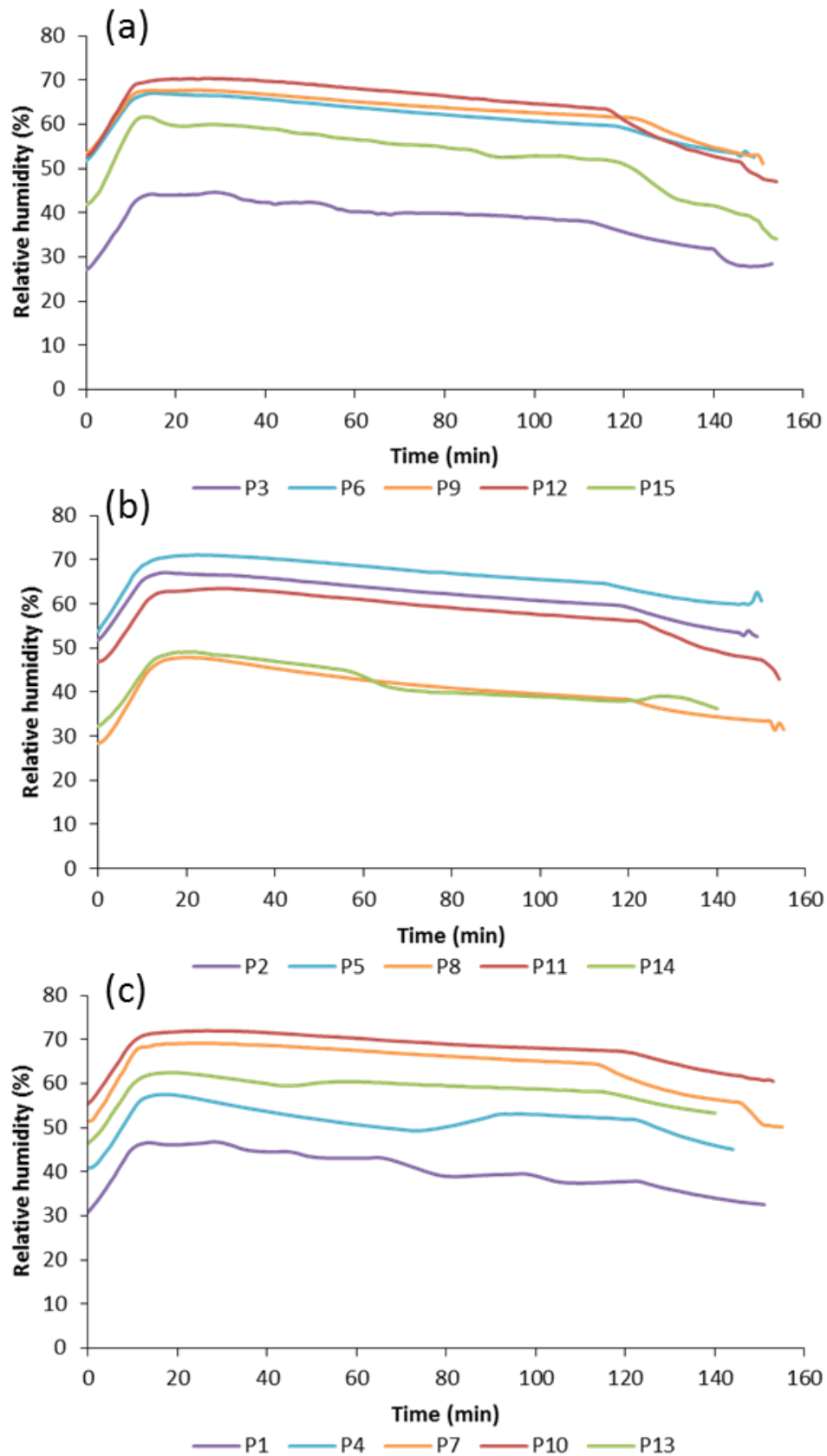
Figure 6.4 Averaged relative humidity traces (from 6 sensors) for (a) the ceiling (b) mid-way up the test room and (c) the floor.
Data generated by Keith Schou (Hygienics Biosecurity).

6.2.2 Estimation of Log Reduction in an Enclosed Space

Using the hydrogen peroxide traces for the different locations within the test room the concentration-time integral was calculated using Simpson's rule (Figure 6.5). The model developed in Chapter 5 was then used to calculate the inactivation based on the concentration-time differential. It was deduced that at least a 7-log$_{10}$ (maximum log reduction investigated) could be achieved in all areas. This is due to the concentration-time integrals being above 1093 ppm min in all areas which is the minimum exposure required for a 7-log$_{10}$ reduction.

