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

A thesis submitted to Cardiff University for the degree of

Doctor of Philosophy

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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_2O_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_2O_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_2O_2 in suspension was ineffective (< 1 $\log_{10}$ reduction). Furthermore, 200 ppm of the flow reactor H_2O_2 prevented formation of *E. coli* K12 JM109 and *B. subtilis* ATCC6633 biofilms.

H_2O_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

H_2O_2 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.

Contents

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Abbreviations

S. aureus – Staphylococcus aureus

E. hirae – Enterococcus hirae

E. coli – Escherichia coli

P. aeruginosa – Pseudomonas aeruginosa

B. subtilis – Bacillus subtilis

S. maltophilia – Stenotrophomonas maltophilia

P. putida – Pseudomonas putida

A. fumigatus – Aspergillus fumigatus

M. tuberculosis – Mycobacterium tuberculosis

G. stearothermophilus - Geobacillus stearothermophilus

D. radiodurans - Deinococcus radiodurans

GI – gastrointestinal

AER – automated endoscope reprocessor

H_2O_2 – hydrogen peroxide

MIC – minimum inhibitory concentration

TSA – tryptone soya agar

TSB – tryptone soya broth

TSC – tryptone sodium chloride

BSA – bovine serum albumin

cfu/mL – colony forming units/mL

pfu/mL – phage forming units/mL

TiO_2 - titanium dioxide

Au – gold

Pd – palladium

$NaHCO_3$ – sodium bicarbonate

MgCl – magnesium chloride

$CaCl_2$ – calcium chloride

$PdCl_2$ - 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_2O_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)

Body system	Name of endoscopy	What is visualized/accessed
Gastrointestinal (GI) tract	Oesophagogastroduodenoscopy (OGD)	Oesophagus, stomach and duodenum
	Enteroscopy	Small intestine
	Colonoscopy	Large intestine (and terminal ileum)
	Sigmoidoscopy	Sigmoid colon, rectum
	Endoscopic UltraSound (EUS)	Upper GI tract and biliary tree
Biliary tree	Endoscopic retrograde cholangiopancreatography (ERCP)	Pancreas, common bile duct, hepatic ducts and gallbladder
	Choledocoscopy	Intraoperative access to bile ducts
	Duodenoscope-assisted cholangiopancreatoscopy	
Respiratory tract	Bronchoscopy/endobronchial ultrasound	Trachea, large and small bronchi
Ear, nose and throat	Rhinoscopy	Nose
	Laryngoscopy	Throat
Urological	Cystoscopy	Bladder, urethra
	Uretoscopy	Ureters
Gynaecological	Hysteroscopy	Uterus
	Falloscopy	Fallopian tubes

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).

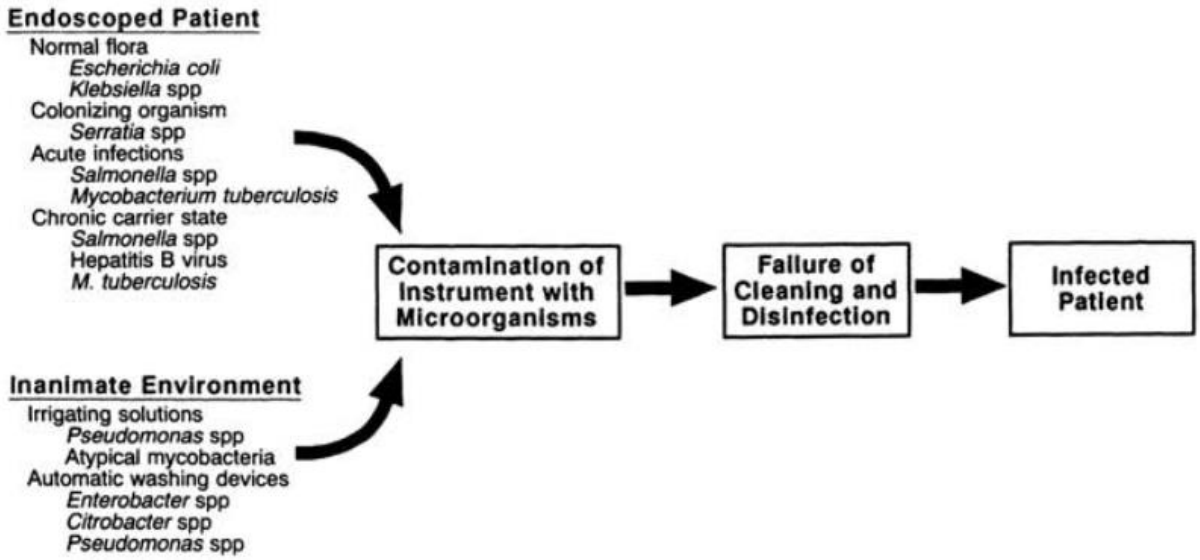
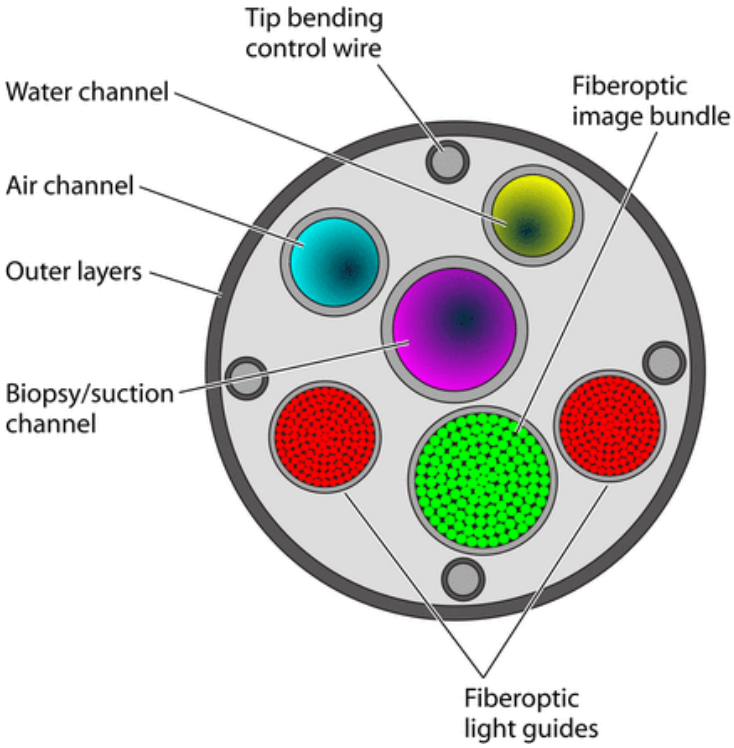


Figure 1.2 A) A schematic of a complex internal design of an endoscope (from Kovaleva et al., 2013). **B)** A photograph of an endoscope with a surgical instrument (from Khatait et al., 2013).

A)



B)

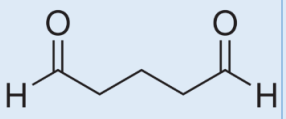
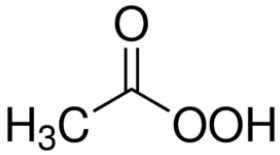
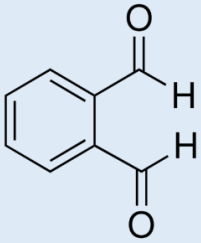
1.2 Disinfection of endoscopes

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).

High-level disinfectant	Advantages	Disadvantages
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 chelonae* 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).

Reference	Publication year	Microorganism	Outbreak or pseudo-outbreak?	Isolates	Infections	Deaths	Source of contamination
Cosgrove et al[10]	2012	*Pseudomonas* sp., *Stenotrophomonas*	Pseudo-outbreak	16	0	0	Irregularities in repair by third-party vendor, nonstandard part replacements
Rosengarten et al[11]	2010	*Burkholderia cepacia*	Pseudo-outbreak	3	0	0	Missing antibacterial filter on washer disinfector
CDC[12]	2009	*Legionella pneumophilia*	Pseudo-outbreak	4	0	0	Nonsterile ice used to cool saline filler syringes for bronchoalveolar lavage
Schuetz et al[13]	2009	*L. pneumophilia*	Pseudo-outbreak	13	0	0	Immersion of uncapped saline-filled syringes in contaminated ice
Chroneou et al[14]	2009	*Mycobacterium chelonae*	Pseudo-outbreak	9	0	0	Contamination of an AER
DiazGranados et al[15]	2009	*Pseudomonas aeruginosa*	Both	12	2	0	Damaged bronchoscope
Schaffer et al[16]	2008	*Fursarium solani*	Pseudo-outbreak	4	0	0	Bronchoscope
Shimono et al[17]	2008	*P. aeruginosa*	Outbreak	7	7	0	Flaw in AER, failure to properly clean and disinfect bronchoscopes
Ahn et al[18]	2007	*Stenotrophomonas maltophilia*	Pseudo-outbreak	7	0	0	Failure to properly clean and disinfect bronchoscopes
Bou et al[19]	2006	*P. aeruginosa*	Outbreak	10	10	0	Failure to properly clean and disinfect bronchoscopes
Corne et al[20]	2005	*P. aeruginosa*	Both	16	4	0	Damaged internal channel caused by defective biopsy forceps
Cêtre et al[21]	2005	Enteric GNR	Both	117	2	0	Bronchoscope: loose port of the biopsy channel
Larson et al[22]	2003	*Mycobacterium tuberculosis*	Pseudo-outbreak	3	1	0	Failure to properly clean bronchoscopes, use of an AER not approved for the type of bronchoscope
Singh et al[23]	2003	*Trichosporon mucoides*	Pseudo-outbreak	6	0	0	Defective bronchoscopes
Silva et al[24]	2003	*P. aeruginosa, Serratia marcescens*	Pseudo-outbreak	41	0	0	Failure to properly clean bronchoscopes
Srinivasan et al[25]	2003	*P. aeruginosa*	Outbreak	97	48	3?	Defective bronchoscopes: loosened biopsy port
Kirschke et al[26]	2003	*P. aeruginosa*	Both	20	1	0	Defective bronchoscopes: loosened biopsy port
Ramsey et al[27]	2002	*M. tuberculosis*	Pseudo-outbreak	10	4	0	Damaged bronchoscope; no leak testing; hole in bronchoscope sheath
Rossetti et al[28]	2002	*Mycobacterium gordonae*	Pseudo-outbreak	16	0	0	AER: failure to replace antibacterial filters, maintenance
Kressel and Kidd[29]	2001	*M. chelonae, Methylobacterium mesophilicum*	Pseudo-outbreak	20	0	0	AER contaminated with biofilm resistant to decontamination
Sorin et al[30]	2001	*P. aeruginosa*	Both	18	3	1	AER: inappropriate channel connectors
Kramer et al[31]	2001	*P. aeruginosa*					AER: disinfectant (0.04% glutaraldehyde) contaminated because of inadequate concentration (concentration mistakenly set too low)
Wilson et al[32]	2000	*Aureobasidium* sp.	Pseudo-outbreak	10	0	0	Reuse of single-use stopcocks
Gillespie et al[33]	2000	*M. chelonae*	Pseudo-outbreak	2	0	0	Contaminated water in AER
Schelenz and French[34]	2000	*P. aeruginosa*	Unknown	8	0	0	AER

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).

