Cardiff School of Pharmacy and Pharmaceutical Sciences

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Catalytic Production of Hydrogen Peroxide for \textit{in situ} Disinfection in Medical Applications

A thesis submitted to Cardiff University for the degree of

Doctor of Philosophy

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I would also like to wholeheartedly thank my parents, sister, grandparents and family, my girlfriend Justyna and friends for their help and support throughout my PhD project.
Abstract

Contaminated endoscopes and infections associated with them have been repeatedly reported in the literature. There have been numerous outbreaks and pseudo-outbreaks associated with poorly disinfected endoscopes. Rinse water poses important problems in endoscope disinfection such as recontamination of endoscopes and contamination of patient samples. Biofilms are also a substantial problem for disinfection of endoscopes. The aim of this project is to explore an integrated system based on catalytic technology to produce H$_2$O$_2$ to provide sterile rinse water for endoscope reprocessing in automated endoscope reprocessors (AER).

The catalytic technology used in this project was based on a gold and palladium catalyst which was tested in batch and flow reactors. Flow reactor treatment was a thousand times more effective at killing *E. coli* K12 JM109 (4 log$_{10}$ reduction) than 200 ppm of commercial and batch reactor H$_2$O$_2$ in suspension (~1 log$_{10}$ reduction). Moreover, flow reactor treatment with 1 w/w% AuPd/TiO$_2$ catalyst was extremely effective against MS2 bacteriophages (8 log$_{10}$ reduction) while 200 ppm of commercial and batch reactor H$_2$O$_2$ in suspension was ineffective (< 1 log$_{10}$ reduction). Furthermore, 200 ppm of the flow reactor H$_2$O$_2$ prevented formation of *E. coli* K12 JM109 and *B. subtilis* ATCC6633 biofilms.

H$_2$O$_2$ did not play a major role in the microbicidal activity of the catalyst. The proposed mechanism of microbicidal action is that in a H$_2$/air mixture, H• initiates a reaction cascade which turns O$_2$ into OOH• which can either attack the microorganisms on its own or can propagate a radical chain with contribution of the
H₂O₂ synthesised in the reactor which can support the flux of free radicals out of the surface of the catalyst.

Ultimately, the system tested in this project has an innovative mechanism of action and showed a high microbicidal activity. However, further studies on its optimisation are necessary for its incorporation into AER.
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<table>
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<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>E. hirae</td>
<td><em>Enterococcus hirae</em></td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>B. subtilis</td>
<td><em>Bacillus subtilis</em></td>
</tr>
<tr>
<td>S. maltophilia</td>
<td><em>Stenotrophomonas maltophilia</em></td>
</tr>
<tr>
<td>P. putida</td>
<td><em>Pseudomonas putida</em></td>
</tr>
<tr>
<td>A. fumigatus</td>
<td><em>Aspergillus fumigatus</em></td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>G. stearothermophilus</td>
<td><em>Geobacillus stearothermophilus</em></td>
</tr>
<tr>
<td>D. radiodurans</td>
<td><em>Deinococcus radiodurans</em></td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>AER</td>
<td>automated endoscope reprocessor</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>TSA</td>
<td>tryptone soya agar</td>
</tr>
<tr>
<td>TSB</td>
<td>tryptone soya broth</td>
</tr>
</tbody>
</table>
TSC – tryptone sodium chloride

BSA – bovine serum albumin

cfu/mL – colony forming units/mL

pfu/mL – phage forming units/mL

TiO₂ - titanium dioxide

Au – gold

Pd – palladium

NaHCO₃ – sodium bicarbonate

MgCl – magnesium chloride

CaCl₂ – calcium chloride

PdCl₂ - palladium chloride

EPR – Electron Paramagnetic Resonance

DMPO - 5,5-Dimethyl-1-pyrroline N-oxide

BSG - British Society of Gastroenterology

FDA - Food and Drug Administration

SEM – scanning electron microscope

HMDS – hexamethyldisilazane
Consumables and equipment

Commercial H$_2$O$_2$ and sodium bicarbonate was provided by Acros Organics, Geel, Belgium.

Catalase, magnesium chloride, calcium chloride and glutathione was provided by Sigma-Aldrich, St. Louis, Missouri, United States.

Sodium thiosulphate, bovine serum albumin and sodium chloride was provided by Fisher Scientific, Waltham, Massachusetts, United States.

Tryptone and TSB was supplied by Oxoid, Basingstoke, UK.

TSA agar plates were supplied by E&O Laboratories Limited, Bonnybridge, UK.

H$_2$/air gas cylinder was supplied by BOC, Guildford, UK.

HPLC pumps were supplied by Agilent Technologies, Santa Clara, California, United States.

Refrigeration unit and temperature control were supplied by Grant Instruments, Shepreth, UK.

Reactor tubes were supplied by Swagelok, Solon, Ohio, United States.

Gas flow control was supplied by Brooks Instrument, Hatfield, Pennsylvania, United States.

Water bath was supplied by Techne, Stone, UK.

Water deioniser was supplied by Elga, High Wycombe, UK.

Shaking incubator was supplied by IKA, Oxford, UK.
DMPO was supplied by Sigma-Aldrich, St. Louis, Missouri, United States.

Scanning Electron Microscope Hitachi TM3030Plus was supplied by Hitachi, Maidenhead, UK.
Publications and conference presentations

Work presented in this thesis contributed to the manuscript of a journal article submitted to *Nature Catalysis* titled ‘A new catalytic approach to water disinfection using *in situ* generation of reactive oxygen species’, poster and oral presentations at the ASM Microbe 2019 conference in San Francisco, USA, an oral presentation at the 9th International Water and Health Seminar in Cannes, France, a poster presentation at the Science Polish Perspectives 2018 conference in Oxford, England, an oral presentation at the Speaking of Science 2017 conference in Cardiff, Wales and a poster presentation at Cardiff Catalysis Institute conference 2018 in Cardiff, Wales.

DISCLAIMER

At time, some of the results presented were performed by other researchers belonging to the CCI group, as the development of the flow reactor is a larger multidisciplinary project. When this occurred, researchers who have contributed to the results have been acknowledged.
1 Introduction

1.1 Endoscopes and their issues

Flexible endoscopy is a common therapeutic and diagnostic procedure which allows doctors to view and operate on internal organs. There are a variety of endoscopes (Table 1.1) each suited for a different part of a body and they can be introduced through natural openings i.e. mouth, anus or through a surgical cut in a keyhole surgery (Schwab and Singh, 2011).

**Table 1.1** A list of endoscopies and their uses (from Schwab and Singh, 2011)

<table>
<thead>
<tr>
<th>Body system</th>
<th>Name of endoscopy</th>
<th>What is visualized/accessed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gastrointestinal (GI) tract</strong></td>
<td>Oesophagogastrroduodenoscopy (OGD)</td>
<td>Oesophagus, stomach and duodenum</td>
</tr>
<tr>
<td></td>
<td>Enteroscopy</td>
<td>Small intestine</td>
</tr>
<tr>
<td></td>
<td>Colonoscopy</td>
<td>Large intestine (and terminal ileum)</td>
</tr>
<tr>
<td></td>
<td>Sigmoidoscopy</td>
<td>Sigmoid colon, rectum</td>
</tr>
<tr>
<td></td>
<td>Endoscopic UltraSound (EUS)</td>
<td>Upper GI tract and biliary tree</td>
</tr>
<tr>
<td><strong>Biliary tree</strong></td>
<td>Endoscopic retrograde cholangiopancreatography (ERCP)</td>
<td>Pancreas, common bile duct, hepatic ducts and gallbladder</td>
</tr>
<tr>
<td></td>
<td>Choledoscopy</td>
<td>Intraoperative access to bile ducts</td>
</tr>
<tr>
<td></td>
<td>Duodenoscope-assisted cholangiopancreatoscopy</td>
<td></td>
</tr>
<tr>
<td><strong>Respiratory tract</strong></td>
<td>Bronchoscopy/endobronchial ultrasound</td>
<td>Trachea, large and small bronchi</td>
</tr>
<tr>
<td><strong>Ear, nose and throat</strong></td>
<td>Rhinoscopy</td>
<td>Nose</td>
</tr>
<tr>
<td></td>
<td>Laryngoscopy</td>
<td>Throat</td>
</tr>
<tr>
<td><strong>Urological</strong></td>
<td>Cystoscopy</td>
<td>Bladder, urethra</td>
</tr>
<tr>
<td></td>
<td>Uretoscopy</td>
<td>Ureters</td>
</tr>
<tr>
<td><strong>Gynaecological</strong></td>
<td>Hysteroscopy</td>
<td>Uterus</td>
</tr>
<tr>
<td></td>
<td>Falloscopy</td>
<td>Fallopian tubes</td>
</tr>
</tbody>
</table>
They are classified as a critical medical instrument and therefore, they should undergo high-level disinfection after each use. Disinfection of endoscopes is of a high importance as infections are the predominant cause of death amongst hospital patients. Contaminated endoscopes and infections associated with them have been recurrently reported in the literature (Kovaleva et al., 2013). A study conducted in USA in 2013 indicated that around 15% of endoscopes in the US hospitals did not achieve acceptable standard of cleanliness using liquid reprocessing, the most common disinfection method carried out between patient procedures. The worst result for disinfection was found in duodenoscopes with a 30% contamination rate, whereas, the best results were found in colonoscopes with a 3% contamination rate (Noronha and Brozak, 2014). Endoscopes can become substantially contaminated with secretions, blood, and microorganisms (Kovaleva et al., 2013).

Infections associated with endoscopy are separated into two types: exogenous and endogenous. Endogenous infections are the most common after endoscopies. These are infections that are caused by patients’ microbial flora. Most commonly isolated species from infected patients are Klebsiella species, enterococci, Enterobacter species and Escherichia coli (E. coli) (Spach, et al., 1993). Examples of these infections are bacteraemia in biliary obstruction patients that underwent endoscopic retrograde cholangiopancreaticography and pneumonia due to a sedated patient’s aspiration of oral secretions at a flexible bronchoscopy procedure. On the other hand, exogenous microorganisms are transmitted from either contaminated reprocessing equipment or patients via contaminated endoscopes or supplementary equipment. This means that infections associated with exogenous microorganisms can be avoided by rigorous disinfection procedures as opposed to the endogenous infections for which...
disinfection does not have an effect (Srinivasan, 2003). Prevention of endogenous infections can be achieved through antibiotic prophylaxis (Nelson, 2003). The most commonly isolated exogenous microorganisms are *Pseudomonas aeruginosa* (*P. aeruginosa*) and mycobacteria from bronchoscopy and *P. aeruginosa* and *Salmonella* species from gastrointestinal (GI) endoscopy (Nelson and Muscarella, 2006) (Figure 1.1). An important issue with endoscope disinfection is that bacteria can form biofilms on the inner channel surfaces of the endoscopes which can play a key role in an unsuccessful decontamination (Kovaleva, *et al.*, 2009). Endoscopes have a complex design (Figure 1.2 A and B), therefore, they are laborious to clean and disinfect. Furthermore, they can easily be damaged due to their complicated architecture with multitude of internal channels and narrow lumens. What is more, heat and steam sterilisation cannot be applied on most modern flexible endoscopes due to their plastic and rubber components and fragile fibre optics (Kovaleva *et al.*, 2013; Srinivasan, 2003).
Figure 1.1 A diagram showing the nosocomial infections caused by contaminated endoscopes (from Spach et al., 1993).
Figure 1.2 A) A schematic of a complex internal design of an endoscope (from Kovaleva et al., 2013). B) A photograph of an endoscope with a surgical instrument (from Khatait et al., 2013).
1.2 Disinfection of endoscopes

The British Society of Gastroenterology (BSG) in UK, the Food and Drug Administration (FDA) in USA and other organisations in the world have created guidelines for endoscope disinfection (FDA, 2015; BSG, 2014). Figure 1.3 shows a comprehensive diagram illustrating this process. Firstly, a manual cleaning procedure is carried out which consists of brushing the removable parts and the external surface followed by immersion in a detergent and then irrigation of internal channels with a detergent. A leak test and inspection of endoscope and its accessory materials should take place prior to disinfection (Alvarado and Reichelderfer, 2000). It has been recommended to use automated endoscope reprocessors (AERs) to process flexible endoscopes as this reduces the contamination and contact with contaminated instruments and chemicals. Documentation of all the steps involved is also key for the correct flexible endoscope reprocessing (Beilenhoff et al., 2008). Most common disinfectants used are ortho-phthalaldehyde, peracetic acid, electrolysed acidic water and superoxidised water and glutaraldehyde (in USA). Table 1.2 lists advantages and disadvantages of these disinfectants. Glutaraldehyde mode of action is by protein denaturation and alkylation of nucleic acids (Maris, 1995). The disinfecting activity of peracetic acid is attributed to the release of active oxygen. Furthermore, it has been suggested that peracetic acid causes rupture or dislocation of cell walls which disorganises the chemiosmotic function of the lipoprotein cytoplasmic membrane. Furthermore, protein denaturation is also a part of peracetic acid mode of action. Moreover, peracetic acid oxidises intracellular enzymes and works on the bases within the DNA (Kitis, 2004). The mechanism of action of ortho-phthalaldehyde is cross-
linking membrane receptors and impairing cell membrane functions, permeabilising it and letting the biocide enter the cell. Ortho-phthalaldehyde compromises cell growth by interacting with RNA and DNA (Simoes et al., 2007). Superoxidised water acts by damaging RNA, DNA and proteins, destroying the covalent bonds in the protein and nucleic acid chains (Zinkevich et al., 2000). Electrolysed acidic water works by disrupting the bacterial cell wall and its internal ultrastructure (Ding et al., 2016).

These disinfectants have high biocidal activities. Peracetic acid has a high biocidal activity at low concentrations and at low temperatures. Electrolysed acidic water and superoxidised water are not toxic to biological tissues and non-irritant to the respiratory tract, skin and eyes (SGNA Practice Committee 2013-2014, 2015; Kovaleva et al., 2013; Park et al., 2013). Further two critical aspects of disinfecting endoscopes are exposure time of the disinfectant and its concentration. Reprocessing of endoscopes can be unsuccessful if these two aspects are not addressed appropriately (Kovaleva et al., 2013). Drying is also crucial in endoscope reprocessing as it substantially decreases the likelihood of endoscope recontamination with waterborne microorganisms such as Acinetobacter species or Pseudomonas species. Furthermore, the risk of pathogens remaining is also substantially reduced (Alfa and Sitter, 1991).

Another factor of high importance is the endoscope storage. ESGE–ESGENA guideline (Beilenhoff et al., 2008) suggests that endoscopes should be dried in a dust free cabinet while hanging vertically. It has been observed that the contamination rate has dropped to 0% after appropriate storage and that this can be maintained providing the appropriate drying procedure was undertaken (Pineau et al., 2008).
Figure 1.3 A diagram showing the steps involved in decontamination of endoscopes (from Public Health Agency of Canada, 2011).
Table 1.2 List of advantages and disadvantages of the most common high-level disinfectants used for endoscope disinfection based on information (from Beilenhoff et al., 2018; Kovaleva et al., 2013).

<table>
<thead>
<tr>
<th>High-level disinfectant</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Glutaraldehyde          | • High biocidal activity.  
                           • Relatively cheap.  
                           • Does not corrode metals.  
                           • Widely published in the literature. | • Biocide resistance development.  
                           • Causes irritation to: skin, respiratory tract and eyes.  
                           • Development of allergic reactions, acute colitis, contact dermatitis, asthma.  
                           • Fixation and coagulation of proteins.  
                           • Action against mycobacteria is slow. |
| Peracetic acid           | • High and quick biocidal activity using low concentrations.  
                           • Works at low temperatures. | • Can be corrosive.  
                           • Causes irritation to eyes and respiratory tract.  
                           • Not highly effective in removing biofilm and killing bacteria within biofilms. |
| ortho-Phthalaldehyde    | • High biocidal activity. | • Acts slowly against bacterial spores.  
                           • Irritates respiratory tract and eyes.  
                           • Stains instruments, skin and clothing.  
                           • Fixes and coagulates proteins. |
| Electrolyzed acidic water and superoxidized water | • High and quick biocidal activity.  
                           • Relatively cheap.  
                           • No toxicity to biological tissues and no irritation to the respiratory tract, skin and eyes. | • Low efficacy when organic soil is present. |
AERs provide many benefits for the disinfection of endoscopes and one of them is the reduction of the cost of the procedure. It has been observed that due to a much decreased need for manual handling, the maintenance costs for repairing endoscopes decreased by 34%. Furthermore, the initial cost of setting up an AER system and servicing is relatively cheap when the gains in revenue enhancements and cost savings are being considered. Set up and services of AER are being estimated at $27,887 in Russia, $22,750 in India and $60,000 in China (two units). The subtotal revenue enhancements and cost savings compared with using only manual reprocessing are estimated at $72,394 in Russia, $16,620 in India and $119,506 in China (two units). The payback time of the investment has been estimated to be 7 months in Russia and 14 months in China (Funk and Reaven, 2014).

Automated reprocessing of endoscopes is a multistep process and has its disadvantages. Disadvantages associated with commonly used disinfectants include emerging biocide resistance and skin, eye and respiratory tract irritation (Kovaleva et al., 2013). There are many mechanisms of biocide resistance in bacteria including modification or degradation of biocides, changes in the composition of membrane lipids, overexpression of efflux pumps and modifications in the outer membrane proteins (Gnanadhas et al., 2013). Other problems include fixation and coagulation of proteins, staining of the instruments, skin and clothing for ortho-phthalaldehyde (Beilenhoff et al., 2018; Kovaleva et al., 2013). Sterilisation with ethylene oxide is expensive and it would damage the endoscopes (Kovaleva et al., 2013). Furthermore, it has been shown that peracetic acid is not effective in biofilm removal and the biofilms of *Bacillus subtilis* (*B. subtilis*) can protect other species such as *Staphylococcus aureus* (*S. aureus*) from peracetic acids biocidal activity (Bridier et
al., 2012). Tracing and prevention of endoscope contaminations and infections after endoscopies is an appropriate measure to take. However, the results of surveillance cultures take at least 24 to 48 hours, hence, the potentially contaminated endoscope could be used on another patient (Buss et al., 2008; Beilenhoff et al., 2007).

1.3 Rinse water, outbreaks and pseudo-outbreaks

An issue of a paramount importance which this PhD project is trying to address is the contamination of rinse water used during/after disinfection in AERs. There are many sources of rinse water contamination including mains water, AER internal pipework, inadequately maintained filters and intermediate tanks. Water is used throughout the AER process for various steps such as for cleaning the instruments, preparation of detergents and the removal of traces of disinfectant that could be detrimental to the health of staff and patients. The main problems associated with rinse water are that mains and tap water are not sterile, hence, they have a high capacity for repeated contaminations and transmission of infections to patients. Furthermore, diagnostic samples can also be contaminated by rinse water which can cause false positive results which in turn can lead to inappropriate application of treatment to healthy patients. Generally, water supply comes in tanks which is also not ideal because the water has been stagnant and not chlorinated enough which in turn increases the risk of contamination. Using sterile water is also a possibility, however, it is too expensive and does not provide protection against contamination from exogenous sources. Therefore, considering all above problems, the main focus of tackling the issue of rinse water has been concentrated on trying to optimise the mains water for the last rinse of
endoscopes in a post disinfectant stage (Department of Health, 2016; Joint Working Group of HIS and PHLS, 2002). The contact of rinse water with the endoscope follows chemical immersion, therefore, if the rinse water is contaminated, the endoscope will also get contaminated regardless of the effectiveness of the disinfectant used (Muscarella, 2006).

There has been numerous outbreaks and pseudo-outbreaks associated with AER rinse water and it has been specified that in the crucial final rinse water the conditions should be ‘bacteria free’. In reality, this aim in most cases did not come into fruition with more than 60% of samples being of inadequate quality. Furthermore, none of the 20 tested endoscopy units achieved a consistent sterility of water during the study (Willis, 2006). The bacterial species that is involved in most outbreaks or pseudo-outbreaks is *P. aeruginosa* (Weber and Rutala, 2012). A list of pseudo-outbreaks and outbreaks linked to bronchoscopy is shown in Table 1.3. One example of an outbreak of a multi-drug resistant *P. aeruginosa* associated with contaminated AER which was not appropriately maintained took place in 1998 in St. Thomas Hospital in London (Schelenz and French, 2000). An example of a pseudo-infection associated with rinse water contamination in an AER caused by *Mycobacterium chelonei* happened in 1999 in a Royal Hospital for Sick Children in Edinburgh (Gillespie *et al.*, 2000).
Table 1.3 A list of pseudo-outbreaks and outbreaks that were linked to bronchoscopy from 2000 to 2012 (from Webber and Rutala, 2012).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Publication year</th>
<th>Microorganism</th>
<th>Outbreak or pseudo-outbreak?</th>
<th>Isolates</th>
<th>Infections</th>
<th>Deaths</th>
<th>Source of contamination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congrove et al10</td>
<td>2012</td>
<td><em>Pseudomonas</em>spp., <em>Stenotrophomonas</em></td>
<td>Pseudo-outbreak</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>Irregularities in repair by third-party vendor, nonstandard part replacements</td>
</tr>
<tr>
<td>Rosengarten et al11</td>
<td>2010</td>
<td><em>Burkholderia cepacia</em></td>
<td>Pseudo-outbreak</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>Missing antibacterial filter on washer disinfector</td>
</tr>
<tr>
<td>CDC7</td>
<td>2009</td>
<td><em>Legionella pneumophila</em></td>
<td>Pseudo-outbreak</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>Nonsteril ice used to cool saline filler syringes for bronchoalveolar lavage</td>
</tr>
<tr>
<td>Schuetz et al13</td>
<td>2009</td>
<td><em>L. pneumophila</em></td>
<td>Pseudo-outbreak</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>Immersion of uncap saline-filled syringes in contaminated ice</td>
</tr>
<tr>
<td>Chronou et al14</td>
<td>2009</td>
<td><em>Mycobacterium chelonae</em></td>
<td>Pseudo-outbreak</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>Contamination of an AER</td>
</tr>
<tr>
<td>DiazGranados et al11</td>
<td>2009</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Both</td>
<td>12</td>
<td>2</td>
<td>0</td>
<td>Damaged bronchoscope</td>
</tr>
<tr>
<td>Schaffer et al10</td>
<td>2008</td>
<td><em>Pantoea agglomerans</em></td>
<td>Pseudo-outbreak</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>Bronchoscope</td>
</tr>
<tr>
<td>Shimono et al17</td>
<td>2008</td>
<td><em>P. aeruginosa</em></td>
<td>Outbreak</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>Fault in AER, failure to properly clean and disinfect bronchoscopes</td>
</tr>
<tr>
<td>Ahn et al18</td>
<td>2007</td>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>Pseudo-outbreak</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>Failure to properly clean and disinfect bronchoscopes</td>
</tr>
<tr>
<td>Bou et al119</td>
<td>2006</td>
<td><em>P. aeruginosa</em></td>
<td>Outbreak</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>Failure to properly clean and disinfect bronchoscopes</td>
</tr>
<tr>
<td>Corne et al20</td>
<td>2005</td>
<td><em>P. aeruginosa</em></td>
<td>Both</td>
<td>16</td>
<td>4</td>
<td>0</td>
<td>Damaged internal channel caused by defective biopsy forceps</td>
</tr>
<tr>
<td>Cètre et al11</td>
<td>2005</td>
<td>Enteric GNR</td>
<td>Both</td>
<td>117</td>
<td>2</td>
<td>0</td>
<td>Bronchoscope: bone port of the biopsy channel</td>
</tr>
<tr>
<td>Larson et al17</td>
<td>2003</td>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Pseudo-outbreak</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>Failure to properly clean bronchoscopes, use of an AER not approved for the type of bronchoscope</td>
</tr>
<tr>
<td>Singh et al10</td>
<td>2003</td>
<td><em>Trichoporus musciolus</em></td>
<td>Pseudo-outbreak</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>Defective bronchoscopes</td>
</tr>
<tr>
<td>Silva et al11</td>
<td>2003</td>
<td><em>P. aeruginosa, Serratia marcescens</em></td>
<td>Pseudo-outbreak</td>
<td>41</td>
<td>0</td>
<td>0</td>
<td>Failure to properly clean bronchoscopes</td>
</tr>
<tr>
<td>Srinivasan et al20</td>
<td>2003</td>
<td><em>P. aeruginosa</em></td>
<td>Outbreak</td>
<td>97</td>
<td>48</td>
<td>3?</td>
<td>Defective bronchoscopes: loosened biopsy port</td>
</tr>
<tr>
<td>Kirschke et al16</td>
<td>2003</td>
<td><em>P. aeruginosa</em></td>
<td>Both</td>
<td>20</td>
<td>1</td>
<td>0</td>
<td>Defective bronchoscopes: loosened biopsy port</td>
</tr>
<tr>
<td>Ramsay et al17</td>
<td>2002</td>
<td><em>M. tuberculosis</em></td>
<td>Pseudo-outbreak</td>
<td>10</td>
<td>4</td>
<td>0</td>
<td>Damaged bronchoscope; no leak testing; hole in bronchoscope sheath</td>
</tr>
<tr>
<td>Rossetti et al10</td>
<td>2002</td>
<td><em>Mycobacterium gordonae</em></td>
<td>Pseudo-outbreak</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>AER: failure to replace antibacterial filters, maintenance</td>
</tr>
<tr>
<td>Kressel and Kidd20</td>
<td>2001</td>
<td><em>M. chelonae, Methylobacterium mesophilicum</em></td>
<td>Pseudo-outbreak</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>AER contaminated with biofilm resistant to decontamination</td>
</tr>
<tr>
<td>Sorin et al18</td>
<td>2001</td>
<td><em>P. aeruginosa</em></td>
<td>Both</td>
<td>18</td>
<td>3</td>
<td>1</td>
<td>AER: inappropriate channel connectors</td>
</tr>
<tr>
<td>Kramer et al11</td>
<td>2001</td>
<td><em>P. aeruginosa</em></td>
<td>Both</td>
<td>18</td>
<td>3</td>
<td>1</td>
<td>AER: disinfectant (0.04% glutaraldehyde) contaminated because of inadequate concentration (concentration mistakenly set too low)</td>
</tr>
<tr>
<td>Wilson et al16</td>
<td>2009</td>
<td><em>Aureobasidium sp.</em></td>
<td>Pseudo-outbreak</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>Reuse of single-use stopcocks</td>
</tr>
<tr>
<td>Gillespie et al20</td>
<td>2009</td>
<td><em>M. chelonae</em></td>
<td>Pseudo-outbreak</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>Contaminated water in AER</td>
</tr>
<tr>
<td>Schellenz and French21</td>
<td>2009</td>
<td><em>P. aeruginosa</em></td>
<td>Unknown</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>AER</td>
</tr>
</tbody>
</table>