Figure 6.5 provides details of the room positions that display the high, mid and low peak concentrations of hydrogen peroxide against the calculated log reduction. As the model could only be validated up to an inactivation of 7-log$_{10}$ this is the highest inactivation shown. For the lowest hydrogen peroxide it takes about 56 min for a 7-log$_{10}$ inactivation to be achieved, this is 38 min longer than for the highest peak hydrogen peroxide concentration (18 min).
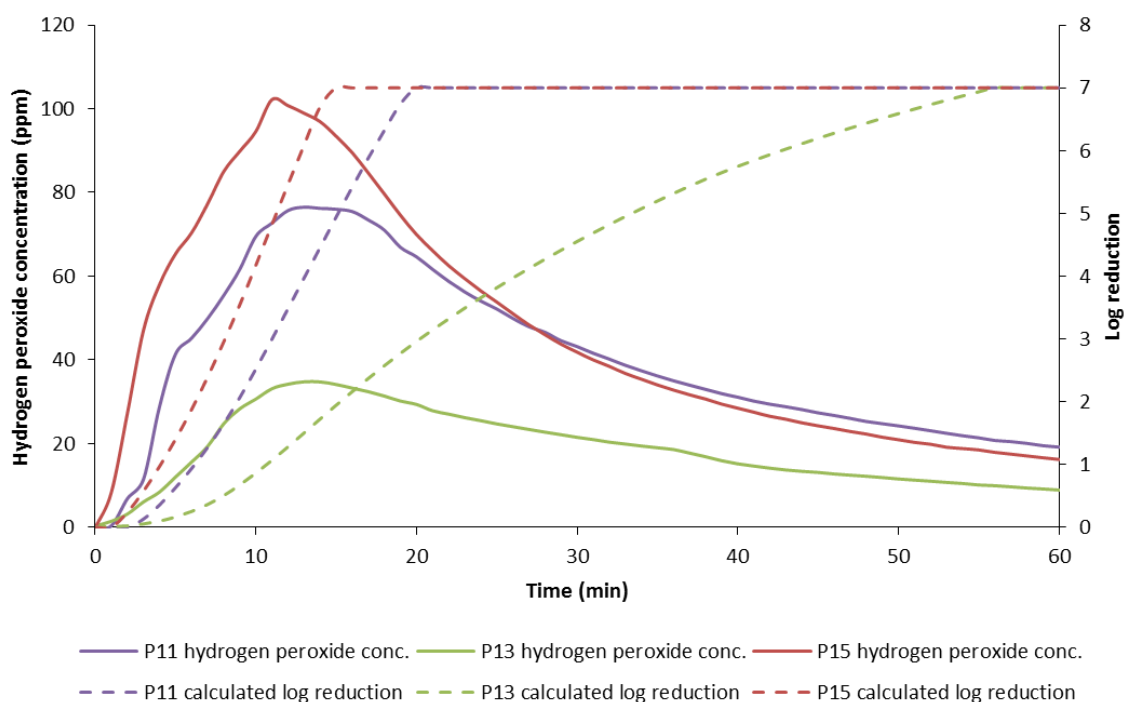
Figure 6.5 High, Mid and Low peak concentration cycles of the hydrogen peroxide decontamination cycle plotted alongside the calculated *C. difficile* log reduction.
Hydrogen peroxide room trace data generated by Keith Schou (Hygienics Biosecurity).

Fu, Gent & Kumar (2012) compared Bioquell (HPV) and ASP (aHP) hydrogen peroxide inactivation cycles, their data was used here to calculate the expected log reduction that could be achieved using the model identified in Chapter 5. A 7-log$_{10}$ reduction for the HPV inactivation cycle is estimated as occurring within 25 min where the cycle peaks at about 125 ppm (Figure 6.6). The concentration profile is comparable to position 15 within the test room used to generate the Hygienics data, although the time to reach a 7-log$_{10}$ reduction is slightly longer; this is due to the HPV cycle taking longer (40 min (HPV) compared to 15 min (aHP)) to reach its peak concentration. Figure 6.6 also shows an ASP hydrogen peroxide concentration trace which is comparable to the lowest concentration trace shown in the Hygienics test room (Position 13). Both cycles show a peak concentration at around 30 ppm, 15 min after the start of the cycle. The main difference between the two cycles however is that in the ASP cycle the hydrogen peroxide concentration within the room drops significantly quicker than with the Hygienics cycle. However, both cycles achieved a 7-log$_{10}$ reduction value in the same time (56 min Hygienics compared to 55 min ASP). Fu, Gent & Kumar (2012) obtained a 5.6-log$_{10}$ reduction of *C. difficile* (complete inactivation of the bioindicators used) within the 50 m^3 test room used in their study. This data shows that a 6-log$_{10}$ reduction required for decontamination is likely to be achieved and the 3-log$_{10}$ reduction required for disinfection should be achieved easily.
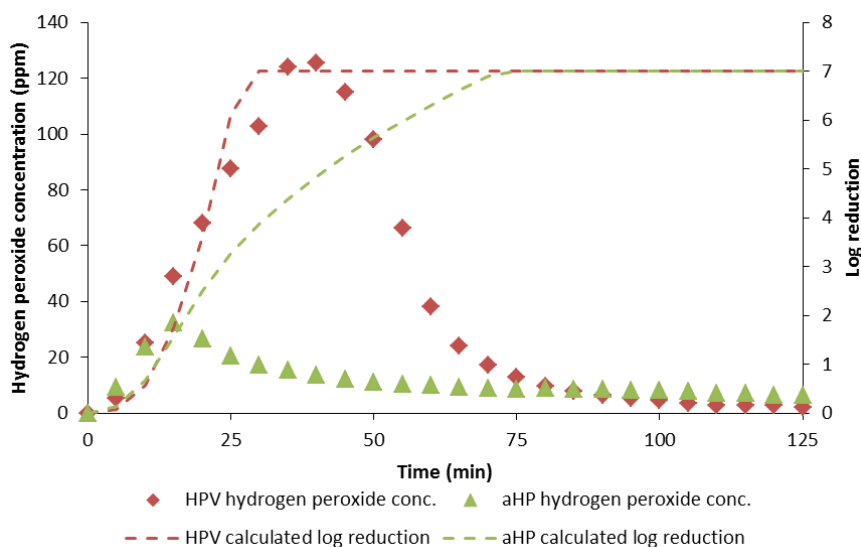
Figure 6.6 Vaporised and aerosolized hydrogen peroxide cycle concentrations plotted alongside the calculated log reduction.
Hydrogen peroxide concentration traces replotted from Fu, Gent & Kumar (2012).