Disinfection step	Reason for disinfection step	Mechanism for failure
Cleaning	Remove bioburden Remove substances that might interfere with disinfection: blood, salt, protein	Inadequate policies; 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
Appropriate disinfectant	Inactivation of contaminating microbes	Ineffective disinfectant (eg, iodides); inadequate concentration; inadequate duration; inadequate temperature
Contact between disinfectant and contaminating microbes	Requirement for killing	AER: failure to use channel connectors; AER: wrong channel connectors; occluded lumen; torn or damaged lumen
Rinse	Remove potentially toxic chemicals (eg, glutaraldehyde, hydrogen peroxide)	Mucous membrane damage to subsequent patient (eg, colitis); contaminated rinse water
Prevention of recontamination	Prevent contamination with environmental microbes	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)

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 (H_2O_2) 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 (TiO_2) support and it was tested in a flow reactor system.

The hypothesis for this project is that microorganisms get killed by H_2O_2 and free radicals produced using a catalyst when passing through the reactor.

The objectives are:

1. Investigation of the microbicidal efficacy of the H_2O_2 and free radicals produced using a catalyst and its limitations against vegetative bacteria, biofilms and bacteriophages.
2. Understanding the biofilm prevention by H_2O_2 produced using a catalyst.
3. Investigating the catalyst performance in terms of H_2O_2 and radical species production.
4. Understanding the microbicidal mechanisms of action of the H_2O_2 and free radicals produced using a catalyst.

2 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_2O_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_2O_2 as a disinfectant in 1891 (Richardson, 1891). H_2O_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_2O_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_2O_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_2O_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_2O_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_2O_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_2O_2 the number of available DNA-associated irons limits the reaction between DNA and H_2O_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_2O_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.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\bullet} + H^{+}$$

$$2\ H_2O_2 \rightarrow OH^{\bullet} + OOH^{\bullet} + H_2O$$

It has been shown that the amino acids that are oxidised by H_2O_2 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 H_2O_2 were enolase, EF-G, alcohol dehydrogenase E, outer membrane protein A and dnaK (Tamarit *et al*., 1998). Oxidative damage caused by H_2O_2 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 H_2O_2, is that these mechanisms are studied in isolation. Even though we know that H_2O_2 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 H_2O_2 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).

H_2O_2 has been studied and used in a variety of formulations. One of such formulations is H_2O_2 with peracetic acid which work synergistically to kill bacterial spores. In this formulation, the H_2O_2 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 H_2O_2 with <2.5% w/v 2-Furoic acid])

containing H_2O_2, 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 H_2O_2. Sensitivity of biofilms towards the different formulations containing H_2O_2 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 H_2O_2 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 H_2O_2) and unstabilised synthesised batch reactor H_2O_2 (batch reactor H_2O_2) against bacteria and MS2 bacteriophages.

The objectives are:

1. Carry out suspension tests on bacteria and MS2 bacteriophages using commercial H_2O_2.
2. Carry out suspension tests on bacteria and MS2 bacteriophages using batch reactor H_2O_2.

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^{o}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^{o}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 $CaCl_2$, transferring it onto TSA agar plates and incubating them at 37^{o}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^{o}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^{o}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 37ºC 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_2O_2 used were: 100, 200, 1,000 and 10,000 ppm. H_2O_2 solutions were titrated periodically to check the concentrations of the H_2O_2 solutions. Commercial H_2O_2 was supplied by Acros Organics. Such low concentrations of H_2O_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_2O_2.

Reaction conditions for the hard water with organic load experiments were 0.3 g/100 mL Bovine Serum Albumin (BSA), H_2O_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 (20ºC). 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_2O_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_2O_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_2O_2 in hard water with organic load on *S. aureus* NCTC 10788 and *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 *et al.*, 2014). The neutraliser used for subsequent suspension tests consisted of 20 g/L sodium thiosulphate and 500 U/mL catalase (Leggett *et al*., 2016). This was because the previous neutraliser did not work for the Gram-negative bacteria tested i.e. *E. coli* NCTC 10418 and *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 *et al.*, 2013; Herigstad *et al.*, 2001) in duplicates and incubated overnight at 37ºC, (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_2O_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 (10^8 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 $MgCl_2$, $CaCl_2$, $NaHCO_3$) (adjusting the initial concentration of bacteria for the test to 10^7 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 1mL of the highest concentration of H_2O_2 used in the experiment (i.e. 100,000 ppm of H_2O_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_2O_2 concentration to 10,000 ppm) followed by a 5 min incubation. The negative control was 8 mL of 10,000 ppm H_2O_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^{o}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^{o}C, (n=3).

2.2.3 H_2O_2 synthesis and titration

A stainless steel Parr autoclave batch reactor was used to synthesise H_2O_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_2O_2 was filtered into a glass vial using a filter paper. After the H_2O_2 was filtered, it was titrated to determine its concentration.

The H_2O_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_2O_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 H_2O_2

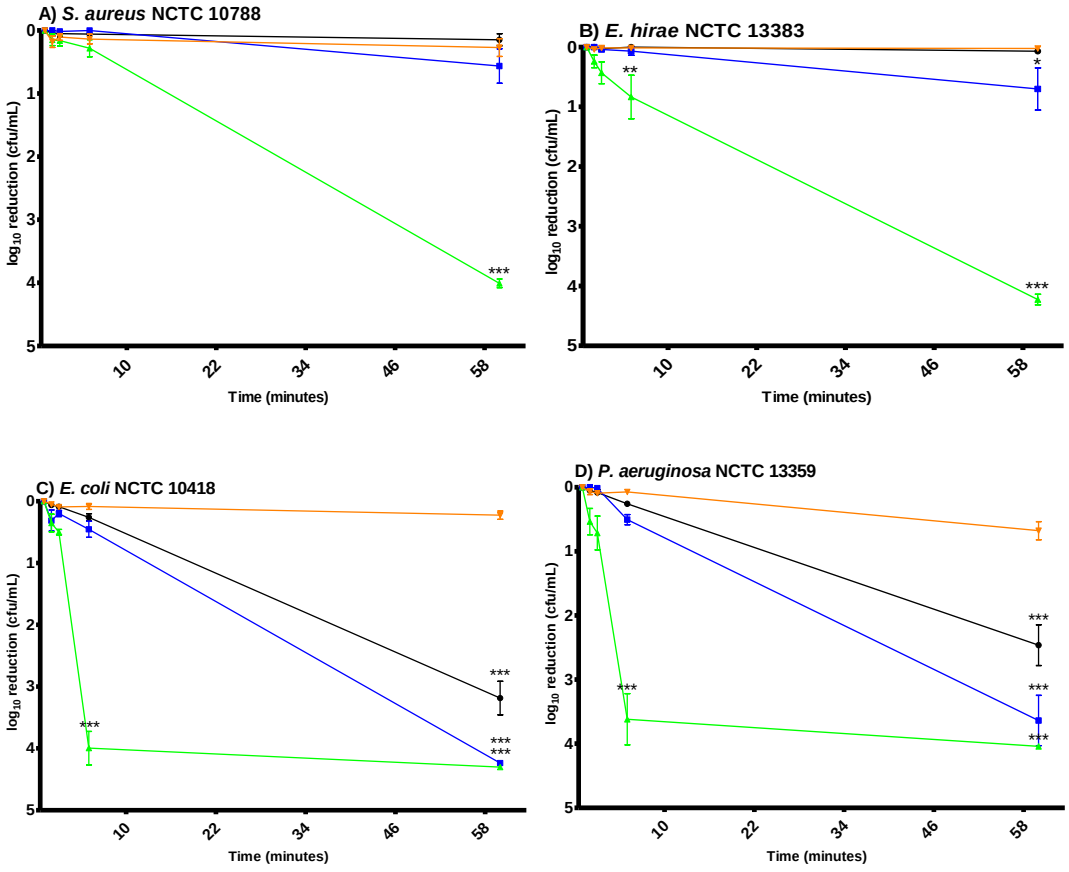
Suspension tests with commercial H_2O_2 were carried out to test its efficacy in terms of log_{10} reductions in cfu/mL against *S. aureus* NCTC10788, *E. hirae* NCTC13383, *E. coli* NCTC10418 and *P. aeruginosa* NCTC13359 in hard water with organic load and clean water conditions.

2.3.1.1.1 Commercial H_2O_2 suspension tests in hard water with organic load conditions

H_2O_2 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 H_2O_2 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 H_2O_2 with inactivation starting to be highly significant (repeated measures ANOVA, $P< 0.001$) at concentrations as low as 200 ppm. Furthermore, a > 4 ± 0.05 log_{10} reduction was observed against *E. coli* at 1,000 ppm after 60 min reaction time and a 3.64 ± 0.68 log_{10} reduction were observed in those conditions

against *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 *E. coli* and a 3.62 ± 0.69 $\log_{10}$ reduction in *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 *E. coli* and a 3.62 ± 0.69 $\log_{10}$ reduction in *P. aeruginosa* which were both significant (repeated measures ANOVA, $P< 0.001$).