Note: AER, automated endoscope reprocessor; GNR, gram-negative rods.
It is alarming that years after a norm for the sterility of rinse water in AERs had been established, outbreaks still occur. An outbreak that occurred between 2011 and 2012 in Hospital das Clínicas, University of São Paulo, Brazil lead to 5 infections with *Mycobacterium abscessus* subsp *bolletii*. Guimaraes et al., (2016) hypothesise that the main reason for this outbreak was inadequate sterility of rinse water in AERs, however, they do not rule out the possibility of the bacterium developing resistance to 2% glutaraldehyde and inappropriate sterilisation and disinfection practice. Furthermore, there has been a pseudo-outbreak of *Stenotrophomonas maltophilia* (*S. maltophilia*) and *Pseudomonas putida* (*P. putida*) in Complexo Hospitalario Universitario de Vigo, Spain. Samples of these bacteria were isolated from 39 patients and this has been traced back to problems with the water lines and the fact that tap water was used in the endoscope reprocessing could also play a role (Botana-Rial et al., 2016). However, there has been a case described by Khalsa et al. (2014) where microbiological surveillance has shown contamination of the rinse water within the AERs by *Aspergillus fumigatus* (*A. fumigatus*) which led to the closure of endoscopy unit, until the issue was resolved. Microbiological surveillance potentially prevented outbreaks from contaminated endoscopes, especially within the immunocompromised group of patients, and is a good example of the benefits that periodic microbiological tests provide.

Rinse water is an important source of outbreaks and pseudo outbreaks associated with AERs, however, there are a multitude of different reasons. One of the most serious outbreaks occurred due to a defect in a bronchoscope of a biopsy port being loosened in Johns Hopkins Hospital in Baltimore, USA between June 2001 and January 2002. This outbreak was attributed to *P. aeruginosa*. 97 isolates were taken and resulted in
47 infections. It was noted that a contaminated bronchoscope could have played a part in a death of three patients during that outbreak (Srinivasan, et al., 2003). Moreover, an outbreak that resulted in 7 *P. aeruginosa* infections happened due to a failure in appropriate cleaning and disinfection of bronchoscopes in Kyushu University Hospital in Fukuoka, Japan between May and June 2003 (Shimono et al., 2008). Another large outbreak of 10 *P. aeruginosa* infections happened in Hospital de La Ribera in Valencia, Spain in 2003 and it was attributed to inappropriate cleaning and disinfection of bronchoscopes (Bou et al., 2006).

Published data shows that a very common cause of outbreaks and pseudo-outbreaks is an inappropriate cleaning and disinfection procedure of endoscopes and that bronchoscopes are most commonly contaminated. There are multitude steps in cleaning and disinfection procedure of endoscopes and even more ways in which these procedures can fail. Table 1.4 outlines how different steps of cleaning and disinfection can fail. The first step in the process is the manual cleaning which is done to eliminate the biological burden and substances that would hinder the disinfection such as: salt, protein or blood. The most common ways to fail in this step are inappropriate supervision and training, not cleaning the endoscope promptly, missing out brushing of some internal channels or their damage. The second step in the procedure is an appropriate disinfection which is done to inactivate contaminating microorganisms. The most common disinfectants used are glutaraldehyde, ortho-phthalaldehyde, peracetic acid, electrolysed acid and superoxidised water. The reasons for failure in this step are due to an ineffective disinfectant, its concentration, temperature and the contact time. The next step in the process is the contact between the contaminating microorganisms and the disinfectant. In this instance it is usually a defective AER or
its inappropriate set up that is the cause of failure of this step. The penultimate step in the procedure is the rinsing stage which is intended to remove the chemicals. Contaminated water is the most common reason of failure in this step. The final step in the procedure is to prevent recontamination from happening. There are many ways in which this step can fail including not rinsing the endoscope with ethanol after rinsing with water, not air-drying the endoscopes, keeping endoscopes in contaminated environments or an inappropriate drying of the endoscopes (Weber and Rutala, 2012). *P. aeruginosa* while being most common bacterial infection through endoscopes, is also a common cause of nosocomial pneumonia. *P. aeruginosa* along with MRSA are the most common pathogens that have been associated with death due to pneumonia (Rello and Diaz, 2003).
Table 1.4 Outline of disinfection steps and how they can fail (Weber and Rutala, 2012).

<table>
<thead>
<tr>
<th>Disinfection step</th>
<th>Reason for disinfection step</th>
<th>Mechanism for failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaning</td>
<td>Remove bioburden</td>
<td>Inadequate policies;</td>
</tr>
<tr>
<td></td>
<td>Remove substances that might interfere with disinfection: blood, salt, protein</td>
<td>Inadequate training or supervision; failure to clean immediately (ie, allowing body fluids to dry); failure to brush all channels; damaged internal channel(s); poorly mated internal components</td>
</tr>
<tr>
<td>Appropriate disinfectant</td>
<td>Inactivation of contaminating microbes</td>
<td>Ineffective disinfectant (eg, iodides); inadequate concentration; inadequate duration; inadequate temperature</td>
</tr>
<tr>
<td>Contact between disinfectant and contaminating microbes</td>
<td>Requirement for killing</td>
<td>AER: failure to use channel connectors; AER: wrong channel connectors; occluded lumen; torn or damaged lumen</td>
</tr>
<tr>
<td>Rinse</td>
<td>Remove potentially toxic chemicals (eg, glutaraldehyde, hydrogen peroxide)</td>
<td>Mucous membrane damage to subsequent patient (eg, colitis); contaminated rinse water</td>
</tr>
<tr>
<td>Prevention of recontamination</td>
<td>Prevent contamination with environmental microbe</td>
<td>Tap water rinse without subsequent alcohol rinse; failure to air-dry endoscope; contaminated AER; reassembly of valves before storage; placement of endoscope in contaminated container; storage in coiled position (rather than hanging straight)</td>
</tr>
</tbody>
</table>

Note. AER, automatic endoscope reprocessor.

1.4 Biofilms as a considerable problem in endoscope disinfection

Biofilms also pose a substantial problem in endoscopes disinfection. Biofilms are clusters of bacterial or fungal cells in a multi-layer that are fixed within an amorphous extracellular material made of exopolysaccharides that originate from bacteria and this is what binds the bacteria to each other and to a surface (Pajkos et al., 2004). There has been a study carried out in 66 Chinese hospitals that indicated 54.6% biofilm contamination of suction and biopsy channels and 76.9% contamination of air and
water channels. The higher contamination rate in the latter has been attributed to these channels being more difficult to clean manually. What is more, it has been noted that in hospitals with the proportion of manual cleaning as high as 92.3% there was a biofilm formation, on the other hand, in the hospitals with 50% manual cleaning proportion, biofilm formation was not observed. This indicates how important AERs are in disinfection of endoscopes (Ren-Pei et al., 2014). Using AERs substantially decreases the formation of biofilms. However, that does not prevent the biofilm formation during the endoscopic procedure (Kovaleva et al., 2009). AERs contaminated with biofilm have been associated with outbreaks including an outbreak of a multi-drug resistant *P. aeruginosa* (Schelenz and French, 2000). *P. aeruginosa* can form biofilms and therefore, they are difficult to remove from the endoscope internal channels, the plumbing and the AERs. Biofilms are substantially more resistant to disinfectants than planktonic bacteria and their decreased metabolic rate is believed to be the reason for that. Moreover, the presence of biofilm protects microorganisms from chemical inactivation and influences from the environment. The risk of failure of the decontamination process is therefore highly increased. A study conducted in Australia showed all of the endoscopes that were tested had biofilm presence after cleaning which means that the current cleaning procedure of endoscopes does not provide a sufficient protection from subsequent infections associated with endoscopies (Pajkos et al., 2004).
1.5 Aim and objectives of the project

The aim of this project is to explore an integrated system based on catalytic technology to produce hydrogen peroxide (H₂O₂) to provide sterile rinse water for endoscope reprocessing in automated endoscope reprocessors.

The catalytic technology used in this project is based on a gold (Au) and palladium (Pd) bimetallic catalyst on a titanium dioxide (TiO₂) support and it was tested in a flow reactor system.

The hypothesis for this project is that microorganisms get killed by H₂O₂ and free radicals produced using a catalyst when passing through the reactor.

The objectives are:

1. Investigation of the microbicidal efficacy of the H₂O₂ and free radicals produced using a catalyst and its limitations against vegetative bacteria, biofilms and bacteriophages.
2. Understanding the biofilm prevention by H₂O₂ produced using a catalyst.
3. Investigating the catalyst performance in terms of H₂O₂ and radical species production.
4. Understanding the microbicidal mechanisms of action of the H₂O₂ and free radicals produced using a catalyst.
Efficacy of hydrogen peroxide in suspension

2.1 Introduction

2.1.1 Hydrogen peroxide as a disinfectant

The British Society of Gastroenterology (BSG) states that one of the features of an ideal disinfectant is effectiveness against a wide variety of microorganisms such as viruses, bacteria, fungi and spores. Furthermore, a disinfectant should be effective against prions proteins, however, there are no such agents compatible with endoscopes. A disinfectant must also be compatible with AER, endoscopes and its accessories. What is more, disinfectants used should be environmentally friendly in terms of disposal and should not be an irritant for the safety of the users (BSG, 2014).

H$_2$O$_2$ was discovered by Louis Jacques Thenard in 1818 by heating barium (Ba) in oxygen (O$_2$) and then dissolving the solid barium peroxide (BaO$_2$) in hydrochloric acid (HCl) (Koppenol, 2018). Benjamin Ward Richardson was a first person to propose H$_2$O$_2$ as a disinfectant in 1891 (Richardson, 1891). H$_2$O$_2$ is a strong disinfectant, bleaching agent and oxidiser (Jiang et al., 2018; McDonnell, 2014). It has no odour and it is colourless (Juven and Pierson, 1996; Baldry 1983). It is also environmentally friendly as it decomposes to water and oxygen (McDonnell, 2014). Furthermore, H$_2$O$_2$ has got a broad spectrum of activity against bacteria, bacterial spores, viruses and yeasts (McDonnell and Russell, 1999).

The mechanism of action of H$_2$O$_2$ has been extensively studied and it is still difficult to pin point which mechanism is the most important for its biocidal effect (Linley et al., 2012). Fenton’s reaction (Figure 2.1) is considered to be the mechanism of H$_2$O$_2$
cytotoxicity and Rapine with his colleagues were first to provide evidence for this (Rapine et al., 1981). In the Fenton’s reaction, higher oxidation states of iron and hydroxyl radicals are generated and these hydroxyl radicals cause biological damage (Winterbourn, 1995). In H$_2$O$_2$ biocidal mechanism due to the Fenton’s reaction, the free hydroxyl radicals cause oxidation of DNA and its subsequent damage (Rohwer and Azam, 2000). Due to the Fenton’s reaction, oxidation of membrane lipids and protein also occurs (McDonnell, 2007; McDonnell and Russell, 1999). Further studies suggest that it is a ferryl radical intermediates that oxidises DNA. This radical is made from DNA-associated iron (Imlay et al., 1988). Furthermore, it has been shown that the concentration of H$_2$O$_2$ does influence its mechanism of action. Under lower concentrations the DNA damage seems to be the predominant mechanism. However, under high concentrations of H$_2$O$_2$ the number of available DNA-associated irons limits the reaction between DNA and H$_2$O$_2$, therefore, the further increase in concentration would not increase the rate of reaction. This suggests that at higher concentrations it is the oxidation of proteins and membrane lipids that would be the predominant mechanism of action (Imlay and Linn, 1986). Oxidation of proteins by H$_2$O$_2$ is a selective process with certain proteins being more prone to oxidation than others (Abreu et al., 2013; Linley et al., 2012).

Figure 2.1 Fenton reaction.

\[
\begin{align*}
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{OH}^\cdot + \text{OH}^{-} \\
\text{Fe}^{3+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{2+} + \text{OOH}^\cdot + \text{H}^+ \\
2 \text{H}_2\text{O}_2 & \rightarrow \text{OH}^\cdot + \text{OOH}^\cdot + \text{H}_2\text{O}
\end{align*}
\]
It has been shown that the amino acids that are oxidised by H₂O₂ in solution are methionine, histidine, glycine, cysteine and lysine (Finnegan et al., 2010). Some of the changes that oxidation of protein amino acids can introduce include small changes in the side-chains of individual residues or total protein backbone cleavage (Linley et al. 2012). It has been observed that in E. coli K12 ECL1, the proteins that were affected after treatment with H₂O₂ were enolase, EF-G, alcohol dehydrogenase E, outer membrane protein A and dnaK (Tamarit et al., 1998). Oxidative damage caused by H₂O₂ has been shown to cause cell membrane damage which lead to an increase of the membrane permeability which caused loss of intracellular material from bacterial cells (Baatout et al. 2006; Brandi et al., 1991).

The biggest limitation of the studies that provide data on mechanisms of action of H₂O₂, is that these mechanisms are studied in isolation. Even though we know that H₂O₂ oxidises DNA, membrane lipids and proteins in vivo, we cannot tell for certain which mechanism is the predominant one. For this to happen, a study would have to be designed that would enable to observe the action of H₂O₂ on all these components at the same time which could then be correlated with its effect on reduction of bacterial cells (Linley et al., 2012).

H₂O₂ has been studied and used in a variety of formulations. One of such formulations is H₂O₂ with peracetic acid which work synergistically to kill bacterial spores. In this formulation, the H₂O₂ role was to compromise the spore coat which then enabled peracetic acid to penetrate through which increases its sporicidal activity (Leggett et al., 2016). In a study conducted in 2014 by Perumal and colleagues, a variety of different hard surface disinfectants formulations (mouthwash/antiseptics and an endoscope reprocessing formulation [588mM H₂O₂ with <2.5% w/v 2-Furoic acid])
containing H₂O₂, were tested against planktonic bacteria and biofilms. It was observed that the tested formulations were effective against planktonic bacteria with a range of minimum inhibitory concentrations (MIC) from 0.5 mM to 20 mM H₂O₂. Sensitivity of biofilms towards the different formulations containing H₂O₂ was up to 266-fold lower than that with planktonic bacteria. This highlights the issue that biofilms pose on the hospital environment and showed that H₂O₂ based formulations may not be effective against them.

2.1.2 Aim and objectives

The aim of this chapter is to measure the efficacy of commercial stabilised (commercial H₂O₂) and unstabilised synthesised batch reactor H₂O₂ (batch reactor H₂O₂) against bacteria and MS2 bacteriophages.

The objectives are:

1. Carry out suspension tests on bacteria and MS2 bacteriophages using commercial H₂O₂.

2. Carry out suspension tests on bacteria and MS2 bacteriophages using batch reactor H₂O₂.
2.2 Materials and methods

2.2.1 Bacterial strains and growth

Bacterial strains used for the investigations were S. aureus NCTC10788, Enterococcus hirae (E. hirae) NCTC 13359, E. coli NCTC10418, P. aeruginosa NCTC13359, E. coli K12 JM109 and E. coli C3000. Suspension tests were also carried out on MS2 bacteriophage which has been used as a surrogate for the poliovirus (Mikel et al., 2016).

A colony of each bacterial strain that was previously grown on Tryptone Soya Agar (TSA) plates and stored in a fridge at 4°C was transferred from the TSA into 20 mL of sterile Tryptone Soya Broth (TSB) using a sterile plastic loop. These strains were then incubated at 37°C overnight in an aerobic atmosphere.

MS2 bacteriophages were propagated by mixing the bacteriophages with E. coli C3000 in a 1:1 ratio with 5 mL of 65% TSA and 5 mM CaCl₂, transferring it onto TSA agar plates and incubating them at 37°C overnight in an aerobic atmosphere. The next day, the agar overlayer containing MS2 bacteriophages in E. coli C3000 was scraped of the plates and centrifuged at 10,000g for 15 min at 4°C. The supernatant was then filtered using a 0.45 µm filter and then filtered using a 0.2 µm filter. The sample of MS2 bacteriophages was then stored in a -20°C freezer.
2.2.2 Suspension tests

Suspension tests were undertaken based on the BS EN 1276:2009 (BSI, 2010). *S. aureus* NCTC 10788, *E. hirae* NCTC 13383, *E. coli* NCTC 10418, *P. aeruginosa* NCTC 13359 were incubated overnight at 37°C in an aerobic atmosphere in sterile TSB. The overnight cultures were centrifuged at 4194 g for 10 min and then were washed in sterile Tryptone Sodium Chloride (TSC). The concentration of bacteria at which the experiment was undertaken was adjusted to $10^7$ cfu/mL in sterile TSC. Concentrations of commercial H$_2$O$_2$ used were: 100, 200, 1,000 and 10,000 ppm. H$_2$O$_2$ solutions were titrated periodically to check the concentrations of the H$_2$O$_2$ solutions. Commercial H$_2$O$_2$ was supplied by Acros Organics. Such low concentrations of H$_2$O$_2$ were used for the suspension tests because the catalytic reaction in the flow through system (described in Chapter 3) can produce around 200 ppm of H$_2$O$_2$.

Reaction conditions for the hard water with organic load experiments were 0.3 g/100 mL Bovine Serum Albumin (BSA), H$_2$O$_2$ diluted in hard water which contained 11.4 mg/100 mL magnesium chloride (MgCl$_2$), 27.6 mg/100 mL calcium chloride (CaCl$_2$) and 28 mg/100 mL sodium bicarbonate (NaHCO$_3$). Reaction was performed in falcon tubes at room temperature (20°C). One mL of bacteria ($10^8$ cfu/mL) was added to a reaction tube containing 1 mL of BSA and a 2 min incubation in room temperature followed. After that, 8 mL of H$_2$O$_2$ was added to the reaction tube (adjusting the initial concentration of bacteria for the test to $10^7$ cfu/mL). Samples were neutralised at 30 s, 1, 5 and 60 min time points.
For testing of the clean water conditions, H\textsubscript{2}O\textsubscript{2} was diluted in sterile deionised water. The rest of the reaction was carried out the same as in hard water with organic load conditions.

The neutraliser used for the suspension tests with formulated H\textsubscript{2}O\textsubscript{2} in hard water with organic load on \textit{S. aureus} NCTC 10788 and \textit{E. hirae} NCTC 13359 consisted of 30 g/L Tween-80, 3 g/L lecithin, 30 g/L saponin, 5 g/L sodium thiosulphate and 1 g/L L-histidine (Lelieveld \textit{et al.}, 2014). The neutraliser used for subsequent suspension tests consisted of 20 g/L sodium thiosulphate and 500 U/mL catalase (Leggett \textit{et al.}, 2016). This was because the previous neutraliser did not work for the Gram-negative bacteria tested i.e. \textit{E. coli} NCTC 10418 and \textit{P. aeruginosa} NCTC 13359. The toxicity and efficacy of the neutralisers were validated according to the BS EN 1276:2009 (BSI, 2010). After neutralisation, samples were serially diluted in sterile TSC and added onto the TSA plates using a drop count method (3 drops of 10 µL) (Naghili \textit{et al.}, 2013; Herigstad \textit{et al.}, 2001) in duplicates and incubated overnight at 37°C, (n=3).

Validations of the reaction conditions were performed to see whether these conditions have a microbicidal activity on their own. The neutraliser was checked whether it has efficacy against the H\textsubscript{2}O\textsubscript{2} and whether it causes toxicity towards the microorganisms tested.