Using the model developed in Chapter 5 it may be concluded that even if hydrogen peroxide cycles with the lowest peak concentration were used, a 7-log$_{10}$ reduction in *C. difficile* could be expected. These results are in agreement with other studies by Otter & French (2009) and Barbut, Yezli & Otter (2012) which both report a greater than 6-log$_{10}$ reduction during an inactivation cycle.

6.3 Discussion

Concentration-time ($\int C_P dt$) data found by integrating the hydrogen peroxide profile were used in conjunction with the regression model in order to predict the log reduction that could be expected. These results suggested that a 7-log$_{10}$ reduction in *C. difficile* spores was achievable even with the cycles or positions showing the lowest peak concentration. These reductions are confirmed by results shown in the literature which detail complete inactivation of bioindicators of a 4.5- to 6-log$_{10}$ reduction in *C. difficile* spores (Barbut *et al.*, 2009; Barbut, Yezli & Otter, 2012; Galvin *et al.*, 2012).

Four studies have been carried out in which the inactivation of bacteria within a confined area has been reported. Of these studies only two give the hydrogen peroxide concentrations within the room; Fu, Gent & Kumar (2012) provide hydrogen peroxide traces for the two different process cycles used,

whereas Tuladhar *et al.* (2012) exposed the spores to a constant hydrogen peroxide concentration of 126 ppm for 1 h before an aeration stage which lasted approximately 45 min. Fu, Gent & Kumar (2012) compared the inactivation efficiencies of the Bioquell (HPV) and ASP (aHP) cycles which show peak hydrogen peroxide concentrations of 125 ppm and 30 ppm respectively in a 50.1 m^3 room with a 13.2 m^3 side room. The results published for the ASP cycle, which shows a similar cycle to position 13 used in this study, suggest a 5.6-$\log_{10}$ reduction (5.6-$\log_{10}$ starting concentration) within the main room. There was failure to inactivate all of the bioindicators within the side room with only a 3.6-$\log_{10}$ reduction being observed in most locations and this reducing to a 0.6-$\log_{10}$ reduction behind the door. The lower reduction in the side room can be explained by the limited diffusion of the aerosol, resulting in the spores having been exposed to a significantly lower hydrogen peroxide concentration. This supports the findings from this study which suggests a 7-$\log_{10}$ reduction should be achieved in a 50 m^3 room using a similar cycle. Although no hydrogen peroxide concentration data is shown, Piskin *et al.* (2011) use the same ASP cycle as Fu, Gent & Kumar (2012); it was shown that in a 53 m^3 room a 4-$\log_{10}$ (initial concentration) reduction of both MRSA and *Acinetobacter* in every area of the room apart from inside a closed drawer.

C. difficile was used in this study as it is a spore-forming obligately anaerobic bacterium, however MRSA has been identified as more resistant to hydrogen peroxide as it produces catalase (Pottage *et al.*, 2012). As previously stated, Piskin *et al.* (2011) identified a 4-$\log_{10}$ reduction in MRSA using a ASP disinfection cycle. Fu, Gent & Kumar (2012) agreed with these findings showing a 5.3-$\log_{10}$ reduction (complete inactivation) in the main room which dropped to a 1.7-$\log_{10}$ reduction in the side room. This compared to a 7.2-$\log_{10}$ (complete inactivation) that was shown in all areas as part of the same study when using the Bioquell cycle. It also indicated that even though the Bioquell cycle produced a higher maximum hydrogen peroxide concentration the aeration cycle appeared shortened in the study by Fu, Gent & Kumar (2012) became a catalyst was used to remove the residual hydrogen peroxide. A greater than 7-$\log_{10}$ reduction for MRSA was also shown by Galvin *et al.* (2012) although no hydrogen peroxide vapour concentration data was given.