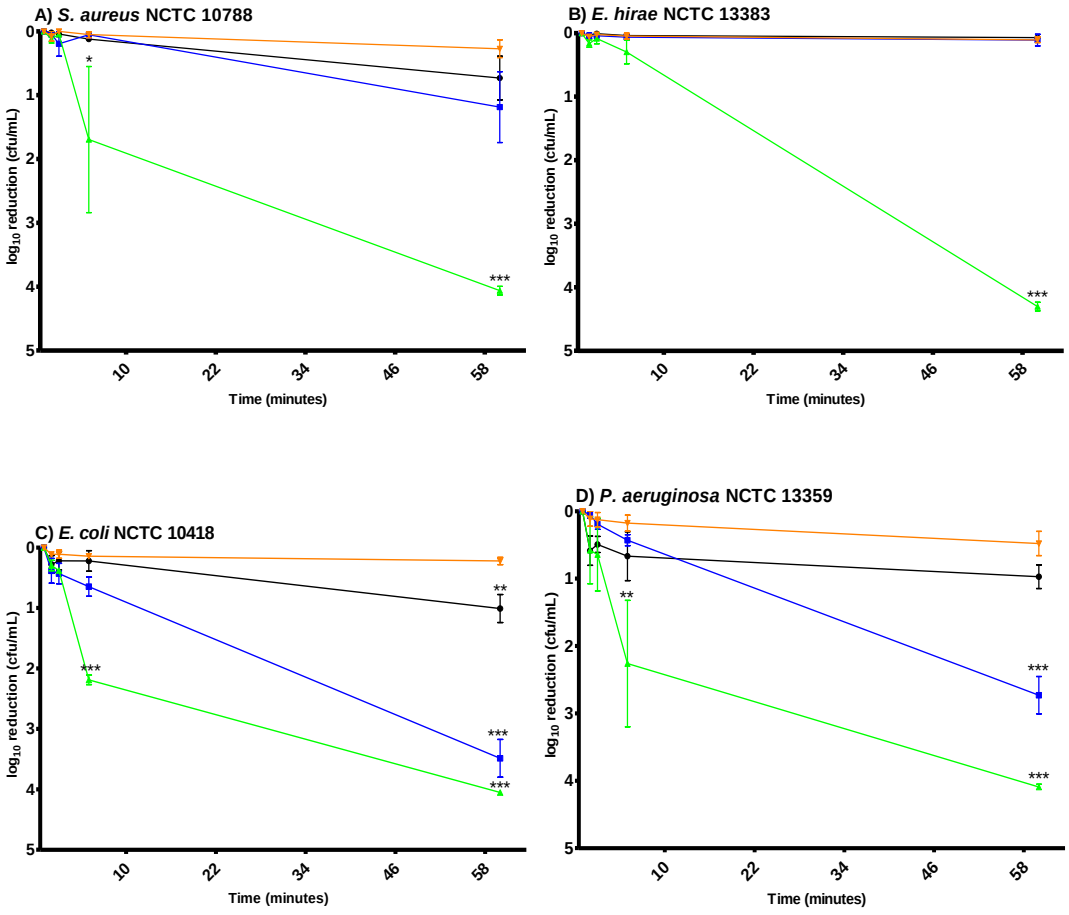
Figure 2.2 Log_{10} reduction results from commercial H_2O_2 in hard water with organic load conditions. Suspension tests were performed with 100, 200, 1,000 and 10,000 ppm commercial H_2O_2 on A) *S. aureus* NCTC10788, B) *E. hirae* NCTC13383, C) *E. coli* NCTC10418 and D) *P. aeruginosa* NCTC13359 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_2O_2, 200 ppm H_2O_2, 1,000 ppm H_2O_2 and 10,000 ppm H_2O_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 H_2O_2 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 $\log_{10}$ reduction of 1.19 ± 0.96 for *S. aureus*. However, the differences between *E. hirae* and *S. aureus* were within 1 $\log_{10}$ difference in majority of cases. Likewise with hard water with organic load conditions, the 10,000 ppm concentration of commercial H_2O_2 caused a highly significant difference (repeated measures ANOVA, $P< 0.001$) in $\log_{10}$ reductions (over 4 $\log_{10}$) 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 $\log_{10}$ 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 $\log_{10}$ 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 $\log_{10}$ reduction for both Gram negatives at a 60 min time point and at 10,000 ppm concentration of commercial H_2O_2 in clean water (SD = 0.05 for *E. coli* and SD = 0.07 for *P aeruginosa*).

Figure 2.3 Log_{10} reduction results from commercial H_2O_2 in clean water conditions. Suspension tests were carried out with 100, 200, 1,000 and 10,000 ppm commercial H_2O_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_2O_2, 200 ppm H_2O_2, 1,000 ppm H_2O_2 and 10,000 ppm H_2O_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)



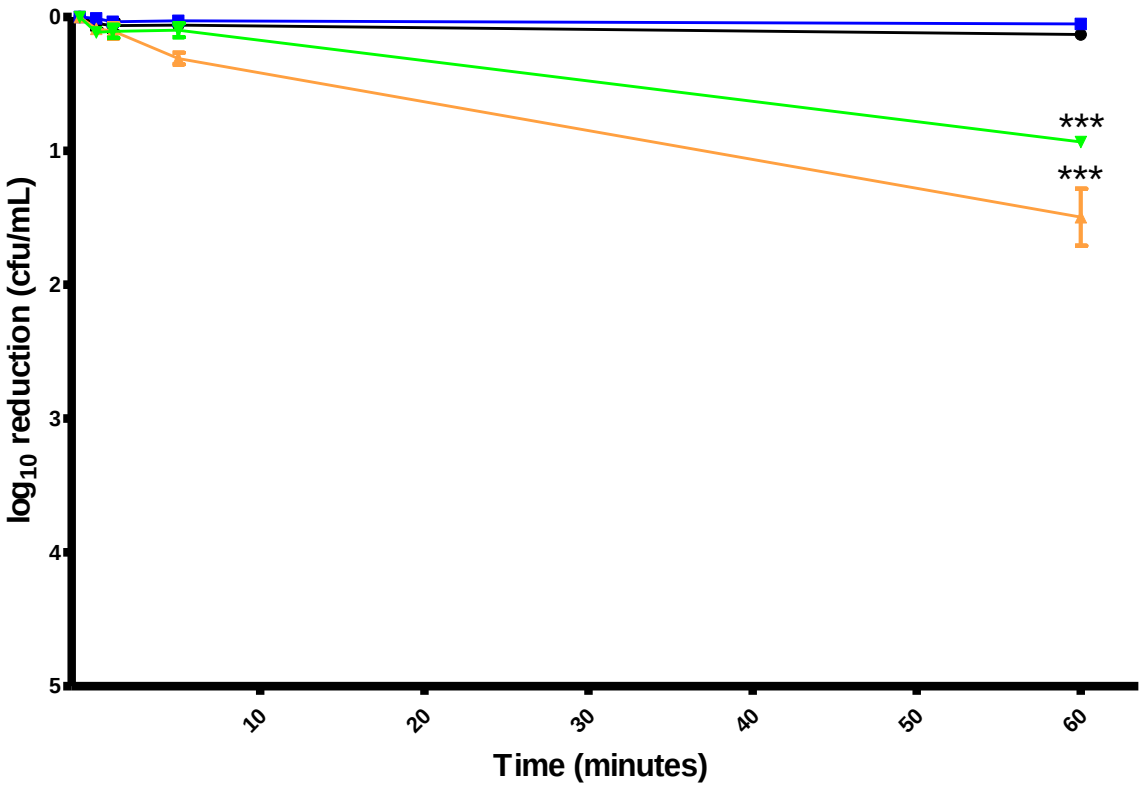
2.3.1.2 Suspension tests using batch reactor H_2O_2

Suspension tests were carried out using 200 ppm of batch reactor H_2O_2 on the same bacteria and conditions described in Section 2.3.1.1.

2.3.1.2.1 Batch reactor H_2O_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.

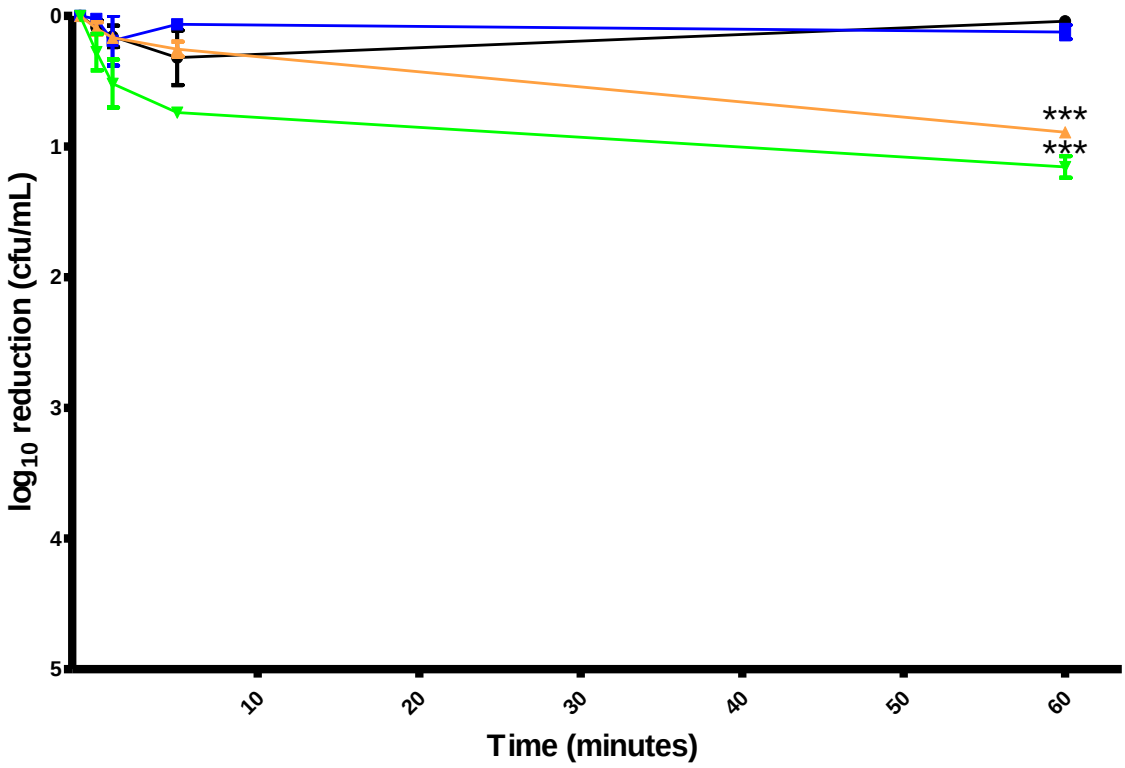
Figure 2.4 Log_{10} reduction results from batch reactor H_2O_2 in hard water with organic load conditions. Suspension tests were performed with 200 ppm batch reactor H_2O_2 on *S. aureus* NCTC10788, *E. hirae* NCTC13383, *E. coli* NCTC10418 and *P. aeruginosa* NCTC13359 all at room temperature. Data 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 H_2O_2 suspension tests in clean water conditions