Validation of reaction conditions for hard water with organic load conditions was performed by adding 1 mL of bacteria (10\textsuperscript{8} cfu/mL) to 1 mL of BSA and 2 min incubation at room temperature followed by addition of 8 mL of hard water (water containing MgCl\textsubscript{2}, CaCl\textsubscript{2}, NaHCO\textsubscript{3}) (adjusting the initial concentration of bacteria for the test to 10\textsuperscript{7} cfu/mL) and incubation for 5 min. Validation of reaction conditions for clean water conditions (sterile deionised water) was performed by adding 1 mL of
bacteria ($10^8$ cfu/mL) to 1 mL of sterile deionised water and 2 min incubation followed by addition of 8 mL of sterile deionised water (adjusting the initial concentration of bacteria for the test to $10^7$ cfu/mL) and incubation for 5 min. Validation of neutraliser toxicity was performed by adding 1 mL of bacteria ($10^8$ cfu/mL) into 8 mL of neutraliser and 1 mL of water (hard with organic load and clean) (adjusting the initial concentration of bacteria for the test to $10^7$ cfu/mL) and incubation for 5 min. Validation of neutraliser efficacy was performed by adding 1 mL of bacteria ($10^8$ cfu/mL) to 8 mL the neutraliser and the 1 mL of the highest concentration of H$_2$O$_2$ used in the experiment (i.e. 100,000 ppm of H$_2$O$_2$ was added to achieve an initial concentration of 10,000 ppm) (adjusting the initial concentration of bacteria for the test to $10^7$ cfu/mL and the H$_2$O$_2$ concentration to 10,000 ppm) followed by a 5 min incubation. The negative control was 8 mL of 10,000 ppm H$_2$O$_2$ with 1 mL of water and 1 mL of bacteria ($10^8$ cfu/mL) (adjusting the initial concentration of bacteria for the test to $10^7$ cfu/mL) incubated for 5 min. All the validations were carried out at room temperature (20°C). Validation experiments samples were serially diluted in sterile TSC and added onto the TSA plates using a drop count method (3 drops of 10 µL) in duplicates and incubated overnight at 37°C, (n=3).

2.2.3 H$_2$O$_2$ synthesis and titration

A stainless steel Parr autoclave batch reactor was used to synthesise H$_2$O$_2$. The reaction conditions were: 8.5 g of water, 10 mg of the 1 w/w% AuPd/TiO$_2$ catalyst, 2.9 MPa of H$_2$/CO$_2$, 1.1 MPa 25% O$_2$/CO$_2$, room temperature, 1200 rpm spinning for
30 minutes. After the reaction, the H$_2$O$_2$ was filtered into a glass vial using a filter paper. After the H$_2$O$_2$ was filtered, it was titrated to determine its concentration.

The H$_2$O$_2$ was titrated using 0.00085M cerium (IV) oxide solution with 2% sulphuric acid and a 0.025M 1,10-phenanthroline iron(II) sulfate aqueous solution. Half a gram of the H$_2$O$_2$ solution was weighted in a vial. 3 drops of 2% sulphuric acid was added to a vial, then a drop of 0.025M 1,10-phenanthroline iron(II) sulfate aqueous solution was added using a glass Pasteur pipette. The solution was titrated with 0.00085M cerium (IV) oxide solution until the colour change and the volume used was recorded.

2.2.4 Statistical analyses

Data from the suspension tests were analysed using repeated measures and One-way ANOVA statistical tests with a Bonferroni post hoc test using GraphPad Prism software.
2.3 Results

2.3.1 Suspension tests against bacteria

2.3.1.1 Suspension tests using commercial H₂O₂

Suspension tests with commercial H₂O₂ were carried out to test its efficacy in terms of \( \log_{10} \) reductions in cfu/mL against *S. aureus* NCTC10788, *E. hirae* NCTC13383, *E. coli* NCTC10418 and *P. aeruginosa* NCTC13359 in hard water with organic load and clean water conditions.

2.3.1.1.1 Commercial H₂O₂ suspension tests in hard water with organic load conditions

H₂O₂ concentrations of 100, 200 and 1,000 ppm had no effect on *S. aureus* and *E. hirae* (Figure 2.2). Furthermore, the contact times of 30 s, 1 min and 5 min did not provide results of statistical significance for these bacteria in aforementioned concentrations. It is only with a 10,000 ppm concentration of commercial H₂O₂ that significant changes (repeated measures ANOVA, \( P < 0.001 \)) can be observed in the Gram-positives with over 4 \( \log_{10} \) reductions at a 60 min time point. In contrast to the observations made with the Gram-positives, *E. coli* and *P. aeruginosa* were more susceptible to H₂O₂ with inactivation starting to be highly significant (repeated measures ANOVA, \( P < 0.001 \)) at concentrations as low as 200 ppm. Furthermore, a > 4 \( \pm 0.05 \) \( \log_{10} \) reduction was observed against *E. coli* at 1,000 ppm after 60 min reaction time and a 3.64 \( \pm 0.68 \) \( \log_{10} \) reduction were observed in those conditions.
against \textit{P. aeruginosa}. These reductions were statistically significant (repeated measures ANOVA, \(P < 0.001\)) compared to the control. What is more, at 10,000 ppm concentration and a 5 min contact time there was \(> 4.00 \pm 0.47 \log_{10} \) reduction in \textit{E. coli} and a \(3.62 \pm 0.69 \log_{10} \) reduction in \textit{P. aeruginosa} which were both significant (repeated measures ANOVA, \(P < 0.001\)). What is more, at 10,000 ppm concentration and a 5 min contact time there was \(> 4.00 \pm 0.47 \log_{10} \) reduction in \textit{E. coli} and a \(3.62 \pm 0.69 \log_{10} \) reduction in \textit{P. aeruginosa} which were both significant (repeated measures ANOVA, \(P < 0.001\)).
Figure 2.2 Log_{10} reduction results from commercial H$_2$O$_2$ in hard water with organic load conditions. Suspension tests were performed with 100, 200, 1,000 and 10,000 ppm commercial H$_2$O$_2$ on A) *S. aureus* NCTC10788, B) *E. hirae* NCTC13383, C) *E. coli* NCTC10418 and D) *P. aeruginosa* NCTC13359 all at room temperature. Data were analysed using a repeated measures ANOVA with a Bonferroni post hoc test using GraphPad Prism software. The significance of results is shown as asterisks above the bars * = P< 0.05, ** = P< 0.01, *** = P< 0.001. 100 ppm H$_2$O$_2$, 200 ppm H$_2$O$_2$, 1,000 ppm H$_2$O$_2$ and 10,000 ppm H$_2$O$_2$. Log$_{10}$ reduction results of the suspension test validations for all bacteria were: Reaction conditions: 0, Neutraliser toxicity: 0, Neutraliser efficacy: 0, Negative control: 4. (n=3)
2.3.1.1.2 Commercial H₂O₂ suspension tests in clean water conditions

Figure 2.3 illustrates results obtained from the clean water suspension tests which show that 100, 200 and 1,000 ppm concentrations had no activity against *E. hirae*. Nevertheless, the 1,000 ppm concentration at a 60 min time point gave a log₁₀ reduction of 1.19 ± 0.96 for *S. aureus*. However, the differences between *E. hirae* and *S. aureus* were within 1 log₁₀ difference in majority of cases. Likewise with hard water with organic load conditions, the 10,000 ppm concentration of commercial H₂O₂ caused a highly significant difference (repeated measures ANOVA, P< 0.001) in log₁₀ reductions (over 4 log₁₀) after 60 min time point for both bacteria (SD = 0.11 for *S. aureus* and SD = 0.12 for *E. hirae*). At 60 min time point and a concentration of 200 ppm, the log₁₀ reduction observed for *E. coli* and *P. aeruginosa* were 1.01 ± 0.40 and 0.97 ± 0.30, respectively. Only the result for *E. coli* was significant (repeated measures ANOVA, P< 0.01) in relation to a 100 ppm concentration considering *E. coli* and *P. aeruginosa*. There were 3.28 ± 0.54 and 2.73 ± 0.48 log₁₀ reductions for *E. coli* and *P. aeruginosa* respectively and both were significant (repeated measures ANOVA, P< 0.001) at a 1,000 ppm concentration and a 60min time point. Moreover, there was > 4 log₁₀ reduction for both Gram negatives at a 60 min time point and at 10,000 ppm concentration of commercial H₂O₂ in clean water (SD = 0.05 for *E. coli* and SD = 0.07 for *P. aeruginosa*).
Figure 2.3 Log\textsubscript{10} reduction results from commercial H\textsubscript{2}O\textsubscript{2} in clean water conditions. Suspension tests were carried out with 100, 200, 1,000 and 10,000 ppm commercial H\textsubscript{2}O\textsubscript{2} on A) *S. aureus* NCTC10788, B) *E. hirae* NCTC13383, C) *E. coli* NCTC10418 and D) *P. aeruginosa* NCTC13359 all at room temperature. Data were analysed using a repeated measures ANOVA with a Bonferroni post hoc test using GraphPad Prism software. The significance of results is shown as asterisks above the bars* = P < 0.05, ** = P < 0.01, *** = P < 0.001.  

100 ppm H\textsubscript{2}O\textsubscript{2}, 200 ppm H\textsubscript{2}O\textsubscript{2}, 1,000 ppm H\textsubscript{2}O\textsubscript{2} and 10,000 ppm H\textsubscript{2}O\textsubscript{2}. Log\textsubscript{10} reduction results of the suspension test validations for all bacteria were: Reaction conditions: 0, Neutraliser toxicity: 0, Neutraliser efficacy: 0, Negative control: 4. (n=3)
2.3.1.2 Suspension tests using batch reactor $\text{H}_2\text{O}_2$

Suspension tests were carried out using 200 ppm of batch reactor $\text{H}_2\text{O}_2$ on the same bacteria and conditions described in Section 2.3.1.1.

2.3.1.2.1 Batch reactor $\text{H}_2\text{O}_2$ suspension tests in hard water with organic load conditions

Figure 2.4 illustrates results obtained from the hard water with organic load suspension tests which show that there were no significant (i.e. $P > 0.05$) $\log_{10}$ reductions of Gram-positive bacteria and the contact times of 30 s, 1 min and 5 min did not provide $\log_{10}$ reductions of significance for Gram-negative bacteria. $\log_{10}$ reductions for $E. coli$ and $P. aeruginosa$ (1.50 ± 0.37 and 0.93 ± 0.06, respectively) were statistically significant (repeated measures ANOVA, $P < 0.001$) only at 60 min time.
**Figure 2.4** Log$_{10}$ reduction results from batch reactor H$_2$O$_2$ in hard water with organic load conditions. Suspension tests were performed with 200 ppm batch reactor H$_2$O$_2$ on *S. aureus* NCTC10788, *E. hirae* NCTC13383, *E. coli* NCTC10418 and *P. aeruginosa* NCTC13359 all at room temperature. Data were analysed using a repeated measures ANOVA with a Bonferroni post hoc test using GraphPad Prism software. The significance of results is shown as asterisks above the bars *** = P< 0.001. *S. aureus* NCTC10788, *E. hirae* NCTC13383, *E. coli* NCTC10418, *P. aeruginosa* NCTC13359. Log$_{10}$ reduction results of the suspension test validations for all bacteria were: Reaction conditions: 0, Neutraliser toxicity: 0, Neutraliser efficacy: 0, Negative control: 0. (n=3)
2.3.1.2.2 Batch reactor H₂O₂ suspension tests in clean water conditions

Figure 2.5 shows the results of the tests with batch reactor H₂O₂ in clean water conditions. The log₁₀ reduction of *P. aeruginosa* at a 60 min time point was higher than that of *E. coli* (1.16 ± 0.14 and 0.89 ± 0.04, respectively) (repeated measures ANOVA, P< 0.001).
Figure 2.5 Log₁₀ reduction results from batch reactor H₂O₂ in clean water conditions. Suspension tests were performed with 200 ppm batch reactor H₂O₂ on *S. aureus* NCTC10788, *E. hirae* NCTC13383, *E. coli* NCTC10418 and *P. aeruginosa* NCTC13359 all at room temperature. Data were analysed using a repeated measures ANOVA with a Bonferroni post hoc test using GraphPad Prism software. The significance of results is shown as asterisks above the bars *** = P< 0.001. *S. aureus* NCTC10788, *E. hirae* NCTC13383, *E. coli* NCTC10418, *P. aeruginosa* NCTC13359. Log₁₀ reduction results of the suspension test validations for all bacteria were: Reaction conditions: 0, Neutraliser toxicity: 0, Neutraliser efficacy: 0, Negative control: 0. (n=3)
2.3.1.3 Comparison of suspension tests using commercial and batch reactor H₂O₂

2.3.1.3.1 Comparison of commercial and batch reactor H₂O₂ in hard water with organic load conditions

Figure 2.6 shows the comparison between the results obtained in hard water with organic load conditions with commercial and batch reactor H₂O₂ at 200 ppm (repeated measures ANOVA, P > 0.05) for both commercial and batch reactor H₂O₂ experiments at every time point. Log₁₀ reductions of Gram-negative bacteria were significantly higher (P< 0.001) when exposed to commercial H₂O₂ than the batch reactor H₂O₂ after 60 min contact time. The reductions for *E. coli* in commercial and batch reactor H₂O₂ were 3.19 ± 0.47 and 1.50 ± 0.37, respectively. The reductions for *P. aeruginosa* in commercial and batch reactor H₂O₂ were 2.46 ± 0.55 and 0.93 ± 0.06, respectively.
Figure 2.6 Log$_{10}$ reduction results from commercial and batch reactor H$_2$O$_2$ in hard water with organic load conditions. Suspension tests were performed with 200 ppm commercial and batch reactor H$_2$O$_2$ on S. aureus NCTC10788, E. hirae NCTC13383, E. coli NCTC10418 and P. aeruginosa NCTC13359 all at room temperature. Data were analysed using a repeated measures ANOVA with a Bonferroni post hoc test using GraphPad Prism software. The significance of results is shown as asterisks above the bars ***) = P < 0.001. S. aureus NCTC10788 commercial H$_2$O$_2$, E. hirae NCTC13383 commercial H$_2$O$_2$, E. coli NCTC10418 commercial H$_2$O$_2$, P. aeruginosa NCTC13359 commercial H$_2$O$_2$, S. aureus NCTC10788 batch reactor H$_2$O$_2$, E. hirae NCTC13383 batch reactor H$_2$O$_2$, E. coli NCTC10418 batch reactor H$_2$O$_2$, P. aeruginosa NCTC13359 batch reactor H$_2$O$_2$. (n = 3)
2.3.1.3.2 Comparison of commercial and batch reactor H$_2$O$_2$ in clean water conditions

Figure 2.7 illustrates the comparison between the results obtained in clean water conditions with commercial and batch reactor H$_2$O$_2$ at 200 ppm concentration. The majority of reductions were less than 1 log$_{10}$ with exceptions of *E. coli* in commercial H$_2$O$_2$ (1.01 ± 0.40 log$_{10}$ reduction) and *P. aeruginosa* in batch reactor H$_2$O$_2$ (1.16 ± 0.14 log$_{10}$ reduction) at 60 min time point. The only difference in log$_{10}$ reductions that was statistically significant (P< 0.05) was between *S. aureus* in commercial and batch reactor H$_2$O$_2$ (0.73 ± 0.59 and 0.04 ± 0.04, respectively).
Figure 2.7 Log$_{10}$ reduction results from commercial and batch reactor H$_2$O$_2$ in clean water conditions. Suspension tests were carried out with 200 ppm commercial and batch reactor H$_2$O$_2$ on S. aureus NCTC10788, E. hirae NCTC13383, E. coli NCTC10418 and P. aeruginosa NCTC13359 all at room temperature. Data were analysed using a repeated measures ANOVA with a Bonferroni post hoc test using GraphPad Prism software. The significance of results is shown as asterisks above the bars* = P< 0.05. S. aureus NCTC10788 commercial H$_2$O$_2$, E. hirae NCTC13383 commercial H$_2$O$_2$, E. coli NCTC10418 commercial H$_2$O$_2$, P. aeruginosa NCTC13359 commercial H$_2$O$_2$, S. aureus NCTC10788 batch reactor H$_2$O$_2$, E. hirae NCTC13383 batch reactor H$_2$O$_2$, E. coli NCTC10418 batch reactor H$_2$O$_2$, P. aeruginosa NCTC13359 batch reactor H$_2$O$_2$. (n = 3)
2.3.1.4 Suspension tests against *E. coli* K12 JM109

Figure 2.8 shows the results of suspension tests carried out on *E. coli* K12 JM109 with 100 ppm and 200 ppm commercial and batch reactor H$_2$O$_2$ after 1 min and 60 min treatments. As can be seen from these results, the 60 min time point results were significantly different when different concentrations of the same H$_2$O$_2$ (i.e. 100 ppm commercial with 200 ppm commercial, 100 ppm batch reactor with 200 ppm batch reactor) were compared (Two-way ANOVA, P< 0.05). However, when comparing same concentrations of different H$_2$O$_2$ (i.e. 100 ppm commercial with 100 ppm batch reactor and 200 ppm commercial with 200 ppm batch reactor) there were no significant differences (Two-way ANOVA, P > 0.05). At 1 min contact time, results were not significantly different (Two-way ANOVA, P > 0.05) from each other when comparing different concentrations of the same H$_2$O$_2$ or same concentrations of different H$_2$O$_2$. All of the results using 100 ppm of both commercial and batch reactor H$_2$O$_2$ gave results below 1 log$_{10}$ reduction with batch reactor H$_2$O$_2$ treatments providing higher log$_{10}$ reductions (0.88 ± 0.08 log$_{10}$ reductions after 1 min and 60 min 0.88 ± 0.28) than the commercial H$_2$O$_2$ treatments (0.42 ± 0.14 and 0.60 ± 0.07 log$_{10}$ reductions after 1 min and 60 min, respectively). When comparing results obtained from 200 ppm treatments, the batch reactor H$_2$O$_2$ continued to cause higher log$_{10}$ reductions than the commercial H$_2$O$_2$. Results obtained with 200 ppm batch reactor H$_2$O$_2$ treatments were 1.16 ± 0.29 and 1.46 ± 0.55 log$_{10}$ reductions after 1 min and 60 min respectively, whereas, results obtained with 200 ppm commercial H$_2$O$_2$ treatments were 0.93 ± 0.26 and 1.18 ± 0.19 log$_{10}$ reductions after 1 min and 60 min, respectively.
**Figure 2.8** Log_{10} reduction results from commercial and batch reactor H_{2}O_{2} in clean water conditions. Suspension tests were carried out with 100 and 200ppm commercial and batch reactor H_{2}O_{2} on *E. coli* K12 JM109 at room temperature. Data were analysed using a Two-way ANOVA with a Bonferroni post hoc test using GraphPad Prism software. The significance of results is shown as asterisks above the bars * = P< 0.05 and it shows the differences between the different concentrations of the same type of H_{2}O_{2} ie. between 100 and 200ppm of commercial H_{2}O_{2} and 100 and 200ppm of batch reactor H_{2}O_{2}.  

100 ppm commercial H_{2}O_{2}, 100 ppm batch reactor H_{2}O_{2}, 200 ppm commercial H_{2}O_{2} and 200 ppm batch reactor H_{2}O_{2}. Log_{10} reduction results of the suspension test validations were: Reaction conditions: 0, Neutraliser toxicity: 0, Neutraliser efficacy: 0, Negative control: 0. (n = 3)
Titrations of the different H$_2$O$_2$ were carried out throughout the suspension test at 10 min intervals. As can be seen in tables 2.1 A) and B), batch reactor H$_2$O$_2$ decomposed faster than the commercial H$_2$O$_2$ which decomposed only by 9 ppm in both 100 and 200 ppm after 60 min. The batch reactor H$_2$O$_2$ dropped from 200 ppm to 135 ppm and from 100 ppm to 74 ppm after 60 min.
Table 2.1 A) and B) Titrations of commercial and batch reactor H₂O₂ at 10 min intervals for 1 hour. H₂O₂ were titrated using cerium (IV) oxide.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Commercial H₂O₂ (ppm)</th>
<th>Batch reactor H₂O₂ (ppm)</th>
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<tbody>
<tr>
<td>A) 100 ppm initial concentration</td>
<td></td>
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<tr>
<td>0</td>
<td>100</td>
<td>100</td>
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<tr>
<td>10</td>
<td>100</td>
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<tr>
<td>20</td>
<td>96</td>
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<td>B) 200 ppm initial concentration</td>
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<td>0</td>
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<tr>
<td>60</td>
<td>191</td>
<td>135</td>
</tr>
</tbody>
</table>
2.3.2 Suspension tests against MS2 bacteriophages

Suspension tests were carried out against MS2 bacteriophages in order to see the efficacy of commercial and batch reactor H₂O₂ against viruses. In this set of experiments, MS2 bacteriophages were used as a poliovirus surrogate (Mikel et al., 2016). As can be seen from Figure 2.9, 100 and 200 ppm commercial and 100 and 200 ppm batch reactor H₂O₂ treatments did not produce any significant activity (P value = 0.60). Moreover, none of them produced a reduction above 1 log₁₀ pfu/mL. A 0.49 log₁₀ reduction caused by 200 ppm of batch reactor H₂O₂ after 60 min was the highest recorded activity.
Figure 2.9 $\log_{10}$ reduction results from commercial and batch reactor $H_2O_2$ in clean water conditions. Suspension tests were carried out with 100 and 200 ppm commercial and batch reactor $H_2O_2$ on MS2 bacteriophages at room temperature. Data were analysed using a One-way ANOVA with a Bonferroni post hoc test using GraphPad Prism software. $\log_{10}$ reduction results of the suspension test validations were: Reaction conditions: 0, Neutraliser toxicity: 0, Neutraliser efficacy: 0, Negative control: 0. (n=3).

2.4 Discussion

The first experiments were carried out in hard water conditions and with an addition of organic load in the form of BSA. According to the United States Geological Survey and Water Quality Association, water is classified as hard at 121–180 ppm $Mg^{2+}$ and $Ca^{2+}$ concentrations (Ahn et al., 2018). In these experiments, the organic load (BSA) may have a non-specific reaction with $H_2O_2$ which would cause its biocidal potency
to decrease (Holah, 1995). What is more, BSA inhibits H₂O₂ in a dose dependent manner (Kouoh et al., 1999). Furthermore, the presence of divalent cations in hard water can cause the formation of insoluble precipitates which can lower disinfectants’ efficacy (West et al. 2018). This knowledge would indicate that H₂O₂ should be more effective in clean water conditions rather than in hard water with organic load.

As can be observed from results of both hard water with organic load and clean water conditions suspension tests, the Gram-negative bacteria were more susceptible to commercial H₂O₂ than the Gram-positive bacteria. A study conducted by (Martin and Maris, 2012) also indicated that Gram-negative bacteria were more susceptible to H₂O₂ than Gram-positive bacteria at different water hardness and organic load conditions. The highest concentration of H₂O₂ used in my study and the longest contact time was necessary to cause an over 4 log₁₀ reduction in majority of tested bacteria (1,000 ppm at 60 min and 10,000 ppm at 5 min was enough to achieve a 4 log₁₀ reduction for E. coli) in hard water with organic load conditions. To note, the usual concentration of H₂O₂ used for high level disinfection is 75,000 ppm (Martin et al., 2008) which is 7.5 times higher than the concentration used in this study. Therefore, it is not surprising that concentrations between 100 and 1,000 ppm exhibit low efficacies in suspension tests carried out and 10,000 ppm shows high efficacies after an hour of exposure. Furthermore, it has been shown previously that the longer the contact time, the more effective the H₂O₂ is (Tote et al., 2009) which is also observed in this study.

The next set of suspension tests was carried out in clean water conditions to see the difference in effectiveness of commercial H₂O₂ without the organic load and salts in the test suspension. Considering the 200 ppm concentration at a 60 min time point, the
log₁₀ reduction observed for *E. coli* and *P. aeruginosa* were 1.01 and 0.97 respectively. The log₁₀ reductions at 200 ppm H₂O₂ at 60 min time point in hard water with organic load conditions for *E. coli* and *P. aeruginosa* were 3.19 and 2.46 log₁₀ reductions respectively. In the study carried out by (Martin and Maris, 2012), water hardness had no interfering effect on the efficacy of hydrogen peroxide, however, no information about the enhancing activity of hard water on the efficacy of H₂O₂ can be found in the literature.