The results presented in this chapter have demonstrated that it is possible to use a model to identify the inactivation that could be achieved by a hydrogen peroxide cycle. The model developed in Chapter 5 was applied to the Hygienics Biogenie cycle and found that a 7-log$_{10}$ reduction should be achieved in all areas in the room. This was substantiated by data shown in other studies using similar hydrogen peroxide cycles. However, the inactivation achievable is reduced in the case of a side room due the limited diffusion of the aerosol. This is important as it shows that limited diffusion is a major issue when using hydrogen peroxide vapour to disinfect rooms with a side room, such as an *en suite* bathroom. A recent study by Unger-Bimczok *et al.* (2011) demonstrated the efficiency of hydrogen peroxide at inactivating *G. stearothermophilus* bioindicators at the end of narrow cavities. However, this study was carried out at much higher concentrations than were employed in these experiments (greater than 400 ppm), making it much more efficient. The use of a model such as described here will provide greater confidence in the decontamination cycle. It could also be used to optimise the current hydrogen peroxide decontamination processes on the market, as to date little to no optimisation appears to have been carried out, as the current standard method of measuring the efficiency of a hydrogen peroxide decontamination cycle is the use of commercially available *G. stearothermophilus* bioindicators (Otter & Yezli, 2012; Malik, 2013).

6.4 Chapter Summary

- The model generated in Chapter 5 was used to estimate the inactivation that can be achieved during a hydrogen peroxide cycle.
- A 7-log$_{10}$ inactivation can be achieved for *C. difficile* using an aerosolized disinfection system in all positions monitored within a 50 m^3 test room.
- The potential inactivation was confirmed by comparison with previous published studies.
- Further investigation is required in order to generate a similar model for other HAI specific organisms.

Chapter 7
Conclusions and Suggestions for Further Work

7.1 Conclusions

In this study an exposure chamber was designed and used to investigate inactivation kinetics of bacterial endospores exposed to a constant concentration of hydrogen peroxide vapour. Spore-forming bacteria used throughout the study were *B. subtilis* as a model microorganism and *C. difficile* as it is the most common cause of healthcare-acquired infection in the UK.

Firstly a study was designed to explore whether the exposure chamber was suitable to yield consistent inactivation kinetics of the bacteria when exposed to hydrogen peroxide (Chapter 4). It was demonstrated that the humidity and hydrogen peroxide concentration within the chamber was reproducible and maintainable. Concentrations between 10 ppm and 90 ppm could be reached and maintained for up to 48 h. The data generated using this method could then be compared to that detailed in published literature to highlight similarities or differences.

A filtration method was developed to produce a uniform distribution of spores on the surface of the membrane allowing for uniform exposure of all the spores to the hydrogen peroxide vapour (as shown in Chapter 3).

Inactivation of *B. subtilis* spores revealed a shoulder period in the inactivation kinetics (Chapter 4). The implication of this is that there is a time where the damage is below the lethal limit and the spore can therefore recover. Three inactivation models were shown to provide a good fit for the data. These were the Series-Event, Baranyi and Weibull inactivation models. The Weibull model provided the best overall fit for the data as well as for data generated by Wang & Toledo (1986) who used hydrogen peroxide to inactivate *B. subtilis* spores on food packaging.

As with the *B. subtilis* results, the *C. difficile* inactivation data could also be predicted using the three models previously discussed, as well as the Chick-Watson model (Chapter 5). The Weibull model again provided the best fit for the data; however, with the results for different titre membranes, the Weibull model predicted an upwardly concave inactivation curve. Although this may provide the best fit for the data it did not represent a realistic fit for the data, which demonstrates a linear trend.

Differences in inactivation of *C. difficile* strains exposed to hydrogen peroxide vapour were investigated (Chapter 5). A difference in the susceptibility of the *C. difficile* strains, with *D*-values ranging between 22 min (014) and 9 min (220), with the clinically relevant strains (014 and 027) being found to be the most resistant was revealed. Further research is warranted to obtain detailed inactivation kinetics of additional strains to ensure that all of the strains would be inactivated in a decontamination cycle.

Using the *C. difficile* inactivation data obtained at steady hydrogen peroxide vapour concentrations; a regression model was developed to investigate the relationship between the integral $\int C_P dt$ and the log reduction achieved. This relationship was then used to identify the log reduction that could be achieved within a room if the time history of the hydrogen peroxide concentration were known. Using the hydrogen peroxide traces for a commercial disinfection system (Hygienics) in a 50 m^3 test facility, it was demonstrated that theoretically a 7-log$_{10}$ inactivation may be achieved anywhere within the room for *C. difficile* spores (Chapter 6).

7.2 Further Work

There are many area highlighted in this work that would be interesting for further study, these include; the spore production method, the susceptibility of different strains of *C. difficile*, the susceptibility of different HAI-causing bacteria and investigation into other deep clean methods that could be used in healthcare environments.

Investigation of the influence of the spore production method on the susceptibility of the spores to hydrogen peroxide vapour is needed. It would be

interesting to identifying any protective effects due to soiling when exposed to hydrogen peroxide, as the spores would potentially be protected by dust and organic matter in the environment. This study also demonstrated potential differences in the susceptibility of different strains, which would provide an interesting area for further study. It would also be beneficial to expose other HAI-causing bacteria using constant concentrations of hydrogen peroxide vapour. MRSA and MSSA, which account for 11.3 % of blood stream infections in the UK, would be important microorganisms for further research. It has recently been shown that MRSA may be more resistant to hydrogen peroxide vapour than the widely available *G. stearothermophilus* bioindicators (Pottage *et al.*, 2012; Davies, 2013). Therefore, it is important to investigate the inactivation kinetics of MRSA when exposed to hydrogen peroxide vapour. These results should then be used to identify an integral $\int C_P dt$ to log reduction model as shown for *C. difficile*.