Figure 2.5 shows the results of the tests with batch reactor H_2O_2 in clean water conditions. The $\log_{10}$ 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 Log_{10} reduction results from batch reactor H_2O_2 in clean water conditions. Suspension tests were performed with 200 ppm batch reactor H_2O_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)

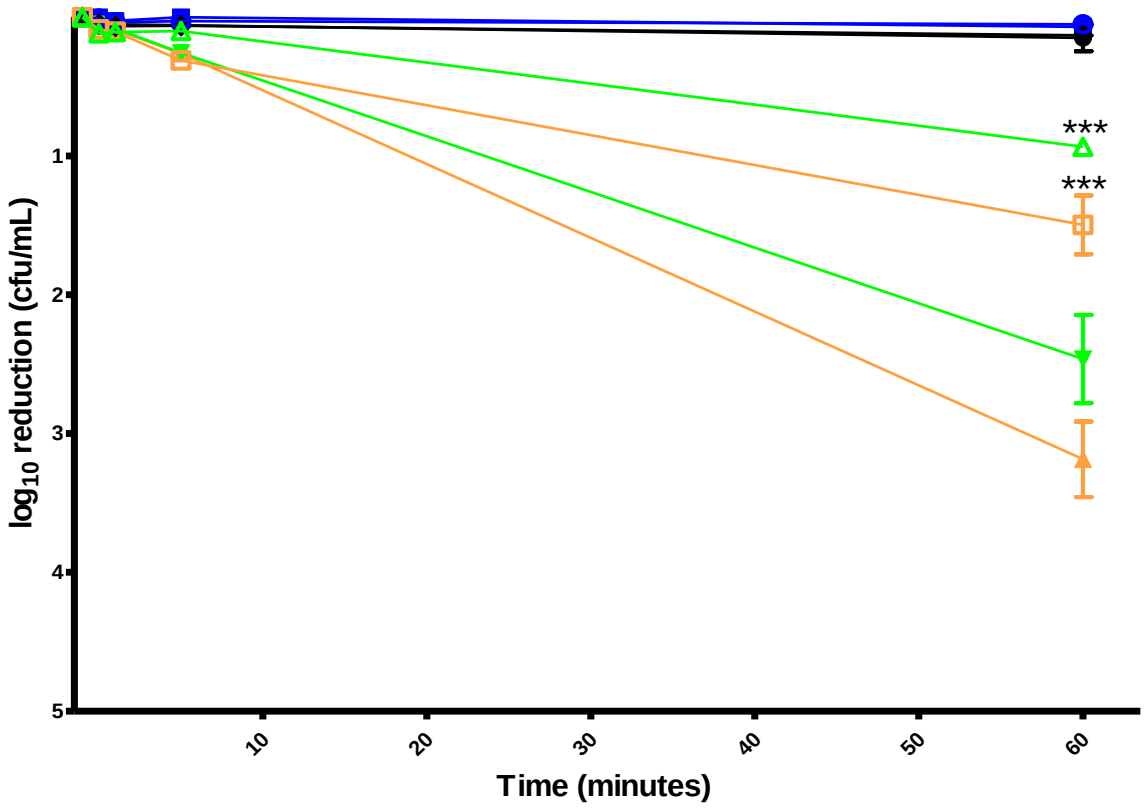


2.3.1.3 Comparison of suspension tests using commercial and batch reactor H_2O_2

2.3.1.3.1 Comparison of commercial and batch reactor H_2O_2 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 H_2O_2 at 200 ppm (repeated measures ANOVA, $P > 0.05$) for both commercial and batch reactor H_2O_2 experiments at every time point. Log_{10} reductions of Gram-negative bacteria were significantly higher ($P< 0.001$) when exposed to commercial H_2O_2 than the batch reactor H_2O_2 after 60 min contact time. The reductions for *E. coli* in commercial and batch reactor H_2O_2 were 3.19 ± 0.47 and 1.50 ± 0.37, respectively. The reductions for *P. aeruginosa* in commercial and batch reactor H_2O_2 were 2.46 ± 0.55 and 0.93 ± 0.06, respectively.

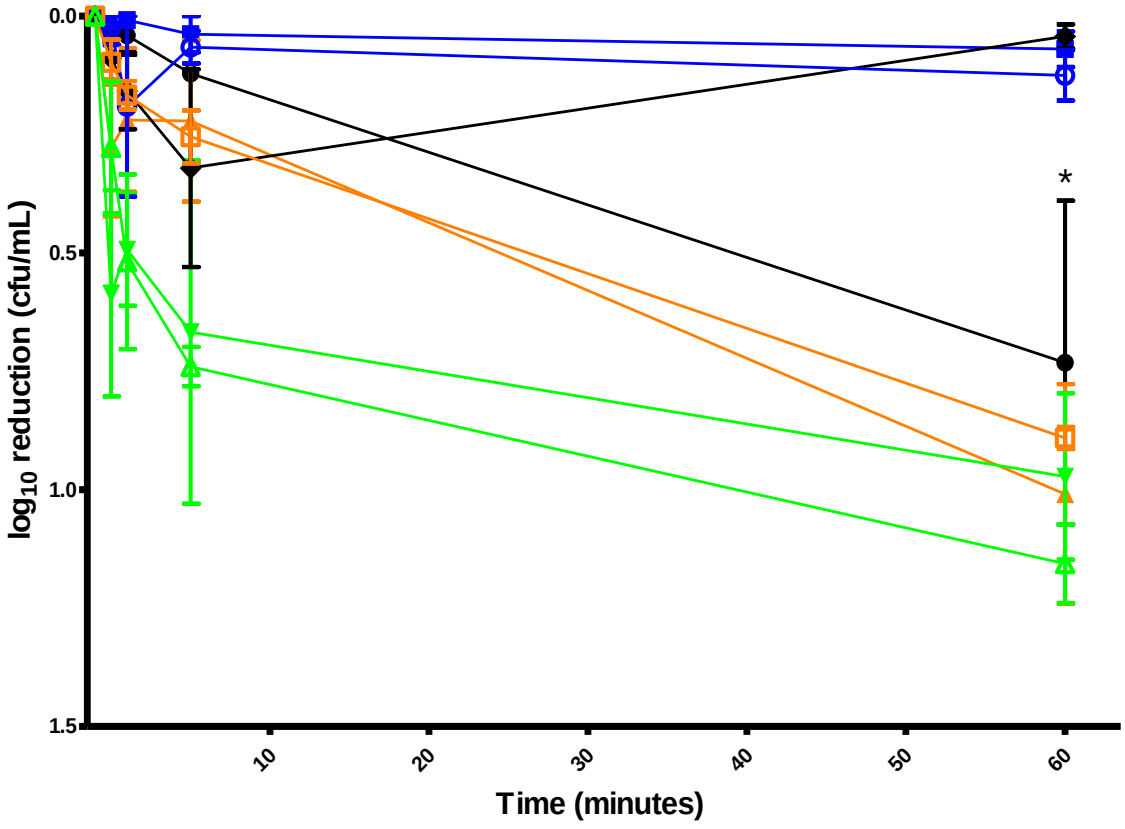
Figure 2.6 Log_{10} reduction results from commercial and batch reactor H_2O_2 in hard water with organic load conditions. Suspension tests were performed with 200 ppm commercial and batch reactor H_2O_2 on *S. aureus* NCTC10788, *E. hirae* NCTC13383, *E. coli* NCTC10418 and *P. aeruginosa* NCTC13359 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_2O_2, *E. hirae* NCTC13383 commercial H_2O_2, *E. coli* NCTC10418 commercial H_2O_2, *P. aeruginosa* NCTC13359 commercial H_2O_2, *S. aureus* NCTC10788 batch reactor H_2O_2, *E. hirae* NCTC13383 batch reactor H_2O_2, *E. coli* NCTC10418 batch reactor H_2O_2, *P. aeruginosa* NCTC13359 batch reactor H_2O_2. (n = 3)



2.3.1.3.2 Comparison of commercial and batch reactor H_2O_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_2O_2 at 200 ppm concentration. The majority of reductions were less than 1 $\log_{10}$ with exceptions of *E. coli* in commercial H_2O_2 (1.01 ± 0.40 $\log_{10}$ reduction) and *P. aeruginosa* in batch reactor H_2O_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_2O_2 (0.73 ± 0.59 and 0.04 ± 0.04, respectively).