Suspension tests were also carried out using 200 ppm of batch reactor H₂O₂ in hard water with organic load and clean water conditions. 200 ppm concentration was chosen as this is the concentration of H₂O₂ that can be produced by the flow reactor (Chapter 3), furthermore, it was not possible to synthesise concentrations as high as 1,000 and 10,000 ppm with the batch reactor to compare with the commercial H₂O₂ experiments. Gram-negative bacteria were more susceptible than the Gram-positive ones as in the (Martin and Maris, 2012) study. Results of hard water with organic load and clean water experiments were similar when using batch reactor H₂O₂.

H₂O₂ at 200 ppm concentration was not highly effective at clean water conditions. However, for both *E. coli* and *P. aeruginosa* the differences in log₁₀ reductions were more than twice as high when commercial H₂O₂ was used compared to the batch reactor H₂O₂ after 60 min time point in hard water with organic load conditions. This would indicate that commercial H₂O₂ is more effective than batch reactor H₂O₂ in hard water with organic load conditions, although it is about the same in clean water conditions. This could be explained by the batch reactor H₂O₂ decomposing to its free radicals more readily (30% of batch reactor H₂O₂ decomposed after 40 minutes) and BSA mopping them up, therefore, making the batch reactor H₂O₂ less effective than
the commercial H₂O₂ when subjected to organic load. This is supported by literature as BSA is a strong reactive oxygen species scavenger (Droge, 2002).

Further suspension tests were carried out on E. coli K12 JM109 using both commercial and batch reactor H₂O₂ to compare efficacies of them both against bacteria in clean water conditions. As could be observed from these data, the batch reactor H₂O₂ caused higher log₁₀ reductions than the commercial H₂O₂, albeit, not statistically significant. What could also be observed was that 30% of batch reactor H₂O₂ was lost after 40 min of reaction. The batch reactor H₂O₂ could decompose to its free radicals more readily than the commercial H₂O₂ which did not decompose at all which corresponded to a higher activity. This finding is novel as studies comparing efficacies of commercial and unstabilised H₂O₂ against bacteria could not be found in the literature.

Suspension test results were unexpected since organic load introduced into the hard water experiment in the form of BSA has a scavenging effect on free radicals which batch reactor H₂O₂ readily makes. Furthermore, the fact that there was more than just bacteria in the suspension would indicate that some of the H₂O₂ would also react with the organic load (BSA) present in the suspension instead of bacteria. Divalent cations found in the hard water have also been found to lower efficacy of a disinfectant. In the case of clean water conditions there were only bacteria and sterile deionised water present so all of the H₂O₂ would react with bacteria, therefore, these results are surprising.

Suspension tests were carried out on MS2 bacteriophages in order to see the efficacy of commercial and batch reactor H₂O₂ against viruses. The time points selected for these suspension tests were 1 min and 60 min. This was because 1 min is the contact time of the bacterial suspension with the catalyst in a flow reactor and 60 min was the
final time point of the previous suspension tests. The results clearly show that 100 and 200 ppm of H$_2$O$_2$ in suspension do not have a high microbicidal effect on MS2 bacteriophages with only 0.49 log$_{10}$ reduction being the highest. This chapter serves as a basis for comparison for flow reactor experiments which produces H$_2$O$_2$ in flow through catalysis (Chapter 4: Microbicidal efficacy of the catalyst).
3 Catalytic production of $\text{H}_2\text{O}_2$ and free radicals

3.1 Introduction

A catalyst is a substance which increases reaction rate and does not alter the overall standard Gibbs energy change during the reaction (Szoke et al., 2003; McNaught and Wilkinson, 1997). Catalysis is the name of the process of increasing the efficiency of the reaction by lowering the activation energy (McNaught and Wilkinson, 1997). The term catalysis was first coined by Jons Jakob Berzelius in 1835, although the first known catalytic reaction was performed by Valerius Cordus in 1552 involving sulphuric acid in order to catalyse the conversion of alcohol to ether (Wisniak, 2010). The first catalytic production of $\text{H}_2\text{O}_2$ industrially was recorded in 1818 and it involved a reaction of $\text{BaO}_2$ with dilute HCl to form $\text{H}_2\text{O}_2$ and barium chloride. This method was discovered by L.J. Thenard (Ranganathan and Sieber, 2018).

There have been numerous advancements in industrial catalytic processes of manufacture of $\text{H}_2\text{O}_2$ since then with the anthraquinone process currently being the most popular (Ranganathan and Sieber, 2018) (explained in more detail in Section 3.1.1 Production of $\text{H}_2\text{O}_2$). However, considering the large scale resource conservation, environmental and economic factors, direct synthesis of $\text{H}_2\text{O}_2$ is the most environmentally friendly alternative that is being researched in the field of catalysis (Samanta, 2008). The first process of catalytic direct synthesis of $\text{H}_2\text{O}_2$ was reported by Henkel and Weber in 1914 and it involved a reaction of gaseous oxygen and hydrogen with water in a pressurised vessel. The catalysts used in this process were noble metals including: Pd, nickel (Ni) and platinum (Pt) (Henkel and Weber, 1914). As with any approach, there are advantages and disadvantages to direct synthesis of
H₂O₂ (Ranganathan and Sieber, 2018). These are summarised in Table 3.1. The steps of the direct synthesis reaction are explained in Section 3.1.1 Production of H₂O₂.

**Table 3.1** Advantages and disadvantages of direct synthesis of H₂O₂ (adapted from Ranganathan and Sieber, 2018).

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lack of organic substrates including organic solvents or anthraquinones.</td>
<td>Simultaneous side products generation other than H₂O₂, such as water due to unselective reactions.</td>
</tr>
<tr>
<td>Green solvents are used such as ethanol, water or methanol.</td>
<td>The process is complex and has mass transfer limitations which involves three phases: liquid (reaction medium), gas (H₂/O₂) and solid (catalyst).</td>
</tr>
<tr>
<td>Fewer downstream operations are needed to produce H₂O₂ making the process more economical.</td>
<td>Hydrogen has a very broad flammability range of 4-94% H₂ in O₂.</td>
</tr>
<tr>
<td>A single reactor system can be used to carry out this process.</td>
<td></td>
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</table>

In recent years Au, Pd and AuPd catalysts were broadly tested for the direct synthesis of H₂O₂. Although there were more studies conducted on Pd catalysts, AuPd catalysts have shown to be more effective at producing H₂O₂ in both experimental and
computational studies (Freakley et al., 2013; Li et al., 2011; Edwards et al., 2005). Another advantage of using AuPd catalysts over monometallic Pd catalysts is that it overcomes the need of halide and acid additives that would need to be used to enhance the H$_2$O$_2$ yields and selectivities of Pd catalysts (Ntainjua et al., 2009). These halide and acid additives cause corrosion of the reaction vessels and increase the cost of the whole process and therefore are undesirable (Freakley et al., 2013).

Edwards et al. (2005) from Cardiff Catalysis Institute compared combinations of TiO$_2$ supported AuPd catalyst with TiO$_2$ supported Pd or Au catalysts for effectiveness of H$_2$O$_2$ production. It has been shown that AuPd catalyst has been significantly more effective at producing H$_2$O$_2$ than the monometallic Au and Pd TiO$_2$ supported catalysts. In this study it was also shown that the AuPd catalyst has a core-shell structure with Pd clustered on the surface. The Au core and Pd shell structure is obtained when the catalyst is calcined at 400°C as heat treatment strongly influences the chemical composition of the metal particles. Calcination process increases metal support interaction therefore the catalyst is more stable than the uncalcined homogenous alloy structure (Edwards and Hutchings, 2008). The increase in activity of the catalysts with an Au core and Pd shell structure is due to Pd atoms withdrawing atoms from the Au core which then shifts the d-band centre of Pd which promotes the O$_2$ adsorption and breaking of the O-O bond (Staykov et al., 2016).
3.1.1 Production of H$_2$O$_2$

There are numerous ways to produce H$_2$O$_2$ and one of them is through water electrolysis. The procedure involves the use of electrodes, carbon felt cathode and a Ruthenium(IV) oxide coated titanium anode through which the current flows. This process is a combination of electrolysis and oxidation from H$_2$O$_2$ allowing for production of H$_2$O$_2$ at high rates. Furthermore, electroperoxidation effectively dissolved organic carbon and removed turbidity from the water (Drogui et al., 2001).

Another way of producing H$_2$O$_2$ is through an ion exchange membrane cell using porous cathodes. This method can be used for in situ production of H$_2$O$_2$ (Asokan and Subramanian, 2009). Moreover, there is also a photocatalytic approach to producing H$_2$O$_2$ where water and oxygen are used along with a heterogeneous catalyst of TiO$_2$, Au and silicon (Si) and the source of energy being UV light only. Photocatalytic activity depends on the interface design and the architecture of the catalyst (Kaynan et al., 2014).

The most common industrial process to produce H$_2$O$_2$ is called an anthraquinone process (Figure 3.1). Alkyl-anthraquinones and Pd catalyst are used in this process with 2-ethylanthraquinone being the most popular choice. In the first step of the reaction, the 2-alkylanthraquinone is hydrogenated to 2-alkylanthrahydroquinone with the help of a Pd catalyst. Then, 2-alkylanthrahydroquinone undergoes oxidation with O$_2$ to form 2-alkylanthraquinone again and produce H$_2$O$_2$. Demineralised water is used to separate H$_2$O$_2$ from the organic working solution, H$_2$O$_2$ is then distilled to purify it and the alkylanthraquinone is recycled (Campos-Martin et al., 2006).
The direct synthesis of H$_2$O$_2$ is a small scale on-site alternative to the large scale industrial anthraquinone process. Direct synthesis of H$_2$O$_2$ is a method which nullifies the need of transportation of highly concentrated solutions of H$_2$O$_2$ and their subsequent dilution. Moreover, it is an atom efficient method of producing H$_2$O$_2$ at
the site of use (Freakley et al., 2013). Atom efficiency takes into account the molecular weights of the desired products and molecular weights of all of the reactants in the reaction (Sheldon, 2000). Figure 3.2 shows the scheme of the direct synthesis reaction which includes the subsequent degradation and hydrogenation of H₂O₂ (Freakley et al., 2013). In the direct synthesis method, the H₂O₂ is formed by a two-step hydrogenation of O₂ that was adsorbed to the surface of the catalyst. Furthermore, all of the reactions taking place in the direct synthesis method of H₂O₂ production have the same intermediate reaction species (Ntainjua et al., 2008). Figure 3.3 shows the steps involved in the direct synthesis of H₂O₂ where the * designates a vacant site of the catalyst surface (Freakley et al., 2013). However, in the direct synthesis of H₂O₂, there are also undesirable hydrogenation reactions of the dissociated surface O₂ species which causes formation of water (Freakley et al., 2013). Figure 3.4 shows the steps involved in water formation during direct synthesis of H₂O₂ where the * designates a vacant site of the catalyst surface (Freakley et al., 2013). Many studies on direct synthesis of H₂O₂ involved an addition of halide and acid additives in order to decrease the reaction rates of the formation of water. This was achieved by halides’ selective poisoning of the sites of the catalyst that were involved in hydrogenation and decomposition pathways involved in the degradation of H₂O₂ (Pashkova et al., 2010; Choudhary and Jana, 2008; Choudhary et al., 2007; Choudhary et al., 2007; Inoue et al., 2007).
Figure 3.2 Direct Synthesis of H₂O₂ diagram (from Freakley et al. 2013).

Figure 3.3 Steps involved in the direct synthesis of H₂O₂ (* designates a vacant site of the catalyst surface) (from Freakley et al., 2013).

\[
\begin{align*}
H_2 + 2* & \rightarrow 2H* \\
O_2 + * & \rightarrow O_2* \\
H* + O_2* & \rightarrow HO_2* + *
\end{align*}
\]

\[HO_2* + H* \rightarrow H_2O_2* + *\]

\[H_2O_2* \rightarrow H_2O_2 + *\]
**Figure 3.4** Steps involved in water formation during direct synthesis of $\text{H}_2\text{O}_2$ (* designates a vacant site of the catalyst surface) (from Freakley et al., 2013).

$$
\text{O}_2 + 2* \rightarrow 2\text{O}^* \text{ or } \text{H}_2\text{O}_2^* + \* \rightarrow \text{H}_2\text{O} + \text{O}^* \\
\text{H}^* + \text{O}^* \rightarrow \text{HO}^* + \* \\
\text{HO}^* + \text{H}^* \rightarrow \text{H}_2\text{O}^* + \* \\
\text{H}_2\text{O}^* \rightarrow \text{H}_2\text{O} + \* 
$$

### 3.1.2 Aim and objectives

The aim of this Chapter is to test efficacies of different catalysts in terms of the $\text{H}_2\text{O}_2$ production and to establish the free radical species produced by those catalysts and their significance.

The objectives are:

1. Produce $0.5 \text{ w/w.}\% \text{ Au-0.5 w/w.}\% \text{ Pd/TiO}_2$ (called $1 \text{ w/w.}\% \text{ AuPd/TiO}_2$ throughout, this Au to Pd w/w% ratio was used throughout the project unless otherwise stated), $1 \text{ w/w.}\% \text{ Au/TiO}_2$ and $1 \text{ w/w.}\% \text{ Pd/TiO}_2$ catalysts using a modified impregnation method.

2. Test the $\text{H}_2\text{O}_2$ production efficacies of these catalysts in a batch reactor.
3. Test the H$_2$O$_2$ production efficacies of these catalysts in a flow reactor.

4. Establish the free radical species produced using these catalysts.

3.2 Materials and methods

3.2.1 Catalyst preparation

HAuCl$_4$·3H$_2$O (Sigma Aldrich) was used as an Au precursor and dissolved in deionised water to a solution with an 8.9 mg/mL concentration of Au. The PdCl$_2$ (Sigma Aldrich) was dissolved in a 0.58 M aqueous HCl solution (concentrated HCl, was diluted with deionized water) with gentle warming and fast stirring forming a solution with a 6 mg/mL concentration of Pd. Once cooled, this solution was the precursor of Pd. The requisite amount of Pd solution and Au solution were added into a 50 mL round-bottom flask with a magnetic stirrer bar. The solution volume was adjusted with deionized water to a 16 mL. Then, the flask was immersed into an oil bath on a magnetic stirrer hot plate (IKA). The solution was stirred at 1,000 rpm and the oil bath temperature was increased from 27 to 60°C in 10 min time. At 60°C, 1.98 g of TiO$_2$ (Degussa Evonik P25) was slowly added in 8–10 min time while constantly stirring forming a slurry. After adding TiO$_2$, the slurry was stirred at 60°C for another 15 min. After that, the oil bath temperature was increased to 95°C, and the slurry was stirred at that temperature for 16 h evaporating the water and leaving a dry solid. Afterwards, the solid powder was ground thoroughly using agate mortar and pestle forming a uniform powder. Then, 400 mg of the catalyst powder was spread over a glass calcination boat (30 cm in length) and was put inside a tube furnace fitted with an inlet and outlet valve (Carbolite Gero). The temperature inside the furnace was
increased from 30 to 400°C at a rate of 10°C/min under a steady flow of 5% H₂ in Ar. The catalyst was reduced at 400°C for 4 h under a steady flow of 5% H₂ in Ar. Then the catalyst was pelletised after reducing the catalyst powder in the furnace. Figure 3.5 shows a diagrammatical representation of this procedure (Sankar et al., 2012). The catalyst was pressed at 10 tons pressure using a hand press. Then, a 425 µm sieve (20 cm diameter, Endecotts LTD) was put on top of a 250 µm sieve (8 cm diameter, Gilson Company) and the catalyst pellet was grinded on top of the first sieve, then, the catalyst powder was collected from the second sieve. The catalyst was packed into the catalyst bed by putting the glass wool into one side of the bed and then inserting 120 mg of the catalyst into the other side.
Figure 3.5 Diagram of the catalyst preparation process and a High-Angle Annular Dark-Field Scanning Transmission Electron Microscopy (HAADF-STEM) image of a catalyst particle (from Sankar et al., 2012). The steps in the catalyst preparation are: mixing of the Au and Pd precursors at 60°C, addition of the TiO₂ support, drying overnight at 90°C and gas reduction at 400°C for 4 hours at a rate of 10°C/min.

3.2.2 Batch reactor tests

Catalysts used in the batch reactor tests were: 1 w/w% AuPd/TiO₂, 1 w/w% Au/TiO₂, 0.75 w/w% Au-0.25 w/w% Pd/TiO₂, 0.25 w/w% Au-0.75 w/w% Pd/TiO₂, 1 w/w% Pd/TiO₂. A stainless steel Parr autoclave batch reactor was used to carry out the tests (Figure 3.6 shows a photograph of the reactor (courtesy of J. Harrhy). The reaction
conditions were: 2.9 g of water, 5.6 g of methanol, 10 mg of the catalyst 2.9 MPa of H$_2$/CO$_2$, 1.1 MPa 25% O$_2$/CO$_2$, 2°C temperature, 1200 rpm spinning for 30 minutes. After the reaction, the H$_2$O$_2$ was filtered into a glass vial using a filter paper. After the H$_2$O$_2$ was filtered, it was titrated to determine its concentration. The H$_2$O$_2$ was titrated using 0.00085 M Cerium(IV) oxide solution with 2% sulphuric acid and a 0.025M 1,10-Phenanthroline iron(II) sulfate aqueous solution. Half a gram of the H$_2$O$_2$ solution was weighted in a vial. 3 drops of 2% sulphuric acid was added to a vial, then a drop of 0.025M 1,10-phenanthroline iron(II) sulfate aqueous solution was added using a glass Pasteur pipette. The solution was titrated with 0.00085M cerium (IV) oxide solution until the colour change and the volume used was recorded, (n=3). Figure 3.7 shows the reaction equations for this titration.
**Figure 3.6** A photograph of the batch reactor used for H$_2$O$_2$ testing (from J. Harrhy).

![Batch Reactor](image)

**Figure 3.7** Titration reaction equations (from J. Harrhy).

$$\text{H}_2\text{O}_2 + 2 \text{Ce(SO}_4)_2 \rightarrow \text{Ce}_2(\text{SO}_4)_3 + \text{H}_2\text{SO}_4 + \text{O}_2$$

wt. % $\text{H}_2\text{O}_2 = \frac{\text{moles of } \text{H}_2\text{O}_2 \times M_r \text{H}_2\text{O}_2}{8.5}$
3.2.3 Initial flow reactor design

The flow reactor was used in this project due to its numerous advantages for the process of direct synthesis of \( \text{H}_2\text{O}_2 \). These advantages include continuous work flow, the properties of the flow are controlled, the surface area to volume ratio is high, flow reactions process safety, improved mass and heat transfer and the throughput can be increased easily (Wegner et al., 2011; Frost and Mutton, 2010; Valera et al., 2010; Webb and Jamison, 2010; Hartman and Jensen, 2009; Wiles and Watts, 2008; Kiwi-Minsker and Renken, 2005; Jahnisch et al., 2004; Fletcher et al., 2002; Jensen, 2001 and DeWitt, 1999).

The flow reactor (Figure 3.8) uses Au-Pd bimetallic nanoparticles catalyst that produce \( \text{H}_2\text{O}_2 \) continuously at 100-200 ppm in water under 10 bar of 2% hydrogen/air (these conditions were optimised by S. Freakley to produce the highest concentration of \( \text{H}_2\text{O}_2 \)). The components of the reactor consist of:

- Gas cylinder consisting of 2% hydrogen/air (BOC, Guildford, UK).
- Gas flow control unit (Brooks Instrument, Hatfield, Pennsylvania, United States) which allows the flow rate between 0 and 50 mL/min. Under the standard reaction conditions, the gas flow rate is 42 mL/min.
- A pressure gauge (Swagelok, Solon, Ohio, United States) which shows the pressure in the system. The pressure under the standard reaction conditions is 10 bar.
- Filters (Swagelok, Solon, Ohio, United States) which let the gas to be pumped in one direction only throughout the reactor (after the gas control units and
before the sample release), there is also a filter which prevents the microorganisms going out of the sample release.

- HPLC pump (Agilent Technologies, Santa Clara, California, United States) which pumps the bacterial suspension into the reactor. The HPLC pump allows the liquid to be pumped at rates between 0.1 mL/min and 5 mL/min. The flow of the bacterial suspension in the reactor under standard conditions is 0.2 mL/min.

- Reactor tubes (Swagelok, Solon, Ohio, United States) and a catalyst bed. The diameter of the catalyst bed is 0.125 inch, it consists of 120 mg of 1 w/w% AuPd/TiO2 catalyst. The 2% hydrogen/air and the bacterial suspension meet in the catalyst bed where the H2O2 and free radicals form and the bacteria get killed.

- Water bath (Techne, Stone, UK) of a 5 L volume and a refrigeration unit (Grant Instruments, Shepreth, UK) are used to cool down the reactor to 2°C which is the optimal temperature for the H2O2 production.
**Figure 3.8** A) Diagram of the initial flow reactor used in this project and B) Annotated photograph of the initial flow reactor used in this project.
3.2.4 Current flow reactor design

Figure 3.9 shows the changes in the flow reactor design. An improvement was an addition of a second HPLC pump and reactor line to be able to carry out two tests simultaneously. This improved both, efficiency and flexibility as twice as much data were able to be generated and two different tests could be carried out at the same time.
Figure 3.9 A) Illustration of the current flow reactor design and B) Annotated photograph of the current flow reactor design.
3.2.5 Bacterial strains and growth

Bacterial strain used in the experiments was *E. coli* K12 JM109. Its growth was considered in detail in Chapter 2 Section 2.2.1.

3.2.6 Flow reactor tests

Catalysts used in the flow reactor tests were: 1 w/w% AuPd/TiO$_2$, 1 w/w% Au/TiO$_2$ and 1 w/w% Pd/TiO$_2$. The gas mixtures used in the flow reactor tests were: 2% H$_2$/air, 5% H$_2$/N$_2$ and air. Control experiments carried out were: passing bacterial suspension through the reactor without catalyst in all of the aforementioned gas mixtures and showed no bacterial kill. There was no neutralisation step after the reaction.

The bacteria were incubated overnight at 37°C in an aerobic atmosphere in sterile TSB. The overnight cultures were centrifuged at 4194 g for 10 min and then were washed in sterile TSC. The concentration of bacteria at which the experiment was undertaken was adjusted to $10^7$ cfu/mL in sterile distilled water and 20 mL of suspension in a falcon tube was used.

The standard reaction conditions for the flow reactor reaction were: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H$_2$/air, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst. Once all of the reaction conditions were set up and achieved, the HPLC capillary tube was inserted into the falcon tube, the top of the tube was sealed with parafilm and the HPLC pump was turned on for the experiment to start. The sample was taken after 30 min of reaction from the sample release at the end of the reactor.
slowly due to a pressure in the reactor. The sample was serially diluted in sterile distilled water and added onto the TSA plates using a drop count method (3 drops of 10 µL) in duplicates and incubated overnight at 37°C, (n=3).

Same protocol and reaction conditions were used for testing the H₂O₂ production, however, sterile distilled water was used instead of bacterial suspension. The sample was taken after 30 min of reaction and titrated in the same way as in 3.2.2. Batch reactor tests, (n=3).