Research is also needed to investigate other disinfection methods that could be used for healthcare decontamination. This is important as current commercially available hydrogen peroxide decontamination systems have very long cycle times and therefore cannot be used regularly; however, the results presented in this thesis have shown that *C. difficile* is highly susceptible to hydrogen peroxide and therefore shorter hydrogen peroxide decontamination cycles maybe possible. A deep clean method that could be completed in a similar time to standard bleach clean (67 min) and required minimal input from healthcare staff would be the ideal solution (Otter *et al.*, 2009). One possible method for this would be the use of plasma, an ionised gas otherwise known as the fourth state of matter (Fridman, 2008). Plasma sterilization is worth considering as it allows high levels of sterilization to be achieved at low temperatures with no residual toxicity. This is as a result of highly reactive-short lived species being generated within plasma; these include electrons, ions, atoms, radicals and UV photons which react with the bacteria thus inactivating them (Yang *et al.*, 2009; von Keudell *et al.*, 2010). To date there are no reports of plasma being used in the healthcare environment; however Tseng *et al.* (2012) recently demonstrated the efficiency of a commercially available plasma jet (Atomflo™ 250) at inactivating *Bacillus* and *Clostridium* spores.

7.2.1 Effect of spore production method and soiling on inactivation of Clostridium difficile by hydrogen peroxide inactivation

Chapter 5 demonstrated a difference in the inactivation of *C. difficile* spores exposed to hydrogen peroxide using two different methods of generating spore laden bioindicators. The main difference in these approaches was the procedure used to produce the spore stocks, more specifically the temperature used for the heat shock. An interesting area for further work would be the effect the temperature used for the heat shock has on the inactivation kinetics. Melly *et al.* (2002) demonstrated that there was a significant effect of the heat shock temperature used to produce spores on the resistance of *B. subtilis* spores to a number of different inactivation methods including liquid hydrogen peroxide. Another difference in the two spore stocks used in this study was the cell debris remaining after purification (shown in Section 3.5). The spore stock containing the most debris proving the most resistant which accords the results presented by Otter *et al.* (2012). This is important as the spores in the environment will not exist in the environment in a purified form and evenly deposited on surfaces and therefore the effects of protection afforded by clumping and organic matter warrant further investigation.

7.2.2 Exposure of different Clostridium difficile strains to decontamination agents

In this work potential differences in the susceptibility of different strains of *C. difficile* were recorded when exposed to hydrogen peroxide vapour. The hospital strains (ribotypes 014 and 027) were identified as the most resistant to inactivation by this method. It would be important to investigate the susceptibility of different clinical strains of *C. difficile* to the decontamination agents used in healthcare facilities, rather than just extrapolating results based on a single strain. More resistant strains can therefore be identified and potentially used as bioindicators to validate disinfection processes e.g. tailored for specific outbreaks.

7.2.2.1 **Further investigation into the regression model for hydrogen peroxide inactivation of *C. difficile***

The regression model developed in this thesis demonstrated that it is possible to estimate the log reduction of *C. difficile* achieved based on the hydrogen peroxide cycle. The model developed does, however, currently overestimate the inactivation achieved at low hydrogen peroxide vapour concentration and underestimated that achieved at high concentrations; therefore further development of the model would be advantageous to identify the range of hydrogen peroxide concentration at which it can be applied.

7.2.3 *Exposure of MRSA to Hydrogen Peroxide Vapour*

As previously discussed MRSA has been found to be far more resistant to hydrogen peroxide vapour inactivation than *C. difficile* spores. Therefore, some preliminary experiments were carried out investigating the inactivation of MRSA exposed to 50 ppm hydrogen peroxide vapour using the same method as with the *C. difficile* spores. Briefly, a fresh solution of 8-$\log_{10}$ MRSA bacteria was produced by Vickesh Patel (PhD student, Loughborough University), from which 2-$\log_{10}$ to 8-$\log_{10}$ dilutions were produced. These solutions were then deposited on the membranes as described in Section 3.5.3. The membranes were exposed to 50 ppm hydrogen peroxide in the exposure chamber for 15 min to 180 min.
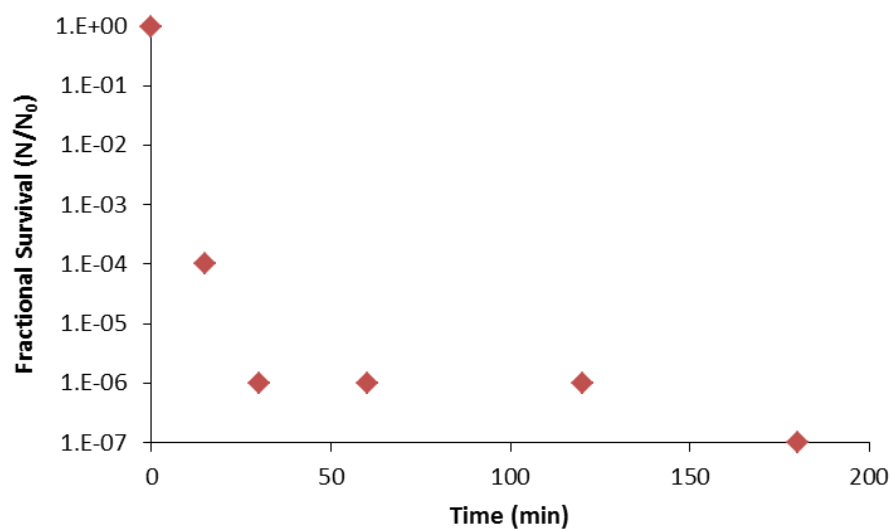
Figure 7.1 MRSA inactivation when exposed to hydrogen peroxide vapour with a concentration of 50 ppm.