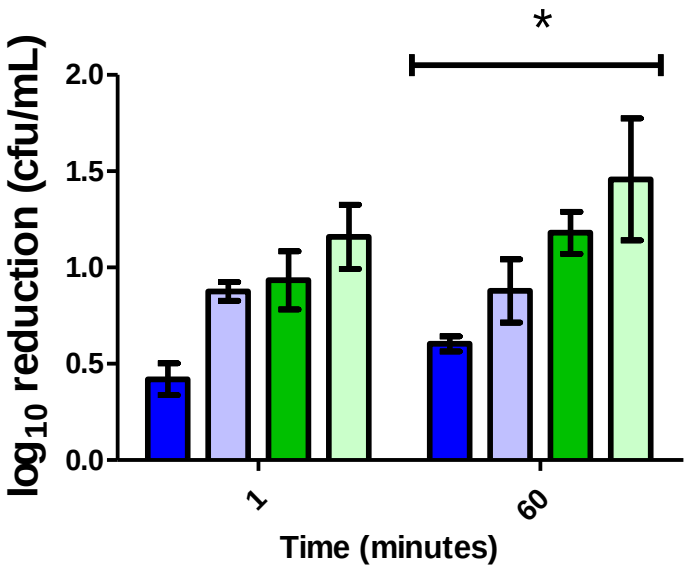
Figure 2.7 Log_{10} reduction results from commercial and batch reactor H_2O_2 in clean water conditions. Suspension tests were carried out with 200 ppm commercial and batch reactor H_2O_2 on *S. aureus* NCTC10788, *E. hirae* NCTC13383, *E. coli* NCTC10418 and *P. aeruginosa* NCTC13359 all at room temperature. 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_2O_2, *E. hirae* NCTC13383 commercial H_2O_2, *E. coli* NCTC10418 commercial H_2O_2, *P. aeruginosa* NCTC13359 commercial H_2O_2, *S. aureus* NCTC10788 batch reactor H_2O_2, *E. hirae* NCTC13383 batch reactor H_2O_2, *E. coli* NCTC10418 batch reactor H_2O_2, *P. aeruginosa* NCTC13359 batch reactor H_2O_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_2O_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_2O_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_2O_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_2O_2 or same concentrations of different H_2O_2. All of the results using 100 ppm of both commercial and batch reactor H_2O_2 gave results bellow 1 $\log_{10}$ reduction with batch reactor H_2O_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_2O_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_2O_2 continued to cause higher $\log_{10}$ reductions than the commercial H_2O_2. Results obtained with 200 ppm batch reactor H_2O_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_2O_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_2O_2 in clean water conditions. Suspension tests were carried out with 100 and 200ppm commercial and batch reactor H_2O_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_2O_2 ie. between 100 and 200ppm of commercial H_2O_2 and 100 and 200ppm of batch reactor H_2O_2. 100 ppm commercial H_2O_2, 100 ppm batch reactor H_2O_2, 200 ppm commercial H_2O_2 and 200 ppm batch reactor H_2O_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_2O_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_2O_2 decomposed faster than the commercial H_2O_2 which decomposed only by 9 ppm in both 100 and 200 ppm after 60 min. The batch reactor H_2O_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 H_2O_2 at 10 min intervals for 1 hour. H_2O_2 were titrated using cerium (IV) oxide.

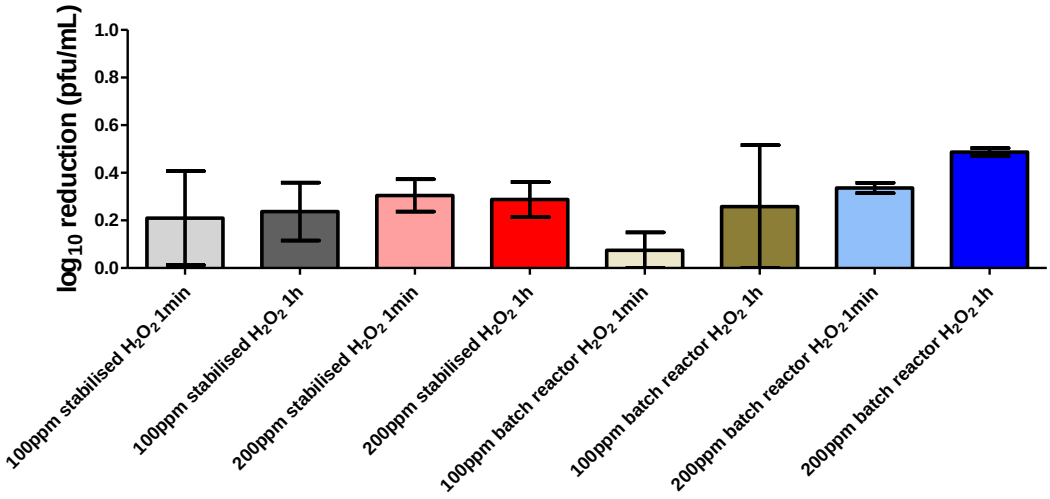
A) 100 ppm initial concentration		
	Concentration of	
Time (min)	Commercial H_2O_2 (ppm)	Batch reactor H_2O_2 (ppm)
0	100	100
10	100	96
20	96	79
30	96	74
40	91	74
50	91	74
60	91	74

B) 200 ppm initial concentration		
	Concentration of	
Time (min)	Commercial H_2O_2 (ppm)	Batch reactor H_2O_2 (ppm)
0	200	200
10	200	157
20	200	149
30	195	144
40	191	140
50	191	140
60	191	135

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 H_2O_2 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 H_2O_2 treatments did not produce any significant activity (P value = 0.60). Moreover, none of them produced a reduction above 1 $\log_{10}$ pfu/mL. A 0.49 $\log_{10}$ reduction caused by 200 ppm of batch reactor H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 than the Gram-positive bacteria. A study conducted by (Martin and Maris, 2012) also indicated that Gram-negative bacteria were more susceptible to H_2O_2 than Gram-positive bacteria at different water hardness and organic load conditions. The highest concentration of H_2O_2 used in my study and the longest contact time was necessary to cause an over 4 $\log_{10}$ reduction in majority of tested bacteria (1,000 ppm at 60 min and 10,000 ppm at 5min was enough to achieve a 4 $\log_{10}$ reduction for *E. coli*) in hard water with organic load conditions. To note, the usual concentration of H_2O_2 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 H_2O_2 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 H_2O_2 without the organic load and salts in the test suspension. Considering the 200 ppm concentration at a 60 min time point, the

$\log_{10}$ reduction observed for *E. coli* and *P. aeruginosa* were 1.01 and 0.97 respectively. The $\log_{10}$ reductions at 200 ppm H_2O_2 at 60 min time point in hard water with organic load conditions for *E. coli* and *P. aeruginosa* were 3.19 and 2.46 $\log_{10}$ 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 H_2O_2 can be found in the literature.

Suspension tests were also carried out using 200 ppm of batch reactor H_2O_2 in hard water with organic load and clean water conditions. 200 ppm concentration was chosen as this is the concentration of H_2O_2 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 H_2O_2 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 H_2O_2.

H_2O_2 at 200 ppm concentration was not highly effective at clean water conditions. However, for both *E. coli* and *P. aeruginosa* the differences in $\log_{10}$ reductions were more than twice as high when commercial H_2O_2 was used compared to the batch reactor H_2O_2 after 60 min time point in hard water with organic load conditions. This would indicate that commercial H_2O_2 is more effective than batch reactor H_2O_2 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 H_2O_2 decomposing to its free radicals more readily (30% of batch reactor H_2O_2 decomposed after 40 minutes) and BSA mopping them up, therefore, making the batch reactor H_2O_2 less effective than

the commercial H_2O_2 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 H_2O_2 to compare efficacies of them both against bacteria in clean water conditions. As could be observed from these data, the batch reactor H_2O_2 caused higher log_{10} reductions than the commercial H_2O_2, albeit, not statistically significant. What could also be observed was that 30% of batch reactor H_2O_2 was lost after 40 min of reaction. The batch reactor H_2O_2 could decompose to its free radicals more readily than the commercial H_2O_2 which did not decompose at all which corresponded to a higher activity. This finding is novel as studies comparing efficacies of commercial and unstabilised H_2O_2 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 H_2O_2 readily makes. Furthermore, the fact that there was more than just bacteria in the suspension would indicate that some of the H_2O_2 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 H_2O_2 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 H_2O_2 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_2O_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_2O_2 in flow through catalysis (Chapter 4: Microbicidal efficacy of the catalyst).

3 Catalytic production of H_2O_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 H_2O_2 industrially was recorded in 1818 and it involved a reaction of BaO_2 with dilute HCl to form H_2O_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 H_2O_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 H_2O_2). However, considering the large scale resource conservation, environmental and economic factors, direct synthesis of H_2O_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 H_2O_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

H_2O_2 (Ranganathan and Sieber, 2018). These are summarised in a (Table 3.1). The steps of the direct synthesis reaction are explained in Section 3.1.1 Production of H_2O_2.

Table 3.1 Advantages and disadvantages of direct synthesis of H_2O_2 (adapted from Ranganathan and Sieber, 2018).

Advantages	Disadvantages
Lack of organic substrates including organic solvents or anthraquinones.	**Simultaneous side products generation other than H_2O_2, such as water due to unselective reactions.**
Green solvents are used such as ethanol, water or methanol.	**The process is complex and has mass transfer limitations which involves three phases: liquid (reaction medium), gas (H_2/O_2) and solid (catalyst).**
Fewer downstream operations are needed to produce H_2O_2 making the process more economical.	
A single reactor system can be used to carry out this process.	**Hydrogen has a very broad flammability range of 4-94% H_2 in O_2**

In recent years Au, Pd and AuPd catalysts were broadly tested for the direct synthesis of H_2O_2. Although there were more studies conducted on Pd catalysts, AuPd catalysts have shown to be more effective at producing H_2O_2 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_2O_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).