3.2.7 Electron paramagnetic resonance (EPR) experiments

The deionised water for the EPR experiments was mixed with 1 mg/ml of 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) and passed through the flow reactor under standard reaction conditions (3.2.5. Flow reactor tests) using different gas mixtures (2% H₂/ air, 5% H₂/N₂ and air) and different catalysts (1 w/w% AuPd/TiO₂, 1 w/w% Pd/TiO₂, and 1 w/w% Au/TiO₂) and for the duration of 10 minutes. For the tests using the 5% H₂/N₂, the deionised water was also degassed overnight using N₂ before the test was carried out. Glutathione of 5 mM concentration was also used in some of the samples. After the reaction, the sample was frozen in liquid nitrogen. The X-band CW-EPR spectra were recorded on a Bruker EMX Micro spectrometer equipped with a Bruker ER4123-D dielectric resonator, operating at room temperature. Before each measurement, samples coming from the flow reactor were thawed and deoxygenated for 20 min under N₂ flow. Spectra were recorded at 298 K using the following instrumental conditions: 5.02 × 10⁴ receiver gain; 100 kHz modulation frequency; 1.5
Gauss modulation amplitude and 6.48 mW microwave power. Experimental spectra were simulated using the EasySpin package (Stoll and Schweiger, 2006) operating within the Mathworks Matlab environment. The EPR experiments on the samples from the flow reactor were carried out by Dr Andrea Folli from the Cardiff Catalysis Institute.

3.2.8 Statistical analyses

Data from the flow reactor tests were analysed using a One-way ANOVA statistical tests with a Bonferroni post hoc test using GraphPad Prism software.

3.3 Results

3.3.1 Efficacy of different catalysts

3.3.1.1 Batch reactor tests

Batch reactor tests were carried out on catalysts with different ratios of Au and Pd in order to see their effectiveness in H₂O₂ production and to compare it to the effectiveness of other catalysts in terms of moles of H₂O₂ per kilogram of catalyst per hour (Productivity/mol H₂O₂ kg⁻¹cat h⁻¹) which is the standard unit of the measure of catalyst productivity (Hammond, 2017).

The productivity of the 1 w/w% AuPd/TiO₂ catalyst used throughout the project was 106 mol H₂O₂ kg⁻¹cat h⁻¹. Results of the productivities of the catalysts with different Au to Pd w/w% ratios were carried out by Alba Santos (Cardiff Catalysis Institute). The
productivity of 1 w/w% Au/TiO$_2$ was 4 mol$_{H_2O_2}$ kg$^{-1}$cat h$^{-1}$ whereas the productivity of 1 w/w% Pd/TiO$_2$ was 80 mol$_{H_2O_2}$ kg$^{-1}$cat h$^{-1}$. The 0.75 w/w% Au-0.25 w/w% Pd/TiO$_2$ catalyst produced 89 mol$_{H_2O_2}$ kg$^{-1}$cat h$^{-1}$. The 0.25 w/w% Au-0.75 w/w% Pd/TiO$_2$ catalyst produced 84 mol$_{H_2O_2}$ kg$^{-1}$cat h$^{-1}$ (Santos et al., 2019) (Table 3.2).

**Table 3.2** H$_2$O$_2$ productivities of 1 w/w% AuPd/TiO$_2$ with different w/w% ratios of Au to Pd in a batch reactor. Standard reaction conditions: 2.9 g of water, 5.6 g of methanol, 10 mg of the catalyst 2.9 MPa of H$_2$/CO$_2$, 1.1 MPa 25% O$_2$/CO$_2$, 2°C temperature, 1200 rpm spinning for 30 minutes. (n=3).

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Mean productivity/mol$_{H_2O_2}$ kg$^{-1}$cat h$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 w/w% Au/TiO$_2$</td>
<td>4</td>
</tr>
<tr>
<td>0.75 w/w% Au-0.25 w/w% Pd/TiO$_2$</td>
<td>89</td>
</tr>
<tr>
<td>1 w/w% AuPd/TiO$_2$</td>
<td>106</td>
</tr>
<tr>
<td>0.25 w/w% Au-0.75 w/w% Pd/TiO$_2$</td>
<td>84</td>
</tr>
<tr>
<td>1 w/w% Pd/TiO$_2$</td>
<td>80</td>
</tr>
</tbody>
</table>

**3.3.1.2 Flow reactor tests**

Flow reactor tests were carried out on the 1 w/w% AuPd/TiO$_2$ catalyst in order to check the effectiveness of the catalyst in production of H$_2$O$_2$ and its antimicrobial efficacy in the flow reactor system used throughout this project. Furthermore, H$_2$O$_2$ productivity and antimicrobial efficacies were also measured for 1 w/w% Au/TiO$_2$ and 1 w/w% Pd/TiO$_2$ catalysts.
Bimetallic 1 w/w% AuPd/TiO$_2$ produced 259 ppm of H$_2$O$_2$ in the flow reactor. The concentrations of H$_2$O$_2$ produced by the monometallic catalysts were 69 and 220 ppm for 1 w/w% Au/TiO$_2$ and 1 w/w% Pd/TiO$_2$ respectively (Table 3.3).

**Table 3.3** H$_2$O$_2$ productivities of 1 w/w% AuPd/TiO$_2$, 1 w/w% Au/TiO$_2$ and 1 w/w% Pd/TiO$_2$ in a flow reactor. Standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H$_2$/air, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst. (n=3).

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>H$_2$O$_2$ productivity (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 w/w% AuPd/TiO$_2$</td>
<td>259</td>
</tr>
<tr>
<td>1 w/w% Au/TiO$_2$</td>
<td>69</td>
</tr>
<tr>
<td>1 w/w% Pd/TiO$_2$</td>
<td>220</td>
</tr>
</tbody>
</table>

Figure 3.10 shows the antimicrobial efficacy of the catalysts used against *E. coli* K12 JM109. 1 w/w% AuPd/TiO$_2$ caused a $4.08 \pm 0.22$ log$_{10}$ reduction in a flow reactor test. Flow reactor experiments using 1 w/w% Au/TiO$_2$ and 1 w/w% Pd/TiO$_2$ caused a $0.75 \pm 0.37$ and $0.54 \pm 0.52$ log$_{10}$ reductions. These results were statistically significant (P>0.0001).
Figure 3.10 Efficacy of flow reactor against *E. coli* K12 JM109 using 1 w/w% AuPd/TiO₂, 1 w/w% Au/TiO₂, 1 w/w% Pd/TiO₂. Standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H₂/air, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst. Data were analysed using a One-way ANOVA with a Bonferroni post hoc test using GraphPad Prism software. The significance of results is shown as asterisks above *** = P< 0.0001. (n=3).
Figure 3.11 shows the efficacy of 1 w/w% AuPd/TiO$_2$ against *E. coli* K12 JM109 with different gas mixtures flowing through the reactor. Experiments with no catalyst in the catalyst bed did not show any log$_{10}$ reduction against *E. coli* K12 JM109 in every gas mixture. The most effective gas mixture was 2% H$_2$/air at which there was a 4.08 ± 0.22 log$_{10}$ reduction. A 1.19 ± 0.42 log$_{10}$ reduction was achieved at 5% H$_2$/N$_2$ gas mixture. A flow reaction in air caused a 0.24 ± 0.12 log$_{10}$ reduction. There were 1.19 ± 0.55 and 0.98 ± 0.11 log$_{10}$ reductions when 100 and 200 ppm of commercial H$_2$O$_2$ was passed through the catalyst with air. One hundred and 200 ppm of batch reactor H$_2$O$_2$ caused 1.13 ± 0.14 and 1.57 ± 0.36 log$_{10}$ reductions when passed through the catalyst with air.
Figure 3.11 Efficacy of flow reactor against *E. coli* K12 JM109 using 1 w/w% AuPd/TiO$_2$ with 2% H$_2$/air, 5% H$_2$/N$_2$ and air gas mixtures. All other reaction conditions were standard: 10 bar pressure, 2°C, 42 mL/min gas flow, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst. Data were analysed using a One-way ANOVA with a Bonferroni post hoc test using GraphPad Prism software. The significance of results is shown as asterisks above *** = P< 0.0001 in comparison to the blank tests without the catalyst in the catalyst bed and *** = P< 0.0001 in comparison to the 2% H$_2$/air gas mixture treatment (the most effective treatment). (n=3).
3.3.2 EPR experiments

The EPR experiments were performed in order to see which free radicals were produced during the flow reactor reactions. Several different reactions were performed under different conditions. Different gas mixtures were used which were: 2% H\textsubscript{2}/air (gas mixture used for standard testing), 5% H\textsubscript{2}/N\textsubscript{2} and compressed air. The water used for the experiments with 5% H\textsubscript{2}/N\textsubscript{2} was degassed overnight in order to see the result of the test without oxygen in the system. Experiments with air were performed with batch reactor and commercial H\textsubscript{2}O\textsubscript{2} to see the difference between the radicals synthesised on the catalyst surface and the radicals from the breakdown of H\textsubscript{2}O\textsubscript{2}. Further EPR experiments were carried out using glutathione, a quencher of oxygen-centred free radicals (Pizzorno, 2014). Experiments with glutathione, were performed using the 2% H\textsubscript{2}/air and 5% H\textsubscript{2}/N\textsubscript{2} gas mixtures. EPR measurements were carried out on the samples from the flow reactor reactions by Dr Andrea Folli (Cardiff University, School of Chemistry).

Figure 3.12 shows the comparison between different spectra obtained from the EPR experiments. As can be observed from the EPR spectra obtained, there was a strong H radical signal when the degassed water with DMPO went through the flow reactor with 5% H\textsubscript{2}/N\textsubscript{2} which shows that the catalyst is able to produce H free radicals in the absence of O\textsubscript{2}. A much less pronounced signal could be observed when batch reactor H\textsubscript{2}O\textsubscript{2} with DMPO went through the flow reactor with the use of the same gas mixture. This indicates that the catalyst synthesises a much higher amount of H free radicals than there are obtained from the breakdown on H\textsubscript{2}O\textsubscript{2}. Experiments carried out with the 2% H\textsubscript{2}/air mixture showed that there were OH free radicals produced and the
signals were much smaller in intensity when the batch reactor and commercial H₂O₂ were passed through the flow reactor in compressed air. This also indicates that the 1 w/w% AuPd/TiO₂ catalyst produces more OH free radicals than there are obtained from the breakdown of H₂O₂.

**Figure 3.12** Comparison of EPR spectra obtained from different flow reactor reactions. Reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of either 2% H₂/air or 5% H₂/N₂ or compressed air, solvent flow rate = 0.2 mL/min, 120 mg of 1 w/w% AuPd/TiO₂ catalyst. DMPO was used as a spin trap in these experiments (Dr A. Folli; Cardiff University, School of Chemistry).
What could be observed from the results shown in Figure 3.13 was that there was no signal when glutathione was passed through the reactor in the 2% H₂/air and that there was a strong H free radical signal when glutathione passed through the flow reactor in the 5% H₂/N₂ gas mixture.

**Figure 3.13** Comparison of EPR spectra obtained from different test reactor reactions with the addition of glutathione. Reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of either 2% H₂/air or 5% H₂/N₂, solvent flow rate = 0.2 mL/min, 120 mg of 1 w/w% AuPd/TiO₂ catalyst. DMPO was used as a spin trap in these experiments (Dr A. Folli; Cardiff University, School of Chemistry).

Figure 3.14 shows EPR spectra obtained from experiments carried out on 1 w/w% Au/TiO₂ and 1 w/w% Pd/TiO₂. As can be observed from the spectra, there were flat lines for experiments carried out on 1 w/w% Pd/TiO₂ with both 5% H₂/N₂ and 2% H₂/air.
H₂/air gas mixtures. There was also a flat line on a spectrum obtained from the experiment on 1 w/w% Au/TiO₂ with a 5% H₂/N₂ mixture, however, there was a signal indicating OH free radicals when 2% H₂/air gas mixture was passed through the catalyst.

**Figure 3.14** Comparison of EPR spectra obtained from experiments on 1 w/w% Au/TiO₂ and 1 w/w% Pd/TiO₂. Reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of either 2% H₂/air or 5% H₂/N₂, solvent flow rate = 0.2 mL/min, 120 mg of 1 w/w% AuPd/TiO₂ catalyst. DMPO was used as a spin trap in these experiments (Dr A. Folli; Cardiff University, School of Chemistry).
3.4 Discussion

The 1 w/w% AuPd/TiO$_2$ catalyst used in this project was made using a modified impregnation method. This method uses an excess of Cl$^-$ which improves the dispersion of Au particles on the support. The reduction treatment at 400°C in a 5% H$_2$/Ar removes the excess Cl$^-$ from the catalyst and improves the Pd incorporation into the 2-5 nm AuPd alloy nanoparticles. The 1 w/w% AuPd/TiO$_2$ catalyst produced using a modified impregnation method has been found to be four times more effective at producing H$_2$O$_2$ from O$_2$ and H$_2$ (106 mol$_{H2O2}$ kg$^{-1}$cat$^{-1}$ h$^{-1}$) than such catalysts made using a conventional impregnation (23 mol$_{H2O2}$ kg$^{-1}$cat$^{-1}$ h$^{-1}$) or sol immobilisation (32 mol$_{H2O2}$ kg$^{-1}$cat$^{-1}$ h$^{-1}$) methods. The modified impregnation method produced catalysts with nanoparticles that have a tight size distribution ranging from 2 to 6 nm which are smaller and better controlled than when using a conventional impregnation method. Furthermore, the composition of the alloys does not differ much between the particles whereas variabilities in size and composition between particles have been reported in conventional impregnation and sol immobilisation methods (Sankar et al., 2012).

Flow reactor standard reaction conditions were optimised by (Freakley et al., 2013). Just like in the batch reactor, the 1 w/w% AuPd/TiO$_2$ catalyst produced more H$_2$O$_2$ than the monometallic 1 w/w% Au/TiO$_2$ and 1 w/w% Pd/TiO$_2$. This also correlated with the higher efficacy of 1 w/w% AuPd/TiO$_2$ in killing bacteria, albeit, the disparity in log$_{10}$ reductions of *E. coli* K12 JM109 between 1 w/w% AuPd/TiO$_2$ and 1 w/w% Pd/TiO$_2$ was much higher (4.08 ± 0.22 and 0.54 ± 0.52 respectively) than disparity in H$_2$O$_2$ productivity (259 and 220 ppm respectively). This provides an indication that
H$_2$O$_2$ productivity is most likely not the only component of the mechanism of microbicidal action.

EPR Spectroscopy is a highly sensitive method used to study materials which contain unpaired electrons. EPR has got numerous applications with the most common one being for identification of free radicals in the system. When a magnetic field is applied, unpaired electron spins can move from one quantum state to another. These transitions happen when the microwave photons energy is matching the splitting between the electron spin states (Sahu, et al., 2013).

Glutathione is a tripeptide consisting of glycine, cysteine and glutamic acid. Figure 3.15 shows the structure of glutathione. The role of glutathione is a direct neutralisation of reactive oxygen species such as hydroxyl and superoxide radicals or singlet oxygen (Pizzorno, 2014). The EPR experiment carried out with glutathione in suspension indicated the importance of free radicals generation in a catalytic reaction for the mechanism of microbicidal action.

**Figure 3.15** Structure of glutathione.
The flat line on the spectrum when glutathione was passed through the reactor with 2% H₂/air reinforces that glutathione quenches the OH free radicals from the system. This result coupled with the fact that when glutathione was added to the bacterial suspension the activity of the flow reactor treatment was lost (Chapter 5 Section 5.3.1), further indicates the importance of the OH free radicals in the mechanism of action of the catalyst. What could also be observed from these data is that, most likely, H free radicals do not play a major role in the mechanism of microbicidal action of the catalyst as glutathione did not mop them up. Moreover, the highest log₁₀ reductions were observed from the flow reactions with 2% H₂/air which corroborates with the highest production of OH free radicals. The flow reaction with 5% H₂/N₂ which correlates with H free radicals production caused a much lower log₁₀ reduction of *E. coli* K12 JM109. This further solidifies that OH• is the main killing factor. What is more, the flow reaction with 2% H₂/air which provided a much stronger signal for OH• than the flow reactions with both commercial and batch reactor H₂O₂ in air, caused a much higher log₁₀ reduction of *E. coli* K12 JM109. This shows that the free radicals are more important than H₂O₂ for the mechanism of microbicidal action of the catalyst. Cho *et al.* (2005) showed that OH• is a free radical involved in the inactivation of MS2 bacteriophages which, combined with the observations made from the flow reactor (Chapter 4 Section 4.3.2) and the EPR experiments, corroborates the importance of free radicals in the mechanism of microbicidal action of the catalyst.

Results of the EPR experiments with 1 w/w% Au/TiO₂ and 1 w/w% Pd/TiO₂ showed a small signal for OH• production by 1 w/w% Au/TiO₂ and flat line spectra for 1 w/w% Pd/TiO₂. It has been shown that Pd is able to directly catalyse production of H₂O₂, however, the free radicals that are produced in the process remain on the surface of
the catalyst (Finkelstein et al., 1982). Au does not have as high productivity towards \( \text{H}_2\text{O}_2 \) formation as Pd, therefore, it produces a low amount of free radicals. However, the fact that \( \text{OH}^- \) was observed in solution indicates that it causes the diffusion of the free radicals from the surface of the catalyst into the solution. Hence, Pd produces a large amount of free radicals and Au is needed in order to release those free radicals into the solution which can then kill the bacteria.
4 Microbicidal efficacy of the catalyst

4.1 Introduction

4.1.1 Different methods of water disinfection

Disinfection is the process by which pathogens, apart from the endospores, are eliminated or destroyed (Yoo, 2018). There are a multitude of ways to disinfect water, the most common being chlorination. Chlorination is readily available, easily applied and it is suitable for use as a primary (Benson et al., 2017) and secondary disinfectant (Tsitsifli and Kanakoudis, 2018). Primary disinfection inactivates or kills pathogenic microorganisms while secondary disinfection provides long-lasting microbicidal treatment of water downstream by having a small residual level of a disinfectant (Environmental Protection Agency, 2011). Another method of water disinfection is ozonation. Ozone is an oxidising agent that effectively destroys viruses, bacteria and cyst-forming protozoan parasites, it does so at a very short contact time (Lazarova et al., 2013). Ultraviolet (UV) light which inactivates viruses, bacteria and protozoa safely and reliably is another alternative in water disinfection (Johnson et al., 2010).

These methods of water disinfection have their disadvantages. The main issue of using chlorine for water disinfection is that many of its disinfection by-products such as haloacetonitriles, trihalomethanes and chlorophenols are dangerous for human health as they are suspected to be carcinogens (Al-Abri et al., 2019). Disadvantages of disinfecting water through ozonation are low solubility and instability in water, and ozone is hazardous if inhaled (Bidhendi et al., 2006). Disadvantage of UV light for water disinfection is that it is not as effective against viruses as chlorine and that it
does not add the residual disinfection effect provided by chemical disinfectants (Zyara et al., 2016).

H$_2$O$_2$ as a disinfectant has been considered in detail in Chapter 2. In this chapter, it will be considered further as part of the microbicidal efficacy of the catalyst used in this project.

4.1.2 Aims and objectives

The aim of this chapter is to elucidate the microbicidal efficacy of the catalyst used in this project.

The objectives are:

1. Test the flow reactor efficacy against bacteria and MS2 bacteriophages.
2. Test the flow reactor efficacy in different reaction conditions.
3. Test the flow reactor efficacy at biofilm prevention.

4.2 Materials and methods

4.2.1 Bacterial strains and growth

Bacterial strains used were *E. coli* NCTC10418, *E. coli* K12 JM109, *E. coli* C3000, *S. aureus* NCTC10788, *B. subtilis* ATCC6633, *B. subtilis* AEWD isolate (Martin et al.,
2008) (vegetative bacteria) and MS2 bacteriophages. The growth of bacterial strains and bacteriophages was considered in detail in Chapter 2.

4.2.2 Suspension tests

Suspension tests based on the BS EN 1276:2009 (BSI, 2010) were described in Chapter 2 Section 2.2.2. These suspension tests were carried out on *E. coli* NCTC10418, *E. coli* K12 JM109 and *S. aureus* NCTC10788 in order to compare the efficacies of H$_2$O$_2$ in suspension and flow reactor. (n=3).

4.2.3 H$_2$O$_2$ synthesis

Batch reactor H$_2$O$_2$ synthesis was described in Chapter 2. Section 2.2.3. H$_2$O$_2$ was synthesised in a batch reactor in order to compare its activity with commercial H$_2$O$_2$.

4.2.4 Flow reactor tests

The bacteria were incubated overnight at 37°C in an aerobic atmosphere in sterile TSB. The overnight cultures were centrifuged at 4194 g for 10 min and then were washed in sterile TSC. The concentration of bacteria at which the experiment was undertaken was adjusted to 10$^7$ cfu/mL in sterile distilled water and 20 mL of suspension in a falcon tube was used.
The standard reaction conditions for the flow reactor reaction were: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H₂/air, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst. Once all of the reaction conditions were set up and achieved, the HPLC capillary tube was inserted into the falcon tube, the top of the tube was sealed with parafilm and the HPLC pump was turned on for the experiment to start. The sample was taken after 30 min of reaction from the sample release at the end of the reactor slowly due to a pressure in the reactor. The sample was serially diluted in sterile distilled water and added onto the TSA plates using a drop count method (3 drops of 10 µL) in duplicates and incubated overnight at 37°C. Spread plate method with 1 mL inoculum was used to enumerate bacteria in the low initial inoculum tests in order to have no limit of detection. Control experiments carried out were: passing bacterial suspension through the reactor without catalyst in 2% H₂/air and showed no bacterial kill. There was no neutralisation step after the reaction. (n=3).

4.2.5 Biofilm prevention tests

Two hundred, 100 and 50 ppm of commercial, batch reactor and flow reactor H₂O₂ was added to the wells of a 96 well plates. *E. coli* K12 JM109, *B. subtilis* ATCC6633 and *B. subtilis* AEWD isolate (concentration of vegetative bacteria adjusted to 1 x 10⁷ cfu/mL) in TSB was inoculated to each well but for the control. Negative control consisted of *E. coli* K12 JM109, *B. subtilis* ATCC6633 and *B. subtilis* AEWD isolate in TSB and took into account any bactericidal activity of H₂O₂ over a 6 hours period i.e. the inoculum for the 200 ppm H₂O₂ (commercial, batch reactor and flow reactor) was adjusted to 5 x 10⁵ cfu/mL for *E. coli* K12 JM109 and *B. subtilis* ATCC6633.
Blank consisted of TSB, 200, 100 and 50 ppm of commercial, batch reactor and flow reactor H$_2$O$_2$. The volume of liquid in every well was 200 µL. The plate was incubated in a shaking incubator at 120 rpm, 37°C for 6 hours. After 6 hours of incubation, crystal violet assay (based on Simoes *et al.*, 2007) was performed to measure the adherence of the bacteria to the well surface. All the wells were emptied after the plate was taken out from the incubator. The wells were washed with 200 µL of 8.5 g/L of sodium chloride. After that, the wells were washed with 200 µL of sterile distilled water. The procedure involved fixing the bacterial biofilm in 250 µL of absolute ethanol for 15 minutes, staining the biofilms with 250 µL of 1% crystal violet for 5 minutes, bacteria were then re-solubilised with 33% acetic acid and then the optical densities at 570 nm wavelengths were measured by a microtiter plate reader. All of the treatments and controls were performed in triplicate and the experiment was performed three times independently. These experiments were also carried out using NaOCl (0.25, 0.5, 1 and 2 ppm av. chlorine) against *E. coli* K12 JM109 in order to compare the efficacy of H$_2$O$_2$ against an established oxidant treatment. (n=3).