Figure 7.1 shows the inactivation of the MRSA when exposed to 50 ppm hydrogen peroxide vapour. A 6-$\log_{10}$ reduction was achieved within the first 30 min, which indicates a higher resistance to hydrogen peroxide vapour than *C. difficile* spores which showed a 7-$\log_{10}$ reduction within 15 min (Section 5.2.2.2) when exposed to the same concentration using the same method. The initial inactivation stage is followed by a period where no further inactivation is achieved between 30 min and 120 min, with a complete 8-$\log_{10}$ reduction not being achieved even after 180 min. This could be attributed to one of two reasons, either the remaining bacteria being protected by the inactivated bacteria or being more resistant to inactivation by the hydrogen peroxide vapour.

Rastogi *et al.* (2009) show inactivation of *B. anthracis* spore bioindicators with a concentration of 8-$\log_{10}$ is much lower than that for 6-$\log_{10}$ or 7-$\log_{10}$ bioindicators. In this study a 5-$\log_{10}$ to 6-$\log_{10}$ reduction is presented for the lower concentration bioindicators compared to a less than 1-$\log_{10}$ reduction for the 8-$\log_{10}$ bioindicators. This shows that if the spore concentration is too high there is a reduction in the inactivation achieved, likely to be caused by spore stacking and therefore increased protection from the disinfectant.

Pottage *et al.* (2010) presented data demonstrating a tailing effect when MS2 bacteriophage was exposed to hydrogen peroxide; suggesting the susceptibility of individual bacteria could vary. In this case the phage which are highly susceptible to the hydrogen peroxide vapour are killed off in an initial phase very quickly; while the bacteria that are left are more resistant and consequently much harder to inactivate.

7.2.4 *Non-thermal Plasma Inactivation of Clostridium difficile*

Although hydrogen peroxide inactivation is becoming widely used within the healthcare environment, it requires significantly longer process times than conventional standard bleach cleaning (Doan *et al.*, 2012). This results in hydrogen peroxide vapour not being used routinely due to a desire to reduce hospital waiting times and the considerable bed pressures faced in the NHS (especially in the winter months). Therefore it is important to investigate other methods that could be used for terminal disinfection. This is important to help

maintain the levels of reductions in HAIs seen over the past few years (Health Protection Agency 2012a).

One possible method for this is the use of non-thermal plasma. Initial experiments were carried out using this method for which the results are shown in Figure 7.2. To expose the *C. difficile* spores to plasma, they were deposited onto membranes using the same method as used for the hydrogen peroxide exposure (Section 3.5.3). The membranes were then placed into a 13 mm Petri dish attached to a specially designed non-thermal plasma generator. The plasma generator was mounted in to the lid of a Petri dish for ease of use, and consisted of a powered plate electrode (~4 cm diameter) attached to a PTFE dielectric barrier (2 mm thick) and a ground electrode consisting of a hexagonal mesh adhered to the opposite side of the PTFE. The device was powered by a high voltage sinusoidal power source with an operating frequency of 20 kHz. The input power was varied between ~0.27 W/cm^2 and 0.77 W/cm^2 to achieve low and high power plasma concentrations respectively. *C. difficile* spores were exposed at two different plasma concentrations (low and high) which were controlled by changing the voltage. The distance from the bioindicators where the plasma was generated was also important, with the low power plasma being exposed at a distance of 5 cm and the high power plasma being exposed at 5 cm and 10 cm distances. The exposure distance was maintained using plastic spacers placed between the bottom of the Petri dish and the plasma generator. Once exposed to the plasma the bioindicators were placed into 2 ml broth (reduced BHI broth containing 0.2 mg catalase) and incubated for 48 h using the growth/no-growth method as used with *C. difficile* exposure to hydrogen peroxide vapour (Section 3.8.4).
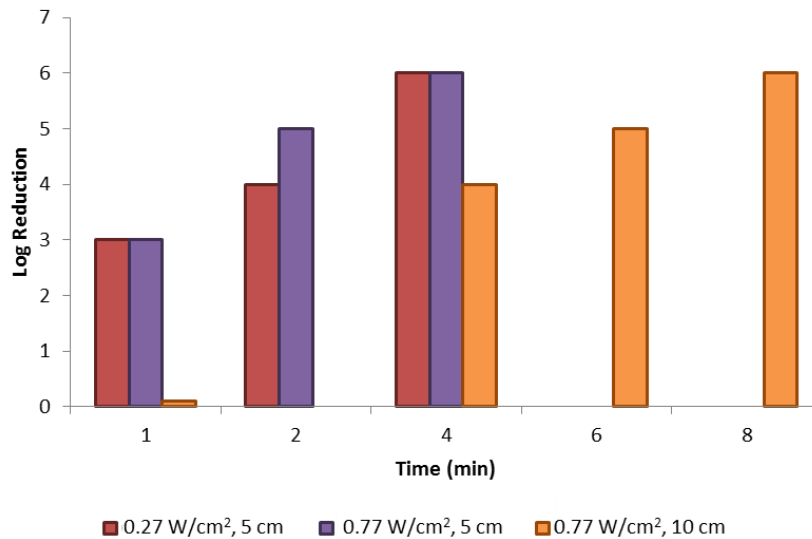
Figure 7.2 *C. difficile* inactivation when exposed to high or low plasmas at low and high distances.