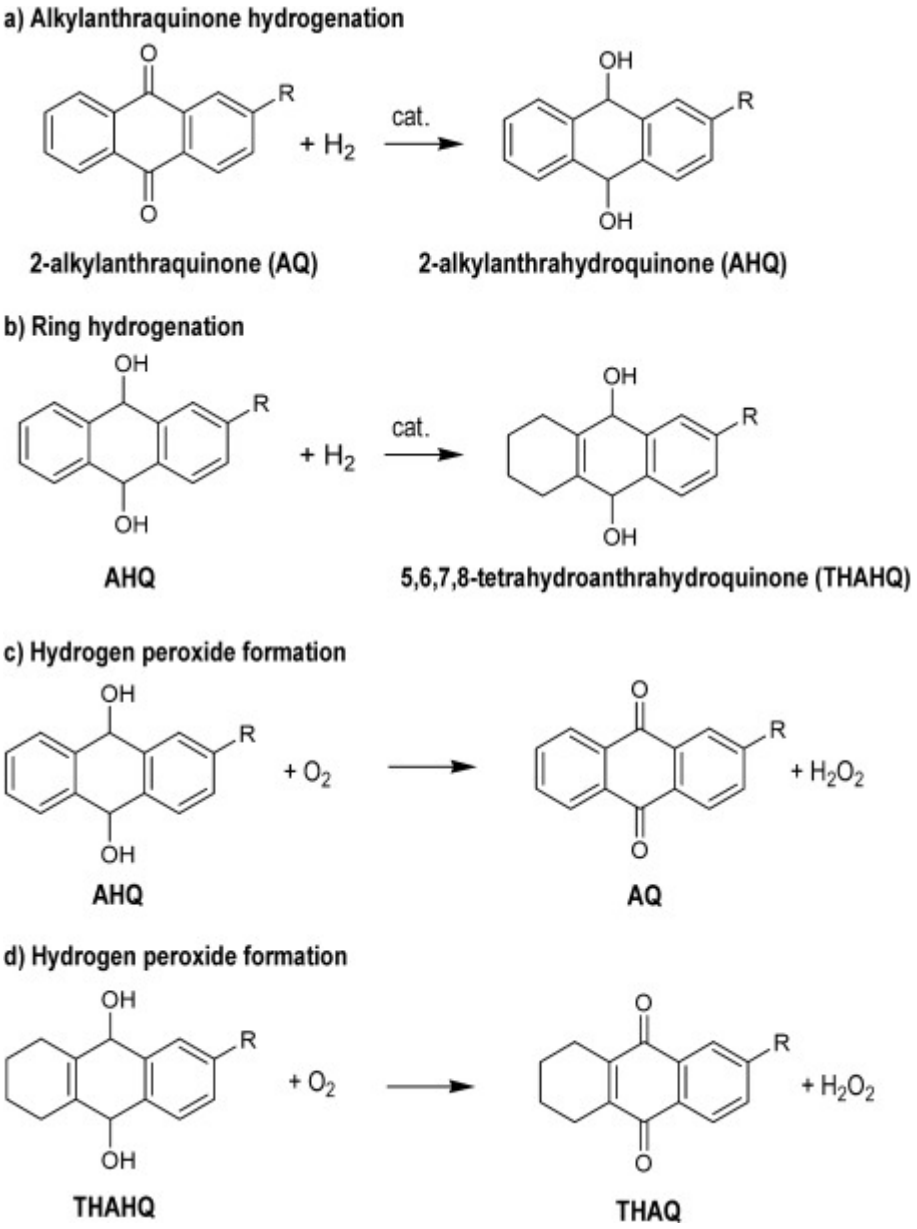
Edwards *et al*. (2005) from Cardiff Catalysis Institute compared combinations of TiO_2 supported AuPd catalyst with TiO_2 supported Pd or Au catalysts for effectiveness of H_2O_2 production. It has been shown that AuPd catalyst has been significantly more effective at producing H_2O_2 than the monometallic 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_2O_2

There are numerous ways to produce H_2O_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_2O_2 allowing for production of H_2O_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_2O_2 is through an ion exchange membrane cell using porous cathodes. This method can be used for *in situ* production of H_2O_2 (Asokan and Subramanian, 2009). Moreover, there is also a photocatalytic approach to producing H_2O_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_2O_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_2O_2. Demineralised water is used to separate H_2O_2 from the organic working solution, H_2O_2 is then distilled to purify it and the alkylanthraquinone is recycled (Campos-Martin *et al*., 2006).

Figure 3.1 Anthraquinone process (Campos-Martin *et al*., 2006).



The direct synthesis of H_2O_2 is a small scale on-site alternative to the large scale industrial anthraquinone process. Direct synthesis of H_2O_2 is a method which nullifies the need of transportation of highly concentrated solutions of H_2O_2 and their subsequent dilution. Moreover, it is an atom efficient method of producing H_2O_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 H_2O_2 (Freakley *et al.*, 2013). In the direct synthesis method, the H_2O_2 is formed by a two-step hydrogenation of O_2 that was adsorbed to the surface of the catalyst. Furthermore, all of the reactions taking place in the direct synthesis method of H_2O_2 production have the same intermediate reaction species (Ntainjua *et al.*, 2008). Figure 3.3 shows the steps involved in the direct synthesis of H_2O_2 where the * designates a vacant site of the catalyst surface (Freakley *et al*., 2013). However, in the direct synthesis of H_2O_2, there are also undesirable hydrogenation reactions of the dissociated surface O_2 species which causes formation of water (Freakley *et al*., 2013). Figure 3.4 shows the steps involved in water formation during direct synthesis of H_2O_2 where the * designates a vacant site of the catalyst surface (Freakley *et al*., 2013). Many studies on direct synthesis of H_2O_2 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 H_2O_2 (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 H_2O_2 diagram (from Freakley *et al*. 2013).

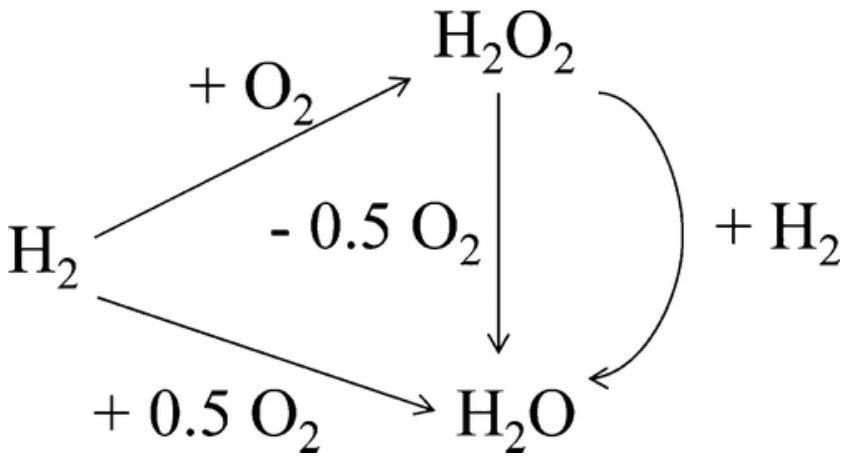


Figure 3.3 Steps involved in the direct synthesis of H_2O_2 (* designates a vacant site of the catalyst surface) (from Freakley et al., 2013).

$$H_2 + 2* \rightarrow 2H*$$

$$O_2 + * \rightarrow O_2*$$

$$H* + O_2* \rightarrow HO_2* + *$$

$$HO_2* + H* \rightarrow H_2O_2* + *$$

$$H_2O_2* \rightarrow H_2O_2 + *$$

Figure 3.4 Steps involved in water formation during direct synthesis of H_2O_2 (* designates a vacant site of the catalyst surface) (from Freakley et al., 2013).

$$O_2 + 2* \rightarrow 2O* \text{ or } H_2O_2* + * \rightarrow H_2O + O*$$

$$H* + O* \rightarrow HO* + *$$

$$HO* + H* \rightarrow H_2O* + *$$

$$H_2O* \rightarrow H_2O + *$$

3.1.2 Aim and objectives

The aim of this Chapter is to test efficacies of different catalysts in terms of the H_2O_2 production and to establish the free radical species produced by those catalysts and their significance.

The objectives are:

1. Produce 0.5 w/w.% Au-0.5 w/w.% Pd/TiO_2 (called 1 w/w% AuPd/TiO_2 throughout, this Au to Pd w/w% ratio was used throughout the project unless otherwise stated), 1 w/w% Au/TiO_2 and 1 w/w% Pd/TiO_2 catalysts using a modified impregnation method.
2. Test the H_2O_2 production efficacies of these catalysts in a batch reactor.

3. Test the H_2O_2 production efficacies of these catalysts in a flow reactor.
4. Establish the free radical species produced using these catalysts.