Biofilm prevention test was also carried out on 1 cm diameter sterile stainless steel discs in a 24 well plates with 200 ppm of commercial, batch reactor and flow reactor H$_2$O$_2$ with the same controls as described above. Crystal violet assay was not carried out on the stainless steel discs as that would not differentiate between the biofilm grown on the stainless steel discs and the rest of the well in the microtiter plate reader. The plate was incubated in a shaking incubator at 120 rpm, 37°C for 6 hours. Afterwards, the stainless steel discs were removed from the 24 well plates using forceps and vortexed in McCartney bottles containing TSB and glass beads for 2 min. Then, the suspension was serially diluted in sterile distilled water and 100 µL was
inoculated onto TSA plates using a spread plate method (Sanders, 2012). These plates were then incubated overnight at 37°C in aerobic atmosphere and colonies were counted, (n=3).

4.2.6 Statistical analyses

Data from the experiments were analysed using an unpaired t-test and One-way ANOVA statistical tests with a Bonferroni post hoc test using GraphPad Prism software.
4.3 Results

4.3.1 Flow reactor efficacy tests against bacteria

4.3.1.1 Results of the flow reactor efficacy tests

The efficacy of the flow reactor H₂O₂ against Gram-positive and Gram-negative bacteria was not significantly different (P value = 0.49). There was a 1.22 ± 0.65 log₁₀ reduction in *S. aureus* and 1.53 ± 0.28 log₁₀ reduction in *E. coli* (Figure 4.1).

**Figure 4.1** Efficacy of flow reactor against *S. aureus* NCTC10788 and *E. coli* NCTC10418 under standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H₂/air, solvent flow rate = 0.2 mL/min, 120mg of the catalyst. Data were analysed using an unpaired t-test using GraphPad Prism software. (n=3)
4.3.1.1.1 Comparison of results of the flow reactor and suspension efficacy tests

Comparing the flow reactor experiments results with the results of suspension tests against *S. aureus* NCTC10788 and *E. coli* NCTC10418 with 200 ppm commercial \( \text{H}_2\text{O}_2 \) at a 60 seconds time point in clean water conditions (Chapter 2 Section 2.3.1.1.2.), the flow reactor treatment is more effective. The results of these suspension tests were 0.02 ± 0.07 and 0.22 ± 0.26 \( \log_{10} \) reductions for *S. aureus* NCTC10788 and *E. coli* NCTC10418 respectively. Figure 4.2 shows these data graphically and an unpaired t-test analysis showed that these differences were statistically significant by P< 0.05 for *S. aureus* NCTC10788, and P< 0.01 for *E. coli* NCTC10418.
**Figure 4.2** Comparison between suspension tests (BS EN 1276:2009) with 200 ppm commercial H₂O₂ in clean water conditions at 1 min time point and flow reactor experiments on *S. aureus* NCTC10788 and *E. coli* NCTC10418. Data were analysed using an unpaired t-test using GraphPad Prism software. The significance of results is shown as asterisks above the bars * = P< 0.05, ** = P< 0.01. (n=3).
4.3.1.2 Flow reactor efficacy tests in different reaction conditions

4.3.1.2.1 Efficacy tests against low bacterial inoculum

Experiments were carried out with a $10^3$ cfu/mL initial bacterial inoculum to test how well the flow reactor works against low bacterial inoculum. Figure 4.3A shows that a $0.33 \pm 0.06 \log_{10}$ reduction was observed with *S. aureus* NCTC10788 and a $1.37 \pm 0.25 \log_{10}$ reduction was recorded for *E. coli* NCTC10418. This difference between the 2 microorganisms was significant ($P < 0.01$). Figure 4.3B shows that there was a higher $\log_{10}$ reduction of *S. aureus* NCTC10788 when the initial inoculum was $10^7$ cfu/mL than $10^3$ cfu/mL, however, the difference in inactivation in *S. aureus* with initial inoculum size of $10^7$ and $10^3$ cfu/mL was not statistically significant ($P > 0.05$). Figure 4.3C shows the results of $10^7$, and $10^3$ cfu/mL initial inoculum of *E. coli* NCTC10418 which were not statistically significant. The experiments with *E. coli* NCTC10418 with both $10^7$ and $10^3$ cfu/mL initial inoculum gave comparable results ($1.53 \pm 0.28$ and $1.37 \pm 0.25 \log_{10}$ reductions for $10^7$ and $10^3$ cfu/mL initial inoculum, respectively).
Figure 4.3 Efficacy of flow reactor with $10^3$ and $10^7$ cfu/mL initial inocula of *S. aureus* NCTC10788 and *E. coli* NCTC10418 under standard reaction conditions: 10 bar pressure, $2^\circ$C, 42 mL/min gas flow of 2% H$_2$/air, solvent flow rate = 0.2 mL/min, 120mg of the catalyst. A) Comparison of results with $10^3$ cfu/mL initial inoculum between *S. aureus* NCTC10788 and *E. coli* NCTC10418. B) Comparison of results with *S. aureus* NCTC10788 between $10^3$ and $10^7$ cfu/mL initial inoculum. C) Comparison between *E. coli* NCTC10418 $10^3$, and *E. coli* NCTC 10418 $10^7$ cfu/mL initial inoculum. Data were analysed using an unpaired t-test using GraphPad Prism software. The significance of results is shown as asterisks above the bars ** = P< 0.01. (n=3)
4.3.1.2.2 Efficacy of flow reactor at room temperature

Experiments at room temperature were carried out with \textit{E. coli} NCTC10418 to determine whether temperature affects catalyst efficacy. Figure 4.4 shows that \textit{E. coli} NCTC10418 at room temperature gave a $1.48 \pm 0.23\log_{10}$ reduction which is virtually identical ($P = 0.82$) to the results obtained in $2\degree C$ (standard reaction condition temperature) ($1.53 \pm 0.28\log_{10}$ reduction).

**Figure 4.4** Efficacy of flow reactor against \textit{E. coli} NCTC 10418 $10^7$ cfu/mL initial inoculum at $2\degree C$ (standard reaction conditions temperature) and room temperature ($20\degree C$). Reaction conditions were otherwise standard i.e. 10 bar pressure, 42 mL/min gas flow of 2% H$_2$/air, solvent flow rate = 0.2 mL/min, 120mg of the catalyst. Data were analysed using an unpaired t-test using GraphPad Prism software. (n=3).
4.3.2 Efficacy of flow reactor against MS2 bacteriophages

Flow reactor experiments were compared with the results of the suspension tests carried out with both commercial and batch reactor H$_2$O$_2$ at 100 and 200 ppm concentration (Chapter 2 Section 2.3.2). The log$_{10}$ reductions of pfu/mL for all of the H$_2$O$_2$ concentrations from suspension tests were below 0.5 whereas the log$_{10}$ reduction pfu/mL for the flow reactor treatment was 8.51 ± 0.28 (Fig 4.5). This result is significantly different (P< 0.001) when compared to the use of H$_2$O$_2$. It has to be noted that the >8 log$_{10}$ reduction was observed on two occasions.
Figure 4.5 Efficacy of 100 ppm and 200 ppm of commercial and batch reactor H$_2$O$_2$ and a flow reactor treatment against MS2 bacteriophages. Data were analysed using a One-way ANOVA with a Bonferroni post hoc test using GraphPad Prism software. The significance of results is shown as asterisks above *** = P < 0.0001. (n=3 for H$_2$O$_2$ in suspension and n=2 for flow reactor).
4.3.3 Flow reactor efficacy tests reproducibility

4.3.3.1 Effects of flow blockage in the catalyst bed on efficacy of flow reactor

Further experiments were carried out with *E. coli* NCTC10418, however, during these experiments, the pressure in the reactor was increased up to 15 bar due to a partial blockage in the catalyst bed (Table 4.1). Increased pressure due to blockage led to a better inactivation of *S. aureus* (Table 4.2). Efficacy against *E. coli* NCTC10418 improved from an average of $1.53 \log_{10}$ reduction to a $4.26$ and $3.30 \log_{10}$ reduction in repeats 4 and 5, respectively. The average activity of the two tests with a partial blockage in a catalyst bed was $3.78 \pm 0.67 \log_{10}$ reduction (Figure 4.6). This activity was significantly different ($P < 0.05$) from the activity without the blockage in the catalyst bed.
Table 4.1 Log$_{10}$ reduction in *E. coli* NCTC10418 under standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H$_2$/air, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst. Results highlighted in yellow are from experiments performed under increased pressure due to a blockage in the catalyst bed. (n=5)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mean log before treatment</th>
<th>Mean log after treatment</th>
<th>Mean log reduction</th>
<th>Catalyst</th>
<th>Run on the catalyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.90</td>
<td>6.37</td>
<td>1.53</td>
<td>GS 2</td>
<td>1st</td>
</tr>
<tr>
<td>2</td>
<td>7.81</td>
<td>6.56</td>
<td>1.25</td>
<td>GS 2</td>
<td>2nd</td>
</tr>
<tr>
<td>3</td>
<td>7.96</td>
<td>6.15</td>
<td>1.82</td>
<td>GS 2</td>
<td>3rd</td>
</tr>
<tr>
<td>4</td>
<td>7.26</td>
<td>3</td>
<td>4.26</td>
<td>GS 5</td>
<td>1st</td>
</tr>
<tr>
<td>5</td>
<td>7.26</td>
<td>3.96</td>
<td>3.30</td>
<td>GS 6</td>
<td>1st</td>
</tr>
</tbody>
</table>

Table 4.2 Log$_{10}$ reduction in *S. aureus* NCTC10788 under standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H$_2$/air, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst. Results highlighted in yellow are from experiments performed under increased pressure due to a blockage in the catalyst bed (n=3).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mean log before treatment</th>
<th>Mean log after treatment</th>
<th>Mean log reduction</th>
<th>Catalyst</th>
<th>Run on the catalyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.59</td>
<td>6.50</td>
<td>1.09</td>
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<td>2</td>
<td>7.24</td>
<td>5.31</td>
<td>1.93</td>
<td>GS 1</td>
<td>2nd</td>
</tr>
<tr>
<td>3</td>
<td>7.77</td>
<td>7.13</td>
<td>0.64</td>
<td>GS 2</td>
<td>4th</td>
</tr>
</tbody>
</table>
**Figure 4.6** Comparison of the flow reactor efficacy against *E. coli* NCTC10418 in standard reaction conditions (10 bar pressure, 2°C, 42 mL/min gas flow of 2% H\textsubscript{2}/air, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst) and with a partial blockage of the catalyst bed. The pressure under partial blockage of the catalyst bed was 15 bar. Data were analysed using an unpaired t-test using GraphPad Prism software. The significance of results is shown as asterisks above the bars * = P< 0.05.

![Graph showing log\textsubscript{10} reduction (cfu/mL) for E. coli (without blockage) and E. coli (with blockage) with a partial blockage of the catalyst bed.](image)

### 4.3.3.2 Efficacy of flow reactor against *E. coli* K12 JM109

Five independent experiments were carried out. The first three in one day and the 4\textsuperscript{th} and 5\textsuperscript{th} repeat were carried out the next day. What could be observed was that the
results of the first three experiments that were carried out on the same day gave consecutively higher \( \log_{10} \) reduction with each repeat: 1.19, 3.52 and 5. Then the 4\(^{th} \) repeat carried out on the next day gave a much lower \( \log_{10} \) reduction (0.74), however, the 5\(^{th} \) repeat carried out on that day (1.76 \( \log_{10} \) reduction) was an improvement from the 4\(^{th} \). The consecutive activity increases with each repeat of the experiment potentially meant that there is an induction period. That means that the catalyst gets activated more after some time to enable it to reach its full potential. Data are illustrated graphically on Figure 4.7 scatter plot.

**Figure 4.7** Scatter plot of 5 independent flow reactor experiments on *E. coli* K12 JM109 under standard reaction conditions: 10 bar pressure, 2\( ^{\circ} \)C, 42 mL/min gas flow of 2\% H\(_2\)/air, solvent flow rate = 0.2 mL/min, 120mg of the catalyst. Median is presented as a red line. Numbers by the arrows represent the order in which the results were obtained. (n=5).
4.3.3.3 Efficacy of flow reactor against *E. coli* K12 JM109 over a 2 hours period

Further flow reactor experiments were carried out on *E. coli* K12 JM109 for 2 hours with 30 minutes time points in order to find out whether there was an induction period in this system. In the first experiment, there was no significant difference (P> 0.05) in activity over a 2 hours period. The bactericidal activity was maintained at around 4 log_{10} reduction. In the second experiment, the starting log_{10} reduction was much lower (1.5 log_{10} reduction) and was also maintained over the course of the experiment (Fig 4.8).

**Figure 4.8** Efficacy of the flow reactor against *E. coli* K12 JM109 over a 2 hours period with 30 min time points under standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H_{2}/air, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst. (n=2).
4.3.3.4 Efficacy of flow reactor against *E. coli* K12 JM109 with artificial turbulent flow

Blockage in the catalyst bed caused an increase in microbicidal activity. Dents in the tubing were created using a crimping tool to attempt to artificially cause a turbulent flow over the catalysts bed based on partial blockage (Fig. 4.9). Two experiments were carried out on two different catalyst beds. The overall efficacy was increased in one instance but not in the other. The log_{10} reduction obtained using one catalyst bed was 3.54 whereas with the other catalyst bed, the log_{10} reduction was 1.21. In both cases the blockage was achieved and the pressure increased. The pressure in the catalyst bed was steadily increasing up to 14 bar and was maintained until the end of experiment, providing a higher log_{10} reduction. In the catalyst bed with a lower log_{10} reduction, the initial increase in pressure diminished with time and eventually reached a normal pressure i.e. 10 bar. This observation provides further evidence that a blockage in the catalyst bed causes an increase in retention time between the bacteria and the catalyst, an introduction of a turbulent flow to the system and an increase of the pressure of the reaction which are increasing the activity of the catalyst. Unfortunately, the use of the crimping tool to create dents did not provide a reproducible way to create partial blockage.
Figure 4.9 Artificial dents were introduced to the catalyst bed using a crimping tool.

4.3.3.5 Catalyst beds timelines

Reproducibility of the inactivation results from the test reactor experiments was identified as an issue. In order to have a holistic picture of the problem, a set of timelines of catalyst beds used in the flow reactor experiments was created (Fig. 4.10).
Figure 4.10 The catalyst beds (120 mg of 1 w/w% AuPd/TiO₂) timelines with microbicidal efficacies of every run on these catalyst beds. Figure legends are present by each timeline.
Catalyst GS 3

E. coli NCTC 10418 $10^7$ cfu/mL
S. aureus NCTC 10788 $10^3$ cfu/mL

Catalyst GS 4

E. coli NCTC 10418 $10^9$ and $10^7$ cfu/mL
room temp
MS2 $10^{10}$ and $10^9$ pfu/mL
Figure 4.10 continued

Catalyst GS 5

- E. coli NCTC 10418 $10^7$ cfu/mL
- Blockage

Catalyst GS 6

- E. coli NCTC 10418 $10^7$ cfu/mL
- E. coli K12 $10^7$ cfu/mL
- MS2 $10^9$ pfu/mL
- E. coli + GSH

Catalyst GS 9

- E. coli K12 $10^7$ cfu/mL
- Blocked HPLC pump

Continuous experiments with time points at 30min intervals
What can be observed from the catalyst beds timelines (Fig. 4.10) for catalyst beds GS1, GS2 and GS3 is that the results were consistent noting that the discrepancy in the MS2 log\textsubscript{10} reductions was due to the fact that the starting inoculum for the first experiment was $10^3$ and for the second experiment was $10^{12}$ pfu/mL. However, catalyst GS4 provided some inconsistencies which were observed in the results of experiments with MS2 bacteriophages. The first experiment on MS2 bacteriophages with high initial inoculum (11.9 log\textsubscript{10}) carried out on catalyst GS3 provided an 8.31 log\textsubscript{10} reduction, whereas, the two experiments in high initial inoculum of MS2 (10.3 and 9.6 log\textsubscript{10}, respectively) carried out on catalyst GS4 provided much lower log\textsubscript{10} reductions of 2.44 and 0.90, respectively. It is worth noting that a fourth experiment on a high initial inoculum of MS2 bacteriophages (9.4 log\textsubscript{10}) on catalyst GS6 provided another high log\textsubscript{10} reduction of 8.71. There were further MS2 bacteriophages experiments carried out on later catalyst beds which provided results of 2.37, 2.87, 5.41 and 6.82 log\textsubscript{10} reductions.

Another example of inconsistencies in results were results obtained in experiments carried out on catalyst bed GS9. The inconsistencies in the first five experiments were the log\textsubscript{10} reductions obtained during the experiments carried out on the same day kept on increasing with each repeat. Another example of inconsistencies from the catalyst GS9 was when 2 hour experiments with 30 minutes time points provided large differences between the average log\textsubscript{10} reductions (3.82 and 1.56).
4.3.4 Biofilm prevention

4.3.4.1 *E. coli* K12 JM109 biofilm prevention on a PVC surface

Figure 4.11 illustrates results of the 6 hour *E. coli* K12 JM109 biofilm prevention tests in 96 well plates. The 200 ppm concentration of commercial and flow reactor H$_2$O$_2$ caused a 96% decrease of biofilm adhesion compared to the negative control biofilm and the batch reactor H$_2$O$_2$ caused a 100% decrease (all P< 0.0001). The 100 ppm concentration of commercial and batch reactor H$_2$O$_2$ caused a 54% and 51% decrease of biofilm adhesion respectively (both P< 0.05) and the flow reactor H$_2$O$_2$ caused a 27% biofilm adhesion decrease (P< 0.01). The 50 ppm concentration of commercial and batch reactor H$_2$O$_2$ caused a 24% and 7% increase in biofilm adhesion and a flow reactor H$_2$O$_2$ caused a 49% increase (P< 0.05).
Figure 4.11 Efficacy of H$_2$O$_2$ on biofilm adhesion prevention experiments against *E. coli* K12 JM109 using 200, 100 and 50 ppm concentration of commercial, batch reactor and flow reactor H$_2$O$_2$. Data were analysed using an unpaired t-test using GraphPad Prism software. The significance of results is shown as asterisks above the bars * = P < 0.05, ** = P < 0.01, *** = P < 0.0001. (n=3).

4.3.4.2 *B. subtilis* ATCC6633 biofilm prevention on a PVC surface

Figure 4.12 shows results of the 6 hour *B. subtilis* ATCC6633 biofilm prevention tests on 96 well plates. The 200 ppm concentration of commercial, batch reactor and flow reactor H$_2$O$_2$ all caused a 96% decrease in biofilm adhesion (P < 0.001). The 100 ppm concentration of commercial H$_2$O$_2$ caused a 93% decrease in biofilm adhesion (P < 0.001). Batch reactor H$_2$O$_2$ of 100 ppm concentration caused a 44% decrease in biofilm adhesion (P < 0.001). The 100 ppm concentration of flow reactor H$_2$O$_2$ caused
a 54% decrease in biofilm adhesion (P< 0.001). The 50 ppm concentration of commercial, batch reactor and flow reactor H$_2$O$_2$ caused a 9%, 7% and 4% decreases in biofilm adhesion respectively which were not statistically significant (P> 0.05).

**Figure 4.12** Efficacy of H$_2$O$_2$ on biofilm adhesion prevention experiments against *B. subtilis* ATCC6633 using 200, 100 and 50 ppm concentration of commercial, batch reactor and flow reactor H$_2$O$_2$. Data were analysed using an unpaired t-test using GraphPad Prism software. The significance of results is shown as asterisks above the bars *** = P< 0.001. (n=3).

4.3.4.3 *B. subtilis* AEWD isolate biofilm prevention on a PVC surface

Figure 4.13 presents results of the 6 hour *B. subtilis* AEWD isolate biofilm prevention tests on 96 well plates using 200 ppm concentration of commercial, batch reactor and
flow reactor H₂O₂. Commercial H₂O₂ caused a 13% decrease in biofilm adhesion which was statistically insignificant. Batch reactor H₂O₂ caused a 29% decrease in biofilm adhesion (P< 0.05). Flow reactor H₂O₂ caused a 28% increase in biofilm adhesion (P< 0.05).

**Figure 4.13** Efficacy of H₂O₂ on biofilm adhesion prevention experiments against *B. subtilis* AEWD isolate using 200 ppm concentration of commercial, batch reactor and flow reactor H₂O₂. Data were analysed using an unpaired t-test using GraphPad Prism software. The significance of results is shown as asterisks above the bars * = P< 0.05. (n=3).
4.3.4.4 Effects of chlorine on *E. coli* K12 JM109 biofilm prevention

Figure 4.14 shows results of the *E. coli* K12 JM109 biofilm prevention experiments using NaOCl. Active chlorine concentration of 0.25 ppm caused a 21% increase of bacterial attachment to the PVC surface. NaOCl at 0.5 ppm of active chlorine caused no change in the bacterial attachment in comparison to the negative control. Active chlorine of 1 ppm and 2 ppm concentration caused a 28 and 20% decrease in bacterial attachment respectively. These differences were statistically insignificant (P> 0.05).

**Figure 4.14** Efficacy of *H₂O₂* on biofilm adhesion prevention experiments against *E. coli* K12 JM109 using NaOCl at 0.25 ppm, 0.5 ppm, 1 ppm and 2 ppm of active chlorine. Data were analysed using an unpaired t-test using GraphPad Prism software. (n=3).
4.3.4.5 *E. coli* K12 JM109 biofilm prevention on a stainless steel surface

200 ppm of commercial, batch reactor and flow reactor H₂O₂ caused a 100% decrease in adhesion to the stainless steel surface (P< 0.01 for commercial and batch reactor H₂O₂ and P< 0.05 for flow reactor H₂O₂) (Fig. 4.15).

**Figure 4.15** Efficacy of H₂O₂ on biofilm adhesion prevention on stainless steel discs experiments against *E. coli* K12 JM109 using 200 ppm concentration of commercial, batch reactor and flow reactor H₂O₂. Data were analysed using an unpaired t-test using GraphPad Prism software. The significance of results is shown as asterisks above the bars * = P< 0.05, ** = P< 0.01. (n=3).
4.4 Discussion

Initial experiments showed a much higher activity from a flow reactor in relation to the commercial and batch reactor H$_2$O$_2$ in suspension. Comparable activity in suspension could be achieved after a 60 min time point when the catalyst reaction took place in 1 min. In this respect, the flow reactor treatment is 60 times more effective than the commercial H$_2$O$_2$. Such a marked difference in results between these two treatments indicates that H$_2$O$_2$ produced in the flow reactor must have an additional mechanism of action or the flow reactor catalyst bed produces additional microbicidal activity alongside the H$_2$O$_2$.