Figure 7.2 shows the inactivation of *C. difficile* spores exposed to low plasma at a distance of 5 cm and high plasma at 5 cm and 10 cm distances for between 1 min and 8 min. It identifies a 6-$\log_{10}$ reduction in the spores exposed at the low distance after 4 min for both the low and high plasmas. This is compared to an 8 min exposure time required to achieve a 6-$\log_{10}$ reduction at the high distance using the high plasma. This initial work has demonstrated that non-thermal plasma could be an effective method for decontamination, with only very short exposure times required to achieve a 6-$\log_{10}$ reduction. Further work would be required to investigate the inactivation kinetics of *C. difficile* exposed to plasma, as well as investigation of possible exposure methods for use in the healthcare environment.

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Appendix 1: Calculation of the *D*-value from the Baranyi Model

Demonstration of the mathematical manipulation of the Baranyi model required to calculate the *D*-value.

$$\frac{N}{N_0} = \exp(-kt)\left(\frac{1+K_C C_P}{1+K_C C_P \exp(-kt)}\right) \tag{A1.1}$$

$\dfrac{N}{N_0} = 0.1$ when $t = D$

$$0.1 = \exp(-kD)\left(\frac{1+K_C C_P}{1+K_C C_P \exp(-kD)}\right) \tag{A1.2}$$

$$\frac{0.1}{\exp(-kD)} = \frac{1+K_C C_P}{1+K_C C_P \exp(-kD)} \tag{A1.3}$$

$$\frac{1+K_C C_P \exp(-kD)}{\exp(-kD)} = 10\left(1+K_C C_P\right) \tag{A1.4}$$

$$\exp(kD) + K_C C_P = 10 + 10 K_C C_P \tag{A1.5}$$

$$\exp(kD) = 10 + 9 K_C C_P \tag{A1.6}$$

$$D = \frac{1}{k}\ln\left[10 + 9 K_C C_P\right] \tag{A1.7}$$

Appendix 2: Hydrogen Peroxide Vapour-Liquid Equilibrium

In order for this method to be successful it is important that the vapour-liquid equilibrium at the different concentrations is known. A relationship for the equilibrium is described in Schumb *et al.* (1955). The first relationship needed is to convert between mole fraction and weight percent as shown in Equation A2.1. This is important as the equations to find the equilibrium are in mole fractions and the experimental work will be carried out in weight/volume percent.

$$W = \frac{34x}{34x + 18(1-x)} \times 100 \tag{A2.1}$$

In order to find the equilibrium concentration the vapour pressures of the different components are required. For an ideal system, as it is a binary mixture this would normally be done using Raoult's law $\left(p_i = x_i p_i^{\circ}\right)$. However, in the case of hydrogen peroxide and water this is not possible as the two components interact strongly with each other via hydrogen bonding (Webb, 2001). It is therefore necessary for Raoult's law to be modified by the addition of activity coefficients (γ_x) to give Equation A2.2 and Equation A2.3.

$$p_W = p_W^{\circ} x_W \gamma_W \tag{A2.2}$$

$$p_H = p_H^{\circ} x_H \gamma_H \tag{A2.3}$$

where p_i is the vapour pressure exerted in the mixture (mmHg), p_i° is the vapour pressure of the pure component (mmHg), x_i is mole fraction (dimensionless) and the subscripts *W* and *H* represent water and hydrogen peroxide respectively.