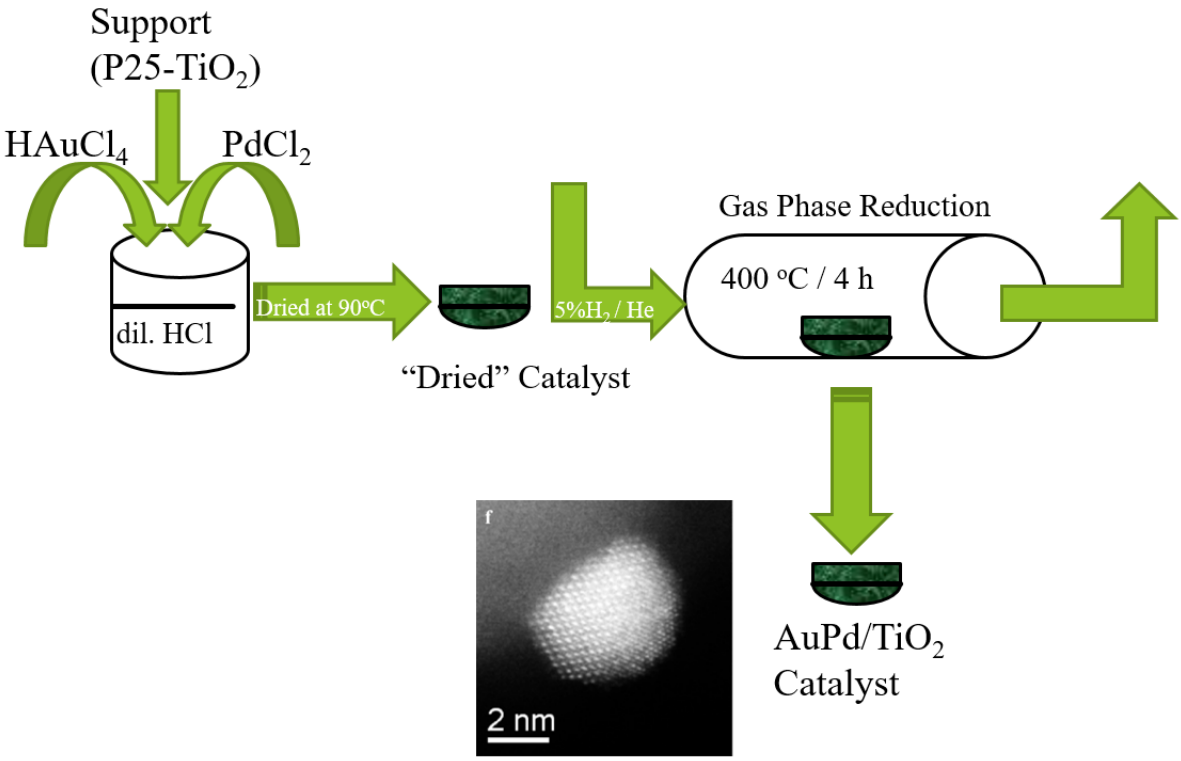
3.2 Materials and methods

3.2.1 Catalyst preparation

$HAuCl_4 \cdot 3H_2O$ (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% H_2 in Ar. The catalyst was reduced at 400°C for 4 h under a steady flow of 5% H_2 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 TiO_2 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% $AuPd/TiO_2$, 1 w/w% Au/TiO_2, 0.75 w/w% Au-0.25 w/w% Pd/TiO_2, 0.25 w/w% Au-0.75 w/w% Pd/TiO_2, 1 w/w% Pd/TiO_2. 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, 2ºC temperature, 1200 rpm spinning for 30 minutes. After the reaction, the H_2O_2 was filtered into a glass vial using a filter paper. After the H_2O_2 was filtered, it was titrated to determine its concentration. The H_2O_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_2O_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_2O_2 testing (from J. Harrhy).

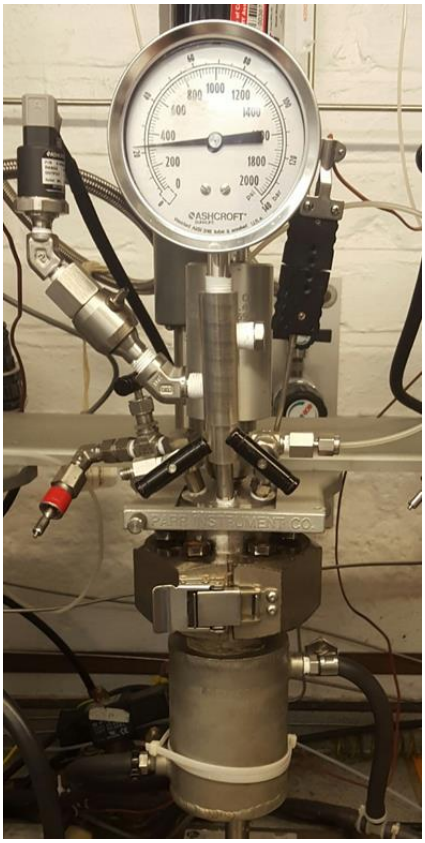


Figure 3.7 Titration reaction equations (from J. Harrhy).

$$H_2O_2 + 2\,Ce(SO_4)_2 \rightarrow Ce_2(SO_4)_3 + H_2SO_4 + O_2$$

$$\text{wt.\% } H_2O_2 = \frac{\text{moles of } H_2O_2 \times M_r\, H_2O_2}{8.5}$$

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 H_2O_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 H_2O_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 H_2O_2). The components of the reactor consist of:

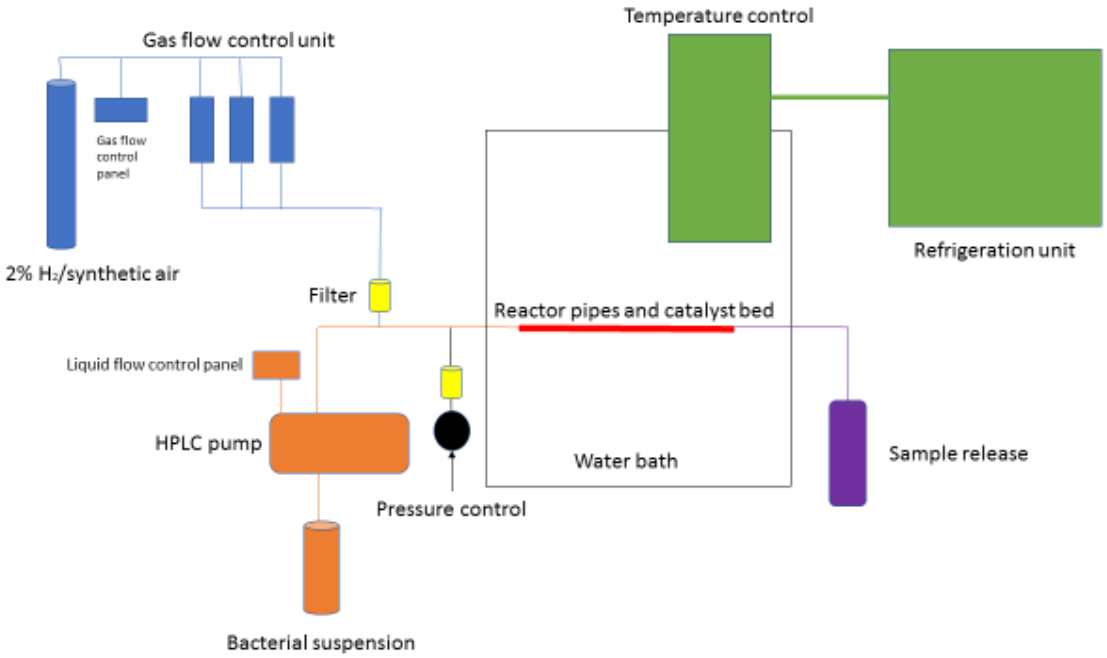
- Gas cylinder consisting of 2% hydrogen/air (BOC, Guildford, UK).
- Gas flow control unit (Brooks Instrument, Hatfield, Pennsylvania, United States) which allows the flow rate between 0 and 50 mL/min. Under the standard reaction conditions, the gas flow rate is 42 mL/min.
- A pressure gauge (Swagelok, 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.

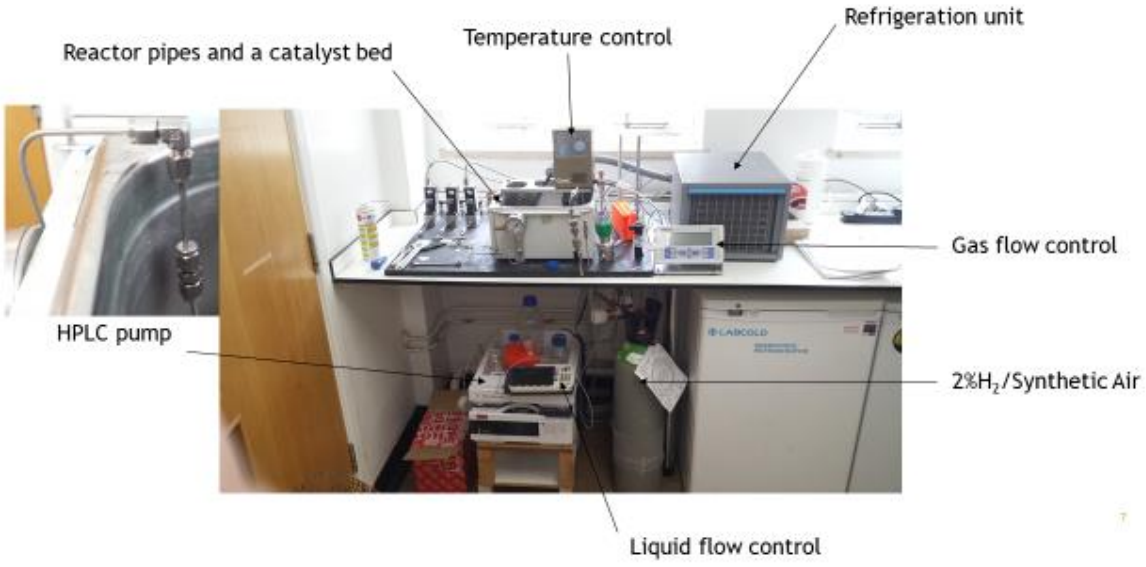
- HPLC pump (Agilent Technologies, Santa Clara, California, United States) which pumps the bacterial suspension into the reactor. The HPLC pump allows the liquid to be pumped at rates between 0.1 mL/min and 5 mL/min. The flow of the bacterial suspension in the reactor under standard conditions is 0.2 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% $AuPd/TiO_2$ catalyst. The 2% hydrogen/air and the bacterial suspension meet in the catalyst bed where the H_2O_2 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^oC which is the optimal temperature for the H_2O_2 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.