Experiments at room temperature that were carried out on *E. coli* NCTC10418 showed that there was no difference between the experiments in room temperature and 2°C. The temperature of 2°C is used under standard reaction conditions as it maximises the concentration of H$_2$O$_2$ produced, therefore, when the temperature of the reaction is higher, it will produce less H$_2$O$_2$. A study conducted by Freakley *et al.* (2013) showed that increasing the reaction temperature from 2°C to 20°C decreased to production of H$_2$O$_2$ by about 30%. This observation adds to the argument that H$_2$O$_2$ plays a lesser part in killing bacteria in the flow reactor. H$_2$O$_2$ is continuously produced by the catalyst indicating that bacteria should always be in contact with H$_2$O$_2$. Furthermore, differences in efficacy between 100 and 200 ppm of H$_2$O$_2$ measured in a suspension test were not statistically significant. This reinforces the point that H$_2$O$_2$ does not play a major role in bacterial kill in this system.
Flow reactor experiments were carried out on MS2 bacteriophages. These experiments showed an over 8 log$_{10}$ reduction (two repeats) and the difference between the suspension and flow reactor experiments was statistically significant (P< 0.001). This result shows that the catalytic technology proposed in this project appears to be a very effective method of killing non-enveloped viruses. In an experiment where 25 ppm of free chlorine was used, a level higher than that used as secondary disinfectant, a 1.35 log$_{10}$ reduction in 5 minutes contact time was observed (Kingsley et al., 2017 and Cervero-Arago et al., 2015). This result observed at a longer contact time is also much lower than the result obtained from the flow reactor experiments. This further reinforces that the flow reactor treatment is very effective against non-enveloped viruses.

Two hours flow reactor experiments carried out on E. coli K12 JM109 showed that there is no induction period in the system as the activity was maintained throughout the 2 hours period. One of the two such experiments gave results of 4 log$_{10}$ reductions for the 2 hours period which shows that our system is more effective than 0.8 ppm chlorine (the most common water disinfectant) at killing E. coli as lower log$_{10}$ reductions were achieved at 1 minute contact time with chlorine (Virto et al., 2005).

The partial blockage of the catalyst bed observed in some experiments meant that the bacterial suspension went through the catalyst bed for longer than it would without the blockage which increased the contact time between the catalyst with its active agents and bacteria. What is more, the partial blockage in the catalyst bed caused an increase in the pressure in the flow reactor which would in turn increase the amount of H$_2$O$_2$ produced by the catalyst. This is because the increase of the reaction pressure decreases the size of the gas bubbles and increases gas solubility (Freakley et al.,
Both of these effects caused by the partial blockage of the catalyst bed meant that the contact time with the killing agents was increased and so was their concentration which had a positive effect on the activity.

Experiments when there was a partial blockage of the catalyst bed showed an increase in activity which lead to an idea to artificially introduce these blockages by creating dents in the catalyst beds. The two experiments carried out with these catalyst beds provided variable results. Furthermore, experiments against MS2 bacteriophages also provided variable results. This shows the need for a more reproducible design of the catalyst bed where the differences in catalyst packing and the contact of the microorganisms with the catalyst are minimised.

Flow reactor, commercial and batch reactor H$_2$O$_2$ at 200 ppm concentration were all highly effective at preventing adhesion of *E. coli* K12 JM109 and *B. subtilis* ATCC6633 to surfaces. Furthermore, concentrations of active chlorine which are permitted as a secondary disinfectant of water i.e. 0.2-0.5 ppm (Cervero-Arago *et al.*, 2015) and higher (1 and 2 ppm) were not effective at preventing biofilm formation of *E. coli* K12 JM109. This finding shows the high effectiveness of our system in biofilm prevention and that it is much more effective than the most commonly used water disinfectant.
5 Mechanisms of microbicidal action

5.1 Introduction

5.1.1 Mechanisms of microbicidal action of different disinfectants

Elucidating the mechanisms of microbicidal action of disinfectants is important to have a better understanding of efficacy, emerging microbial resistance, toxicity, and to help developing new formulations (Denyer, 1990). There are many groups of disinfectants, one of them being oxidising agents which include H₂O₂, peracetic acid, isothiazolones and hypochlorite. Their microbicidal activity is predominantly on proteins, particularly on thiol groups of cysteine residues and oxidation of those leads to inhibition of microorganisms’ metabolism (Collier et al., 1990; Collier et al., 1990 and Thurman and Gerba, 1988). Alkylating and halogenating agents are the next group of disinfectants which include glutaraldehyde and formaldehyde. These chemicals react with protein and nucleic acid residues by alkylation which is an irreversible reaction that causes an inhibition of cell division and metabolism. Many chemical groups can react with aldehydes such as hydroxyl, amino, thiol, carboxyl, amide and imino substituents. Formaldehyde cross-links proteins which leads to their aggregation (Jiang and Schwendeman, 2000; Rossmore and Sondossi, 1988). Protein denaturants such as alcohols and phenols are another group of disinfectants and they bind to amino acid residues and displace water molecules causing protein structure denaturation (Ingram and Buttke, 1984). Concentration of phenols and alcohols determine their effects on bacteria. At lower concentration, enzymes are inhibited. More pronounced conformational changes to the membrane proteins cause damage of the membrane and leakage of the components of the bacterial cell. Complete denaturation causes
coagulation of the cytoplasmic proteins (Lucchini et al., 1990). Some disinfectants such as Quaternary Ammonium Compounds (QAC), cationic detergents and bisbiguanides interact with anionic lipids in the membrane of Gram-negative bacteria (Russell, 1986; Salton, 1968). Low concentrations of these agents cause disruption of the interactions between proteins and lipids in the membrane leading to membrane damage and leakage of the constituents of the cytoplasm. At high concentrations coagulation of the cytoplasm happens. Chitosan which is a cationic polysaccharide damages the Gram-positive bacterial cytoplasmic membrane by binding to lipoteichoic acid (Raafat et al., 2008).

As mentioned in Chapter 3, the catalyst in the flow reactor used in this project produces free radicals. A free radical is a molecule or an atom that contains unpaired electrons in their outer orbit and can exist independently (Cheeseman and Slater, 1993). The fact that free radicals contain unpaired electrons makes them short lived, unstable and highly reactive. Free radicals attack other molecules to obtain electrons and gain stability which makes the attacked molecule unstable which then causes a chain reaction cascade which leads to damaging a living cell (Phaniendra et al., 2015).

Free radicals consist of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Pham-Huy et al., 2008). The four main ROS are H$_2$O$_2$, superoxide (O$_2$•-), hydroxyl radical (OH•) and singlet oxygen (¹O$_2$). The effects of H$_2$O$_2$ and O$_2$•- are less acute than effects of OH• and ¹O$_2$. This is because the formers are less reactive and can be detoxified by enzymatic and non-enzymatic antioxidants that are endogenous and are induced during an oxidative stress. Hence, H$_2$O$_2$ and O$_2$•- can be detoxified more easily by antioxidants. OH• and ¹O$_2$ cannot be detoxified by enzymes making them extremely toxic (Vatansever et al., 2013). As was considered in Chapter 3, the
The catalyst in the flow reactor used in this project catalyses the production of \( \text{OH}^\bullet \) which is important for the microbicidal efficacy.

5.1.2  Aims and objectives

The aim of this chapter is to elucidate the mechanisms of microbicidal action of the catalyst used in this project.

The objectives are:

1. Test the flow reactor efficacy with an antioxidant glutathione and catalase, an enzyme that degrades \( \text{H}_2\text{O}_2 \). This is to see the importance of free radicals and \( \text{H}_2\text{O}_2 \) for the mechanisms of microbicidal action.

2. Test flow reactor and \( \text{H}_2\text{O}_2 \) efficacy against *Deinococcus radiodurans* (*D. radiodurans*), a bacterium that is highly resistant against oxidative damage, to see the importance of free radicals and \( \text{H}_2\text{O}_2 \) for the mechanisms of microbicidal action.

3. Incubate bacteria after treatment on minimum media to see if they can repair themselves after treatment.

4. Microscopic imaging of bacteria to observe their ultrastructure after treatment and the extent of the damage inflicted by the flow reactor and \( \text{H}_2\text{O}_2 \) treatments.
5.2 Materials and methods

5.2.1 Bacterial strains and growth

Bacterial strains used in the investigations were *E. coli* NCTC10418, *E. coli* K12 JM109 and *D. radiodurans*. Growth of *E. coli* NCTC10418 and *E. coli* K12 JM109 was considered in detail in Chapter 2 section 2.2.1. *D. radiodurans* was grown as per other bacteria, the difference was that it was incubated for five days at 32°C prior to and post testing. Furthermore, the initial inoculum of *D. radiodurans* was $10^5$ cfu/mL. The medium used for the minimum media experiments to observe bacteria ability to repair themselves was R2A.

5.2.2 Suspension tests

Suspension tests were carried out in accordance with BS EN 1276:2009 and were considered in detail in Chapter 2 Section 2.2.2. These suspension tests were carried out against *D. radiodurans* and on *E. coli* K12 JM109 in order to compare the effects of flow reactor free radicals treatment and H$_2$O$_2$ in suspension. Spread plate method with 1 mL inoculum was used to enumerate *D. radiodurans* in order to have no limit of detection, otherwise the drop count method was used. (n=3).
5.2.3 H$_2$O$_2$ synthesis

H$_2$O$_2$ synthesis was considered in detail in Chapter 2 Section 2.2.3. H$_2$O$_2$ was synthesised in a batch reactor in order to compare its activity with commercial H$_2$O$_2$.

5.2.4 Flow reactor tests

Flow reactor tests and reaction conditions were considered in detail in Chapter 3 Section 3.2.6. Standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H$_2$/air, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst. Five mM glutathione and 500 U/mL of catalase were added to the bacterial suspensions of *E. coli* NCTC10418 and *E. coli* K12 JM109 in order to see the effect that free radicals and H$_2$O$_2$ on their own have in a flow reactor treatment. Control experiments carried out were: passing bacterial suspension through the reactor without catalyst in 2% H$_2$/air and showed no bacterial kill. There was no neutralisation step after the reaction. Spread plate method with 1 mL inoculum was used to enumerate *D. radiodurans* in order to have no limit of detection, otherwise the drop count method was used. (n=3).

5.2.5 Scanning Electron Microscopy

One mL samples of $10^9$ cfu/mL *E. coli* K12 JM109 suspension treated with 200 ppm of commercial or batch reactor H$_2$O$_2$ for 2 hours or subjected to the flow reactor were added to 9 mL of a fixative solution (2% glutaraldehyde in 0.1 M sodium cacodylate
buffer – pH 7.4). The suspension was then incubated for two hours at room temperature to fix the cells, then transferred to a 0.2 micron polycarbonate filter membrane using a vacuum/pump filtration system. These filter membranes were transferred into 0.1 M sodium cacodylate buffer (pH 7.4) for three minutes in order to wash off excess glutaraldehyde. In order to dehydrate the samples, the filter membrane was transferred to ascending series of ethanol solutions (50%, 70%, 80%, 95%, 100% and 100%) for 3 minutes. After that, the filter membrane was transferred to the drying agent hexamethyldisilazane (HMDS) for 5 minutes. The filter membrane was then placed into a petri dish and then into a bell jar containing silica gel beads overnight in order for the HMDS to dry. One cm squares of the filter membrane were cut the next day and mounted onto stainless steel stubs using adhesive discs. These were then sputter coated with 7 nm of 80:20 Au/Pd and placed into the microscope vacuum chamber for imaging. Scanning Electron Microscopy (SEM) pictures were taken using Hitachi TM3030Plus.

5.2.6 Statistical analyses

Data from the experiments were analysed using an unpaired t-test and One-way ANOVA statistical tests with a Bonferroni post hoc test using GraphPad Prism software.
5.3 Results

5.3.1 Flow reactor efficacy tests with glutathione and catalase

Flow reactor experiments were carried out with glutathione added to the bacterial suspension to test the activity of the flow reactor catalyst bed in presence of an antioxidant (Bajic et al., 2019). Furthermore, flow reactor experiments were carried out with catalase added to the bacterial suspension to test activity in presence of an enzyme that degrades H$_2$O$_2$ into oxygen and water (Alfonso-Prieto et al., 2009).

Results of the flow reactor experiments against E. coli NCTC10418 was 1.53 ± 0.28 log$_{10}$ reduction. Results of the flow reactor experiments with 5 mM glutathione and 500 U/mL catalase were 0.06 ± 0.11 and 1.19 ± 0.39 log$_{10}$ reductions respectively. A statistically significant difference in activity (P< 0.01) was observed in the presence of 5 mM glutathione but not in the presence of catalase 500 U/mL (Fig 5.1).
**Figure 5.1** Efficacy of the flow reactor free radicals on *E. coli* NCTC10418, *E. coli* NCTC10418 with 5mM glutathione and *E. coli* NCTC10418 with 500 U/mL catalase. $10^7$ cfu/mL initial inoculum under standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H$_2$/air, solvent flow rate = 0.2 mL/min, 120mg of the catalyst. Data were analysed using an unpaired t-test using GraphPad Prism software. The significance of results is shown as asterisks above the bars ** = P< 0.01. (n=3).
A significant reduction in activity (P< 0.001) against *E. coli* K12 JM109 was observed in the presence of 5 mM glutathione (0.03 ± 0.05 log_{10} reduction for the reaction with glutathione compared to a 4.08 ± 0.22 log_{10} reduction for the reaction without glutathione) (Fig 5.2).

**Figure 5.2** Efficacy of the flow reactor free radicals on *E. coli* K12 JM109 and *E. coli* K12 JM109 with 5 mM glutathione. 10^7 cfu/mL initial inoculum under standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H\textsubscript{2}/air, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst. Data were analysed using an unpaired t-test using GraphPad Prism software. The significance of results is shown as asterisks above the bars *** = P< 0.001. (n=3).
5.3.2 Flow reactor efficacy tests against *Deinococcus radiodurans*

*D. radiodurans* is a bacterium that is highly resistant to oxidative damage (Krisko and Radman, 2013). Experiments were carried out against this bacterium to compare the activity of flow reactor free radicals and H$_2$O$_2$ in suspension.

Figure 5.3 shows results of suspension tests against *D. radiodurans* at 1 minute and 1 hour contact times with commercial and batch reactor H$_2$O$_2$. Log$_{10}$ reductions of the commercial H$_2$O$_2$ experiments were 0.06 ± 0.08 for both 1 min and 60 min time points. For experiments with the batch reactor H$_2$O$_2$, log$_{10}$ reductions were 0.10 ± 0.18 and 0.28 ± 0.31 at 1 min and 60 min time points respectively. These results were statistically insignificant (P> 0.05).
Figure 5.3 Efficacy of commercial and batch reactor H$_2$O$_2$ on *D. radiodurans* at 1 minute and 1 hour contact times. Suspension tests in accordance with BS EN 1276:2009 were carried out on $10^5$ cfu/mL initial inoculum. The bacteria were incubated for five days at 32°C in aerobic conditions. Data were analysed using a One-way ANOVA with a Bonferoni post hoc test using GraphPad Prism software. 200ppm commercial H$_2$O$_2$ and 200ppm batch reactor H$_2$O$_2$. Log$_{10}$ reduction results of the suspension test validations were: Reaction conditions: 0, Neutraliser toxicity: 0, Neutraliser efficacy: 0, Negative control: 0. (n=3).

Experiments showed a mean log$_{10}$ reduction of 1.26 ± 1.12 in the viability of *D. radiodurans* from the flow reactor free radicals treatment as opposed to a 0.06 and 0.1 log$_{10}$ reductions caused by the 200 ppm commercial and batch reactor H$_2$O$_2$ respectively at 1 minute contact time. The differences in log$_{10}$ reductions between all of the treatments were statistically insignificant (P> 0.05). (Fig 5.4).
Figure 5.4 Comparison of log$_{10}$ reductions between the flow reactor free radicals experiments and the suspension tests against *D. radiodurans*. Data were analysed using a One-way ANOVA with a Bonferoni post hoc test using GraphPad Prism software. Log$_{10}$ reduction results of the suspension test validations were: Reaction conditions: 0, Neutraliser toxicity: 0, Neutraliser efficacy: 0, Negative control: 0. (n=3).

![Bar graph showing log$_{10}$ reduction (cfu/mL) for different treatments](image)

5.3.3 Observing bacterial ability to repair after treatment

Five independent experiments were carried out against *E. coli* K12 JM109 using an R2A media to grow bacteria after treatment. The experiments on R2A media were carried out alongside the experiments on TSA media. The mean log$_{10}$ reductions of *E.*
coli K12 JM109 recorded on TSA and R2A plates were 2.44 ± 1.78 and 1.76 ± 0.79.
The median results recorded on TSA and R2A plates were 1.76 and 1.66 respectively.
The differences between the results were not statistically significant (P> 0.05) meaning that there was no evidence that E. coli K12 JM109 could repair injuries after treatment. Data are illustrated graphically on Figure 5.5 scatter plot.

**Figure 5.5** Efficacy of flow reactor free radicals on E. coli K12 JM109 under standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H₂/air, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst. Medians are presented as red lines. Bacteria were inoculated on TSA and R2A media. Data were analysed using an unpaired t-test using GraphPad Prism software. (n=5).
5.3.4 Microscopic imaging

Scanning Electron Microscopy (SEM) was used to show microbial ultrastructure (Golding et al., 2016) following flow reactor and H₂O₂ exposure. Figure 5.6 shows the effects of using 200 ppm of commercial and batch reactor H₂O₂ on *E. coli* K12 JM109 on SEM pictures (n=3). The flow reactor treatment caused an over the limit of detection log₁₀ cfu/mL reductions (>7 log₁₀ reductions) and no intact bacteria were visible under microscope (n=1).
Figure 5.6 SEM visualisation of the effects of using 200 ppm of commercial and batch reactor H₂O₂ at 2 hours contact time in clean water conditions suspension test based on the BS EN 1276:2009 against *E. coli* K12 JM109. Both treatments caused a 1 log₁₀ reduction. Flow reactor experiment was carried out using standard reaction conditions. The initial inoculum for the experiments was 10⁹ cfu/mL. A) control sample (n=3), B) 200 ppm of commercial H₂O₂ treatment (n=3), C) 200 ppm of batch reactor H₂O₂ treatment (n=3) and D) flow reactor treatment (n=1). SEM taken with a Hitachi TM3030Plus. 25 fields were observed for each treatment.
Figure 5.6 continued.

B)

C)
5.4 Discussion

Glutathione (5 mM) was used to see the effect of quenching free radicals on the efficacy of the catalyst against *E. coli* NCTC 10418 and *E. coli* K12 JM109. Glutathione protects cells against oxidative damage by interacting with reactive oxygen and nitrogen species and it does so with its cysteine residue thiol group (Lushchak, 2012). Experiments were also carried out with 500 U/mL of catalase to measure the effect of degrading of H$_2$O$_2$ on efficacy of the catalyst against *E. coli* NCTC 10418.

There was hardly any reduction in *E. coli* viability when glutathione was added to the bacterial suspension (0.06 log$_{10}$ reduction compared to 1.53 log$_{10}$ reduction without
glutathione) which gives a strong indication that free radicals play an important role in the mechanism of action for killing the bacteria. Further experiments with the same bacteria were performed with the use of 500 U/mL catalase. Catalase is an enzyme that causes degradation of H$_2$O$_2$ into H$_2$O and O$_2$ (Iwase et al., 2013). There was no significant difference in activity between the experiments carried out on E. coli with or without catalase which suggests that H$_2$O$_2$ does not play a major role in the mechanism of kill of bacteria, further emphasising the role of free radicals in this process.

Experiments against D. radiodurans showed that a 1 min contact time treatment with flow reactor caused an over 1 log$_{10}$ higher reduction than 1 hour treatment with 200 ppm commercial and batch reactor H$_2$O$_2$. This considerable difference provides another evidence that free radicals generated by the flow reactor catalyst bed are more important for the mechanism of action than the H$_2$O$_2$ itself. A study conducted by (Baatout et al., 2006) showed that a 1 hour treatment with 3% H$_2$O$_2$ against D. radiodurans provided a strong enough oxidative stress in order to cause an increase in cell permeability indicating membrane damage.

Flow reactor experiments were also carried out on E. coli K12 JM109 and the samples were then inoculated on TSA and R2A plates. R2A is a medium that allows the detection of slow-growing bacteria and better recovery of sub-lethally damaged or stressed microbes (Reasoner and Geldreich, 1985). Furthermore, the low nutritional content of this medium enables the recovery of a larger number of bacteria including the stressed bacteria that would not grow otherwise (Horgan et al., 1999). The log$_{10}$ reductions were smaller on the R2A plates than on the TSA plates, albeit, not
significantly and the medians were very similar. This indicates that bacteria cannot recover from damages caused following the flow reactor treatment.

Looking at the SEM pictures, clear cell wall damage of *E. coli* K12 JM109 could be observed from 200 ppm commercial and batch reactor H$_2$O$_2$. The reduction of *E. coli* K12 JM109 after flow reactor treatment was too high (> 7 log$_{10}$ reduction) to be able to observe the sample under SEM. This would indicate that the H$_2$O$_2$ at a concentration produced by the flow reactor causes a cell wall damage and when combined with the free radicals, cause a severe destruction of the bacterial cells beyond detection under SEM after just 1 min contact time. The SEM pictures were taken from one repeat experiment, therefore, more repeats would have to be carried out in order to confirm this. There are numerous studies indicating cell wall damage using oxidising agents. In a study conducted by (Nishida *et al.*, 2018), it is shown using SEM visualisations that the cell wall structure was affected by disinfection treatments such as with sodium hypochlorite. A study carried out by (Patil *et al.*, 2011) indicated a slight roughening of *E. coli* cell surface structure after ozone treatment. (DeQueiroz and Day, 2007) studied an effect of combination of hydrogen peroxide and sodium hypochlorite on *P. aeruginosa* and also showed cell wall alterations through SEM images.

In summary, it can be clearly seen from the experiments using glutathione and catalase that the free radicals produced by the flow reactor play a major role in the mechanism of action. SEM visualisations further support this as there was no bacteria visible after the flow reactor free radical treatment indicating complete destruction of bacteria (n=1). Moreover, it was observed that bacteria cannot recover after the flow reactor treatment. The strength of the flow reactor free radical treatment was further reinforced
by results of experiments against *D. radiodurans* where a $1.26 \log_{10}$ reduction was achieved against a bacterium that is highly resistant to oxidative damage.
6 General Discussion

6.1 Key findings

It was demonstrated that the 1 w/w% AuPd/TiO$_2$ can catalyse production of H$_2$O$_2$ at around 200 ppm in the flow reactor system. The difference in H$_2$O$_2$ production between 1 w/w% AuPd/TiO$_2$ and 1 w/w% Pd/TiO$_2$ was very small (259 and 220 ppm respectively). However, 1 w/w% AuPd/TiO$_2$ was a thousand times more effective at killing *E. coli* K12 JM109 than 1 w/w% Au/TiO$_2$ or 1% Pd/TiO$_2$ (4 log$_{10}$ reduction using 1 w/w% AuPd/TiO$_2$ compared to ~ 1 log$_{10}$ reduction using 1 w/w% Au/TiO$_2$ or 1 w/w% Pd/TiO$_2$). Furthermore, it was shown that the flow reactor treatment with 1 w/w% AuPd/TiO$_2$ catalyst was a thousand times more effective at killing *E. coli* K12 JM109 than 200 ppm of commercial and batch reactor H$_2$O$_2$ in suspension (4 log$_{10}$ reduction with the flow reactor treatment and ~ 1 log$_{10}$ with the H$_2$O$_2$ in suspension) at the contact time of 1 minute. Moreover, the flow reactor treatment with 1 w/w% AuPd/TiO$_2$ catalyst was extremely effective against MS2 bacteriophages (8 log$_{10}$ reductions in two repeats) while 200 ppm of commercial and batch reactor H$_2$O$_2$ in suspension was ineffective (< 1 log$_{10}$ reduction) at the 1 minute contact time. What is more, there was no activity against *E. coli* K12 JM109 when 5 mM glutathione (OH free radicals quencher) was added to the bacterial suspension, and there was no significant change of activity when catalase (enzyme that breaks down H$_2$O$_2$) was added to the suspension. SEM visualisations showed that commercial and batch reactor H$_2$O$_2$ caused membrane damage, and that the flow reactor treatment completely destroys the bacteria (no identifiable bacterial cells, however, observed after a single experiment only). These findings suggested that H$_2$O$_2$ is most likely not
the only and also not the major part of the mechanism of microbicidal action. It was also found that 200 ppm of commercial, batch reactor and flow reactor H₂O₂ prevented formation of *E. coli* K12JM109 and *B. subtilis* ATCC6633 biofilms.