Equations for the vapour pressure of the pure components and the activity coefficients are required to find the partial pressures. A polynomial equation for the vapour pressure of water is given by Webb (2001) and is shown as Equation A2.4. The other equations are given by Schumb *et al.* (1955) and are shown by Equations A2.5 to Equation A2.7.

$$\log p_W^\circ (T) = G + \frac{A}{T} + B\log T + CT + DT^2 + ET^3 + FT^4 \qquad \text{(A2.4)}$$

where p_W° is the vapour pressure of pure water (mmHg), T is absolute temperature (K), A = -2892.4, B = -2.89, C = -4.94 x 10^{-3}, D = 5.61 x 10^{-6}, E = -4.65 x 10^{-9}, F = 3.79 x 10^{-12} and G = 19.3.

$$\log p_H^\circ (T) = D + \frac{A}{T} + B\log T + CT \qquad \text{(A2.5)}$$

where p_H° is the vapour pressure of hydrogen peroxide (mmHg), A = -4025.3, B = -13.00, C = 4.61 x 10^{-3} and D = 44.58.

$$\gamma_W = \exp\left(\frac{(1-x_W)^2}{RT} \left[B_0 + B_1(1-4x_W) + B_2(1-2x_W)(1-6x_W) \right] \right) \qquad \text{(A2.6)}$$

$$\gamma_H = \exp\left(\frac{x_W^2}{RT} \left[B_0 + B_1(3-4x_W) + B_2(1-2x_W)(5-6x_W) \right] \right) \qquad \text{(A2.7)}$$

where B_0 = -1017 +0.97T, B_1 = 85, B_2 = 13, R is the universal gas constant (1.987 cal/molK) (Hill, 2010) and T is the temperature in K.

Expressions for the total vapour pressure and vapour composition can be found and are shown in Equations A2.8 and Equation A2.9.

$$P = p_W + p_H \qquad \text{(A2.8)}$$

$$y_H = \frac{p_H}{P} \qquad \text{(A2.9)}$$

where P is the total pressure and y_H is the mole fraction of hydrogen peroxide in the vapour.

The results of these calculations are shown in Table A2.1.

Table A2.1. Hydrogen peroxide vapour-liquid equilibrium data calculations from excel

x_H	x_W	weight %	γ_W	γ_H	p_W	p_H	Total vapour Pressure of HP-W solution		Vapour Composition		Vapour concentration	
							Pa	mmHg	y_H	y_W	mol/kg	ppm
0	1	0.00	1.00	0.25	2.31	0.00E+00	2.31	17.36	0	1	0	0
0.01	0.99	1.87	1.00	0.26	2.29	0.000	2.29	17.18	0.000	1.000	0.000	5.36
0.02	0.98	3.71	1.00	0.27	2.26	0.001	2.27	17.01	0.000	1.000	0.000	11.06
0.03	0.97	5.52	1.00	0.28	2.24	0.001	2.24	16.82	0.001	0.999	0.001	17.11
0.04	0.96	7.30	1.00	0.28	2.21	0.002	2.22	16.63	0.001	0.999	0.001	23.51
0.05	0.95	9.04	1.00	0.29	2.19	0.003	2.19	16.44	0.001	0.999	0.001	30.28
0.06	0.94	10.76	0.99	0.30	2.16	0.003	2.16	16.25	0.001	0.999	0.001	37.43
0.07	0.93	12.45	0.99	0.31	2.13	0.004	2.14	16.05	0.002	0.998	0.001	44.96
0.08	0.92	14.11	0.99	0.32	2.11	0.005	2.11	15.84	0.002	0.998	0.002	52.89
0.09	0.91	15.74	0.99	0.33	2.08	0.005	2.08	15.63	0.003	0.997	0.002	61.21
0.1	0.9	17.35	0.98	0.34	2.05	0.006	2.05	15.42	0.003	0.997	0.002	69.94
0.2	0.8	32.08	0.94	0.44	1.74	0.016	1.76	13.18	0.009	0.991	0.005	181.20
0.3	0.7	44.74	0.87	0.55	1.42	0.029	1.44	10.84	0.020	0.980	0.010	338.68
0.4	0.6	55.74	0.79	0.65	1.10	0.047	1.15	8.61	0.041	0.959	0.016	541.77
0.5	0.5	65.38	0.70	0.76	0.81	0.068	0.88	6.62	0.077	0.923	0.023	783.41
0.6	0.4	73.91	0.61	0.85	0.57	0.091	0.66	4.95	0.138	0.862	0.031	1050.82
0.7	0.3	81.51	0.53	0.92	0.37	0.115	0.48	3.62	0.238	0.762	0.039	1327.75
0.8	0.2	88.31	0.45	0.97	0.21	0.138	0.35	2.61	0.396	0.604	0.047	1597.78
0.9	0.1	94.44	0.39	0.99	0.09	0.160	0.25	1.87	0.639	0.361	0.054	1847.54
1	0	100.00	0.34	1.00	0.00	0.179	0.18	1.34	1.000	0.000	0.061	2069.32

Appendix 3: Publications

Published Papers

Malik, D.J., Shaw, C.M., Rielly, C.D. & Shama, G., 2013. The inactivation of *Bacillus subtilis* spores at low concentrations of hydrogen peroxide vapour. *Journal of Food Engineering*, 114(3), pp.391–396.

Submitted Papers

Malik, D.J., Shaw, C.M., Shama, G., Clokie, M.R.J., Reilly, C.D., (Submitted). An in-vitro investigation into the inactivation kinetics of hydrogen peroxide vapour against *Clostridium difficile* endospores. *Applied Microbiology and Biotechnology.*

Malik, D.J., Walsh, J.L., Shaw, C.M., Clokie, M.R.J., Shama, G., (Submitted). Investigation of the inactivation efficacy of non-thermal atmospheric air plasma against *Clostridium difficile* endospores. *Journal of Hospital Infection.*