It has been suggested that the direct synthesis of H₂O₂ on clusters of Pd follows a non-Langmuirian mechanism with first order kinetics with respect to H₂ and zero order kinetics with respect to O₂ at partial pressures close to the ones used throughout this project (Wilson and Flaherty, 2016). Considering this, the surface sites of the catalyst would most likely get saturated by intermediates derived from O₂. This would be attributed to a high heat of adsorption of O₂ on Pd surfaces saturated with water (Ford *et al.*, 2010). H₂O₂ formation is a sequential proton-electron transfer to OOH• and O₂ with the driving thermodynamic force being the chemical potential of H₂ oxidation (Flaherty, 2018).

Findings obtained from the EPR experiments coupled with the microbicidal efficacy data from flow reactions using different catalysts elucidate the mechanisms of microbicidal action further. From the EPR spectrum taken from a reaction in a 5% H₂/N₂ gas mixture H• was observed in a solution. This proves that a homolytic cleavage of H₂ happens on a surface of the catalyst and a free radical diffuses into the solution. Moreover, when H₂O₂ was passed through the catalyst in a 5% H₂/N₂ gas mixture, only H• was detected. This suggests that the cleavage of H₂ does not initiate the production of reactive oxygen species (ROS) from H₂O₂. The radicals in solution during synthesis of H₂O₂ over supported AuPd catalysts have not been previously reported in the literature. It can be argued that activation of H₂ would induce a reaction with O₂ species adsorbed to the catalyst surface when both O₂ and H₂ are available. H• was not detected in solution when a reaction was carried out in a 2% H₂/air gas mixture.
mixture. This is the gas mixture where the highest microbicidal activity was observed, therefore, it can be concluded that H• does not play a direct role in the microbicidal activity. This is further evidenced by a low microbicidal activity against *E. coli* K12 JM109 with a 5% N₂/H₂ gas mixture (1.19 ± 0.42 log₁₀ reduction). The EPR spectra also show a lower amount of OH• produced when commercial or batch reactor H₂O₂ was passed through the reactor in air than when 2% H₂/air was passed through the reactor which correlates with lower microbicidal activity of H₂O₂ than the flow reactor treatment.

EPR experiments showed that ROS are able to desorb from the surface of the catalyst when H₂ and O₂ react and when H₂O₂ passes through the catalyst. This is however not the case with H₂ and H₂O₂. When H₂O₂ is synthesised in the flow reactor, the newly formed H₂O₂ will be enriched with a mix of O-centred free radicals which will be available to attack microorganisms. The free radicals produced during this reaction can be categorised into short range, short lived and highly oxidative OH• and a longer range OOH•/O₂•⁻. It was also observed that when commercial and batch reactor H₂O₂ were passed through the flow reactor, the double integration from the EPR spin trapping suggested a concentration of free radicals in solution which equals only 18-27% of the amount that was measured when O₂ and H₂ were passed through the reactor. When glutathione was passed through the reactor, H• was visible in a 5% H₂/N₂ mixture and no free radicals were seen in 2% H₂/air. This correlated with a lack of microbicidal action of the flow reactor treatment with glutathione further evidences that H• does not have a microbicidal effect and that it is the O-centered free radicals that are mostly responsible for killing the microorganisms.
Considering the above, it is therefore proposed that the difference in the flux of free radicals and hence a significantly increased microbicidal activity of the reaction in a 2% H₂/air gas mixture is dependent on the initiation steps of the free radical flux. In the case when the 2% H₂/air gas mixture is passed through the reactor, the H• in the solution from the H₂ homolytic dissociation initiates a cascade of reactions by turning the O₂ adsorbed to the catalyst surface into OOH• which can either attack the microorganisms on its own or can propagate a radical chain with contribution of the H₂O₂ synthesised in the reactor which can support the flux of free radicals out of the surface of the catalyst. Considering the preformed H₂O₂, the initiation step for the flux of free radicals can only occur from cleaving the O-O bond which is kinetically slower than the O-O bond cleavage in OOH• (Wilson and Flaherty, 2016), therefore, the flux of free radicals is hindered when using the preformed H₂O₂. Hence, the catalyst being more microbicidal in a shorter time period.

The significantly improved microbicidal activity achieved in a catalysed reaction of H₂ and O₂ instead of using commercial H₂O₂ has the potential to enhance water disinfection technologies in medical applications. This project puts forward a novel process in which, apart from the catalyst, contaminated water and the H₂ and O₂ gasses are needed to achieve disinfection. Notably, water disinfection using a flow through catalysis has not been published before. There are many advantages that this system would bring to the water disinfection in medical applications. Crucially, this system shows potential for a fast water disinfection at contact times at which the current microbicidal methods are not effective. Furthermore, this method uses direct synthesis of H₂O₂ which means that there is no formation of disinfection by-products that are hazardous to health which means that it can be incorporated to water disinfection
systems for medical applications safely. Moreover, the H₂O₂ produced in a catalysed flow through reaction inhibits the growth of biofilms which are an important issue in disinfection of waterlines due to their persistence on the surfaces and contamination of water and medical equipment.

The system proposed in this project has potential to be used for disinfection of rinse water in AERs. As mentioned in the Introduction chapter, the rinse water used during reprocessing of endoscopes is not sterile and is currently disinfected using filters (Department of Health, 2016; Joint Working Group of HIS and PHLS, 2002) which need to be disinfected by passing water with a germicide such as ortho-phalaldehyde at 70-80°C (Department of Health, 2016; Williams, 2010). Manufacturers of AERs recommend to change filters periodically which adds costs of maintenance (Funk and Reaven, 2014). One of important issues with disinfection by filtration is that it only physically separates microorganisms and does not kill them. Moreover, a proportion of water passing through the filter will carry microorganisms which can cause contamination downstream. Furthermore, biofouling is a limiting factor of using filtration method for disinfection. If filters are not periodically replaced, the biomass that builds up on the filters causes changes to their hydraulic permeability and selectivity which decreases their effectiveness (Bodzek and Rajca, 2012). Potential incorporation of the catalytic flow through system explored in this project would be beneficial in that the microorganisms passing through the catalyst would be killed and that the water passing through the catalyst would be continually disinfected. Moreover, biofilm formation on the surfaces of the AER pipes would be inhibited and there would be no bacterial build up in the system.
This project contributes to the wider body of research in many ways. Firstly, the free radicals in solution during synthesis of H₂O₂ over supported AuPd catalysts have not been previously reported in the literature. Disinfection of water while directly synthesising H₂O₂ from O₂ and H₂ in a flow through reactor system was not previously reported in the literature. The mechanism of microbicidal action in a flow through reactor was primarily based on free radical production, not on H₂O₂. It was demonstrated that a packed bed reactor design is not effective in terms of reproducibility of microbial kill. The H₂O₂ concentration produced during the flow reaction was sufficient for prevention of biofilm formation.

6.2 Limitations and future experiments

The main limitation of this study was that there were some issues with reproducibility of the experiments. Furthermore, it has been found that a partial blockage of the flow down the catalyst bed increased the microbicidal activity, however, attempts at artificially introducing the partial blockage to the catalyst bed by making dents in the tubes did not provide a reproducible effect.

Packed bed reactor set up was used throughout the project. The catalyst was packed into the catalyst bed with glass wool at the bottom for support. The advantages of such approach are easy quantification and characterisation of the catalyst, high catalyst loading compared with other methods, a large range of catalyst supports can be used and the catalytic device can be easily fabricated filling the channels with functional catalytic particles. This approach has its disadvantages such as fluid dynamics being
uncontrolled, significant pressure drops developing along the microchannel (which happened during this project) and limited heat transfer (Munirathinam et al., 2015). Therefore, exploring different designs for the catalyst bed would be an important topic of future experiments. Apart from the packed bed reactors, there are other ways in which catalysis can be performed in a continuous-flow microreactor such as monolithic and wall-coated microreactors. Figure 6.1 shows schematic representations of microchannel cross sections using different approaches (Munirathinam et al., 2015).

Monolithic microreactors are usually made of inorganic (Walsh et al., 2012) or polymeric (Viklund et al., 1996) material that are filled with interconnected large flow-through pores and micro- or mesopores (Munirathinam et al., 2015). Monolithic materials do not have interstitial spacing and are porous which provides them with some advantages over packed bed reactors such as achieving higher back pressures due to efficient mass transfer through the pores and a better tolerance to high rates of flow (Sachse et al., 2011; Kirschning et al., 2006). Catalysts are immobilised on the monolithic materials which can then be used to perform catalytic flow reactions (Anderson and Buchmeiser, 2011). This approach, however, has some disadvantages such as that the sites of the catalyst that are buried deep inside the monolithic micropores have reduced accessibility, non-uniformity of radial permeability and pore clogging (Munirathinam et al., 2015).

Wall-coated microreactors minimise the mass transfer resistance substantially and provides a smooth reagents inflow that do not cause a blockage of the microchannels or adverse pressure drops (Munirathinam et al., 2015). Furthermore, prediction of mass and heat transfer properties and fluid dynamics in the reactor are enabled by
well-defined flow geometries (Lopes et al., 2013). The disadvantage of this approach is that, generally, the catalyst loading is lower in comparison to the packed bed and monolithic approaches. This is because in the wall-coated microreactor there is a thin film of the catalyst supported on the reactor’s inner walls (Munirathinam et al., 2015). Methods have been developed in order to increase loading of the catalysts such as inorganic porous material deposition on the surface of the microchannel or tethering of polymer brushes (Munirathinam et al., 2015; Kieviet et al., 2014).
Another interesting avenue to explore is producing H₂ from electrolysis of water to feed this gas into the flow reactor system which would be an eco-friendly alternative (Kumar and Himabindu, 2019). Water electrolysis is an electrochemical process in which water molecules split into O₂ and H₂ gases. This technique uses DC electricity to produce energy without pollution emission. Splitting of water is low (approximately $10^{-7}$ moles/litre) in room temperature. This is because water is a poor electricity
conductor. Sodium hydroxide (NaOH), potassium hydroxide (KOH) and sulphuric acid are mainly used with water in order to improve conductivity. The solution splits into negative and positive ions which readily conduct electricity in water by flowing from one electrode to another. The general principle for how electrolysis cells work is that when voltage is applied in the presence of water, $O_2$ is generated at the anode and $H_2$ is generated at the cathode (Rashid et al., 2015). The voltage of water thermodynamic decomposition is 1.23 V, however, the reaction starts to happen at 1.65-1.7 V. Mostly, the electrolysis cells work at 1.8-2.6 V (Nikolic et al., 2010).

Alkaline water electrolysis, Polymer Electrolysis Membrane (PEM) and high temperature water electrolysis using solid oxide electrolysis are the most commonly researched methods of $H_2$ production using water electrolysis (Rashid et al., 2015). However, high temperature water electrolysis requires temperature of 700-900°C (Ferrero et al., 2013), therefore, it would not be feasible for this system. Figure 6.2 shows a schematic of each of the aforementioned methods of water electrolysis (Schmidt et al., 2017).

Alkaline electrolysis cells (AEC) is an established and generally used technology for applications of large scale since 1920s (Zhang et al., 2015). AEC are durable and easily available and relatively cheap to make as there is no need to use noble metals and its stack components are relatively mature (Schmidt et al., 2017). Even though this technology is mostly used on industrial scale (Schmidt et al., 2017), it has been used on a small laboratory scale for research purposes (Ju et al., 2018). Alkaline electrolyser decomposes water to $OH^-$ and $H_2$ at a cathode. The $OH^-$ then moves through the electrolyte and a separating membrane/diaphragm and then is released at the anode and $O_2$ is generated and liberated (Rashid et al., 2015).
PEM works on the concept of solid polymer electrolyte (SPE) for the electrolysis of water and was introduced by General Electric in 1960s in order to improve on the AEC systems (Zhang et al., 2015). PEM technology is mostly used in small scale applications (Schmidt et al., 2017). The most important advantages of PEM are that this system has a small size and mass, allows flexible operation and easy maintenance, lower consumption of power, high conductivity of protons, low gas crossover and that highly compressed and pure H₂ is generated (Carmo et al., 2013; Grigoriev et al., 2006). The disadvantages of this system are that it requires expensive components such as fluorinated membrane and a Pt catalyst, it requires high water purity and pressures, and it has shorter life time in comparison with AEC (Carmo et al., 2013). PEM uses acidic solid polymer instead of liquid electrolyte, and deionised water is used without electrolytic additives such as KOH. The PEM membrane works as both the electrolyte and gas separator. The main component of PEM cell is a membrane electrode assembly which combines cathode, anode, membrane and an electrocatalyst (Rashid et al., 2015).
Figure 6.2 A schematic representation of the most commonly researched methods of using water electrolysis for H$_2$ production. AEC: Alkaline Electrolysis Cells; PEMEC: Proton Exchange Membrane Electrolysis Cells; SOEC: Solid Oxide Electrolysis Cells (from Schmidt et al., 2017).

A small variety of microorganisms tested during this project is one of the limitations of this study. Further microbiological testing of this system would also constitute an important part of the future experiments. Waterborne organisms such as mycobacteria, *Pseudomonas* and *Legionella* cause significant mortality and morbidity especially in patients that are immunocompromised (Decker and Palmore, 2014). Other Gram-negative bacteria that are common causes of waterborne infections are *Acinetobacter*, *Stenotrophomonas*, *Burkholderia*, *Sphingomonas*, *Achromobacter*, *Chryseomonas* and *Klebsiella*. Furthermore, fungal spores of organisms found in water such as *Fusarium*, *Phialemonium*, and *Aspergillus* have been found to cause nosocomial infections. Waterborne protozoan organisms such as *Cryptosporidium* was found to cause infections in immunocompromised people (Decker and Palmore, 2013). Testing of the flow reactor microbicidal activity against these organisms would provide
another strong indication for its viability to be used in AER for disinfection of water in these systems.

This technology has an innovative mechanism of action and showed a high microbicidal activity, however, further studies on its optimisation are necessary for its incorporation into AER. During this project, 6 mL of water was disinfected in 30 minutes at the standard reaction conditions. In a normal AER cycle, 14 L of water needs to be disinfected in 30 minutes. Therefore, 2,335 times faster rate of water disinfection is required in order to disinfect all of the rinse water in a cycle. That would require many changes in the design of the catalytic reactor such as significantly increasing the size of the currently 0.125 inch diameter tubes. AERs use peristaltic pumps, hence, the catalytic reactor would have to be supplied with water by peristaltic pumps at a much higher flow rate (467 ml/min in order to achieve 14L in 30 min). As mentioned earlier, a packed bed reactor design was not effective in terms of reproducibility. It would also not be viable at such high liquid flow rates, therefore, a different reactor bed design would be necessary. Furthermore, the reactor bed would have to be much larger and contain more catalyst. As demonstrated, the flow reaction at 20°C caused a very similar log₁₀ reduction of E.coli as a flow reaction in a standard 2°C temperature, therefore, this should be further explored in the new optimised design.
7 References


BSI (2010) BS EN 1276:2009 Chemical disinfectants and antiseptics. Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas. Test method and requirements (phase 2, step 1). CEN.

BSI (2010) BS EN 1276:2009 Chemical disinfectants and antiseptics. Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas. Test method and requirements (phase 2, step 1). CEN.


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Appendix 1: ASM Microbe 2019 poster

Catalytic Production of Hydrogen Peroxide for in situ Disinfection in Medical Applications

**Introduction & Aims**
- There have been numerous outbreaks and pseudo-outbreaks associated with poorly disinfected endoscopes. Drop in water poses an important problem during endoscopy, reprocessing notably with recontamination of endoscopes. This project aims to explore the development of an integrated system that would use a novel catalytic technology to produce hydrogen peroxide ($\text{H}_2\text{O}_2$) to control and eradicate microbial contamination in rinse water at an automated endoscope washer disinfectant.

**Catalytic Reactor Design**
- 1 wt. % Au/Pd catalyst was prepared using a modified impregnation method (Fig. 3).
- The catalyst bed uses Au-Pd bimetallic nanoparticles catalyst that produces $\text{H}_2\text{O}_2$ continuously at 1000 ppm in water under 10 bar of 95% hydrogen/air (Fig. 7).

**Prevention of biofilm adhesion to surface**
- Prevention of E. coli K12 JM109 attachment to PVC surface by $\text{H}_2\text{O}_2$ in 96 well plate measured by crystal violet assay (based on 1).
- Plates were inoculated in a shaking incubator at 120 rpm, 37°C for 6 hours.
- $\text{H}_2\text{O}_2$ did not decompose significantly for the duration of the test.
- 200 and 100 ppm of $\text{H}_2\text{O}_2$ caused an 96-100% and 27-54% reduction in attachment of E. coli K12 JM109 to surface compared to untreated control (Fig. 4).
- 50 ppm of $\text{H}_2\text{O}_2$ treatments caused a 7-9% increase in bacterial attachment compared to untreated control (Fig. 4).

**Key findings**
- 1 wt. % Au-Pd catalyst prepared using a modified impregnation method can produce 100-200 ppm of $\text{H}_2\text{O}_2$.
- 200 ppm of $\text{H}_2\text{O}_2$ synthesized by the catalytic reactor prevented bacterial attachment to PVC surface.
- Low concentrations of $\text{H}_2\text{O}_2$, produced by catalysis, produces a severe bactericidal effect linked to massive structural damages. Similar damages have been observed with other oxidizing agents albeit at higher concentrations.
- $\text{H}_2\text{O}_2$ is usually used at 75,000 ppm in sterilization applications.

**References**

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Appendix 2: Science Polish Perspectives 2018 poster

Catalytic Production of Hydrogen Peroxide for in situ Disinfection in Medical Applications

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Introduction and aims
- There have been numerous outbreaks and pseudo-outbreaks associated with poorly disinfected endoscopes. [1]
- Recent outbreaks have been linked to poorly disinfected endoscopes, notably with R. norvegicus endoscopes. [2]
- This project aims to explore the development of an integrated system that would use a novel catalytic technology to produce hydrogen peroxide (H₂O₂) in situ to control and eliminate microbial contamination in water in automated endoscope washer disinfectors.

Catalytic Reactor design
- 1 wt. % Au/Fe₂O₃ catalyst was prepared using a modified impregnation method (Fig. 1). [3]
- The catalyst bed uses Au-Pd bimetallic nanoparticles catalyst that produces H₂O₂ continuously at 100-200 ppm in water under 1 bar of 2% H₂O₂ solution (Fig. 2).

Suspension tests
- Use of a suspension test based on EN1276 to determine bactericidal efficacy of commercial formulation of H₂O₂ against Staphylococcus aureus NCTC 10886, Enterococcus hirae NCTC 13685, Enterococcus faecalis NCTC 10421 and Pseudomonas aeruginosa NCTC 13359.
- A 1 mL 100mL 0.9% NaCl, CaCl₂, NaHCO₃ and 1.0% (NaCl) water solution was used with 100, 200, 1000 and 10000 ppm concentration of commercial formulated H₂O₂.

Fig. 1. Catalytic reactor set up.

Fig. 2. Catalytic reactor set up.

Fig. 3. Catalytic reactor set up.

Fig. 4. Prevention of biofilm adhesion by synthesized H₂O₂ (m=5)

SEM imaging
- E. coli K12 IM100 samples treated with 200 ppm of commercial stabilized and synthesized H₂O₂, fixed in glutaraldehyde and dehydrated in ascending ethanolic concentration and HMH, dried on SEM, examined with Hitachi TM3000Plus SEM.
- Clear cell wall damage can be observed in 200ppm H₂O₂ regardless of the treatment (Fig. 5). Both treatments caused a 1 log₁₀ reduction.

Fig. 5. SEM images of E. coli K12 IM100 a) untreated, b) treated with 200ppm of commercial stabilized H₂O₂ and c) treated with 200ppm of synthesized H₂O₂.

Conclusions
- 1 wt. % Au/Fe₂O₃ catalyst prepared using a modified impregnation method can produce 100-300ppm of H₂O₂.
- 200 ppm of H₂O₂ synthesized by the catalytic reactor prevented bacterial attachment to PVC surface.
- Low concentrations of H₂O₂ produced by catalysis produces a severe bacterial effect linked to mass-scale structural damages and can prevent the attachment of bacteria to surfaces. Similar damages have been observed with other oxidizing agents albeit at higher concentrations. [4]
- H₂O₂ is usually used at 75,000 ppm in sterilization applications. [5]

Fig. 6. Prevention of biofilm adhesion by synthesized H₂O₂ (m=5)

References
Appendix 3: Cardiff Catalysis Institute conference 2018 poster

Cardiff University School of Pharmacy and Pharmaceutical Sciences and Cardiff Catalysis Institute

Catalytic Production of Hydrogen Peroxide for *in situ* Disinfection in Medical Applications

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Introduction and aims

- There have been numerous setbacks and pseudo-achievements associated with poorly distributed endemics.
- Inadequate water quality is an important problem during endemics, representing a vital challenge to our understanding of the endemics and potential contamination of water samples.
- This project aims to explore the development of an integrated system that would use a novel catalytic technology to produce hydrogen peroxide (H2O2) to control and eliminate microorganisms in water using solar energy under endemics water distribution.

Catalytic Reactor design

- 2 ml Nafion®-H2O2 solute is prepared using a modified impregnation method (Fig. 2).
- The catalyst bed uses a fluid biocompatible nitrosites catalyst that produces H2O2 continuously at 100-200 ppm in water under 10 bar of 2% hydrogen peroxide (Fig. 1).

Reproducibility

- A scatter plot of the test rig experiments shows that there are reproducibility issues (Fig. 0).
- Blanks of the catalyst bed resulted around 5-log higher reductions compared to the standard reactions.

Catalytic reactor bactericidal activity with artificially introduced blockages

- Dyes were introduced into the catalyst beds in order to artificially create a partial blockage in the catalyst bed (Fig. 0).
- This was done to determine whether the bacteria could be effectively removed in the reactor.

Figure 1: Scatter plot of the test rig experiments with different microorganisms.

Catalytic reactor bactericidal activity with artificially introduced blockages

Conclusions

- There are some benefits in microbial efficacy such as the catalytic reactor in suspension. However, the catalytic reactor caused a similar reduction in pH in both sets (Fig. 4).
- Differences in the catalytic rate consistently increase microbial activity, providing a means to increase microbial efficacy.
- Better understanding of the hydrodynamics of the reactor and improvement of catalyst bed design is necessary to gain reproducibility in microbial activity.
- A more robust design of the catalyst bed will provide a strong foundation for future scaling up of the process.

References