A)



B)

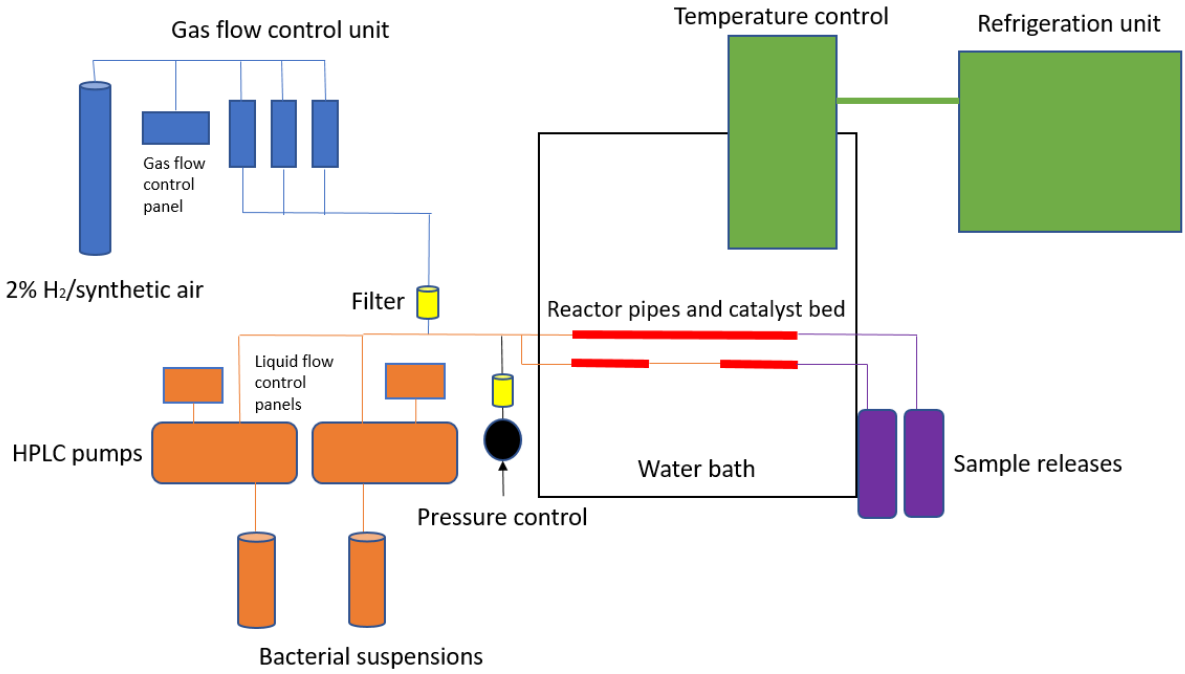


3.2.4 Current flow reactor design

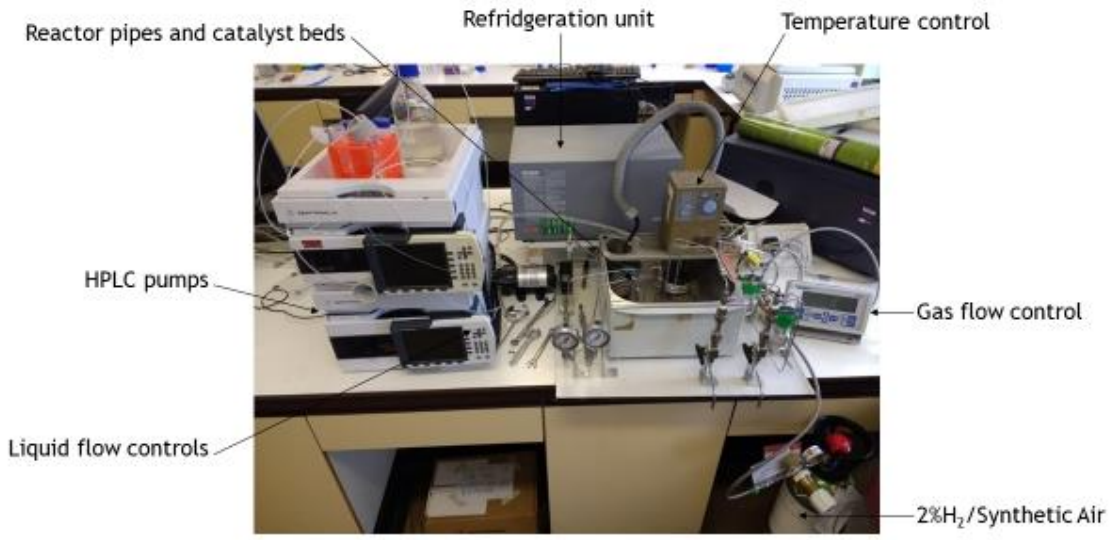
Figure 3.9 shows the changes in the flow reactor design. An improvement was an addition of a second HPLC pump and reactor line to be able to carry out two tests simultaneously. This improved both, efficiency and flexibility as twice as much data were able to be generated and two different tests could 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.

A)



B)



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 H_2O_2 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% H_2/ air, 5% H_2/N_2 and air) and different catalysts (1 w/w% $AuPd/TiO_2$, 1 w/w% Pd/TiO_2, and 1 w/w% Au/TiO_2) and for the duration of 10 minutes. For the tests using the 5% H_2/N_2, the deionised water was also degassed overnight using N_2 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 N_2 flow. Spectra were recorded at 298 K using the following instrumental conditions: 5.02 10^4 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 H_2O_2 production and to compare it to the effectiveness of other catalysts in terms of moles of H_2O_2 per kilogram of catalyst per hour (Productivity/mol_{H2O2} kg^{-1}cat h^{-1}) which is the standard unit of the measure of catalyst productivity (Hammond, 2017).

The productivity of the 1 w/w% AuPd/TiO_2 catalyst used throughout the project was 106 mol_{H2O2} kg^{-1}cat 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_{H2O2} $kg^{-1}cat$ h^{-1} whereas the productivity of 1 w/w% Pd/TiO_2 was 80 mol_{H2O2} $kg^{-1}cat$ h^{-1}. The 0.75 w/w% Au-0.25 w/w% Pd/TiO_2 catalyst produced 89 mol_{H2O2} $kg^{-1}cat$ h^{-1}. The 0.25 w/w% Au-0.75 w/w% Pd/TiO_2 catalyst produced 84 mol_{H2O2} $kg^{-1}cat$ h^{-1} (Santos *et al*., 2019) (Table 3.2).

Table 3.2 H_2O_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, 2ºC temperature, 1200 rpm spinning for 30 minutes. (n=3).

Catalyst	Mean productivity/mol_{H2O2} $kg^{-1}cat$ h^{-1}
1 w/w% Au/TiO_2	**4**
0.75 w/w% Au-0.25 w/w% Pd/TiO_2	**89**
1 w/w% $AuPd/TiO_2$	**106**
0.25 w/w.% Au-0.75 w/w% Pd/TiO_2	**84**
1 w/w% Pd/TiO_2	**80**

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_2O_2 and its antimicrobial efficacy in the flow reactor system used throughout this project. Furthermore, H_2O_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_2O_2 in the flow reactor. The concentrations of H_2O_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_2O_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).

Catalyst	H_2O_2 productivity (ppm)
1 w/w% AuPd/TiO_2	**259**
1 w/w% Au/TiO_2	**69**
1 w/w% Pd/TiO_2	**220**

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 ± 0.22 $\log_{10}$ reduction in a flow reactor test. Flow rector experiments using 1 w/w% Au/TiO_2 and 1 w/w% Pd/TiO_2 caused a 0.75 ± 0.37 and 0.54 ± 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% $AuPd/TiO_2$, 1 w/w% Au/TiO_2, 1 w/w% Pd/TiO_2. 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. 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).

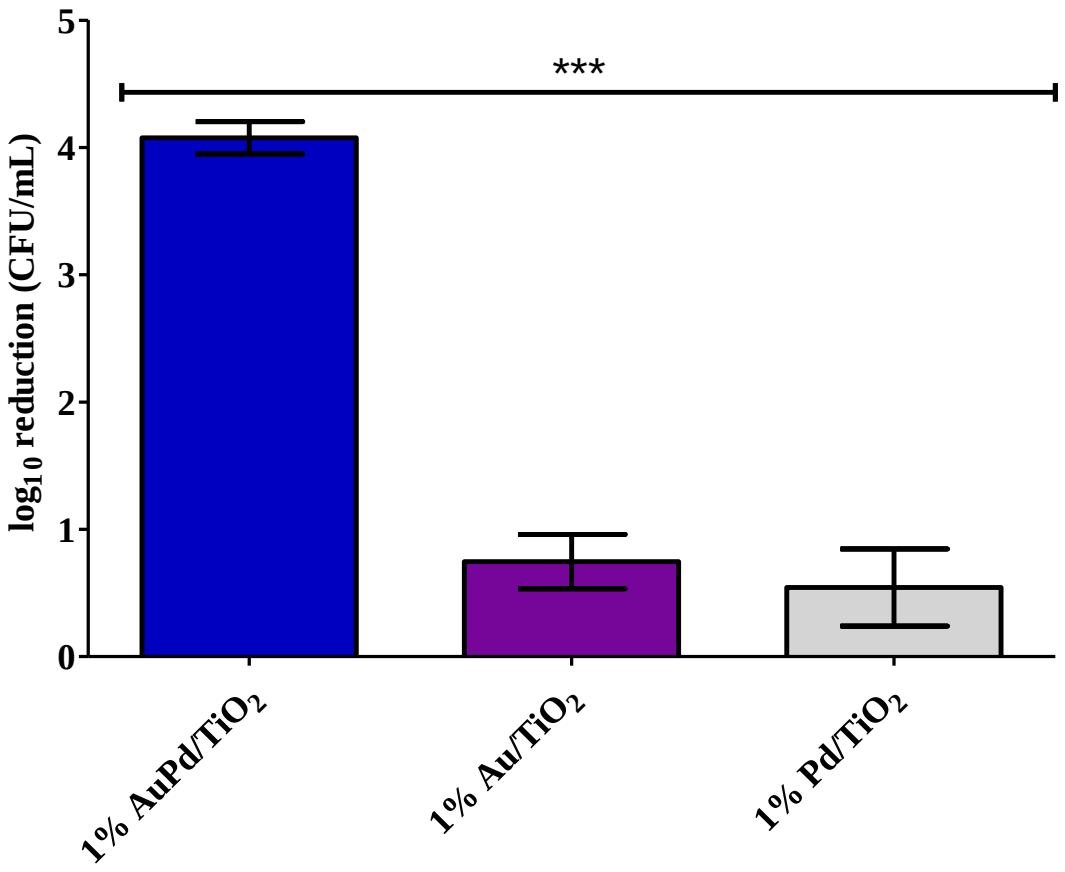
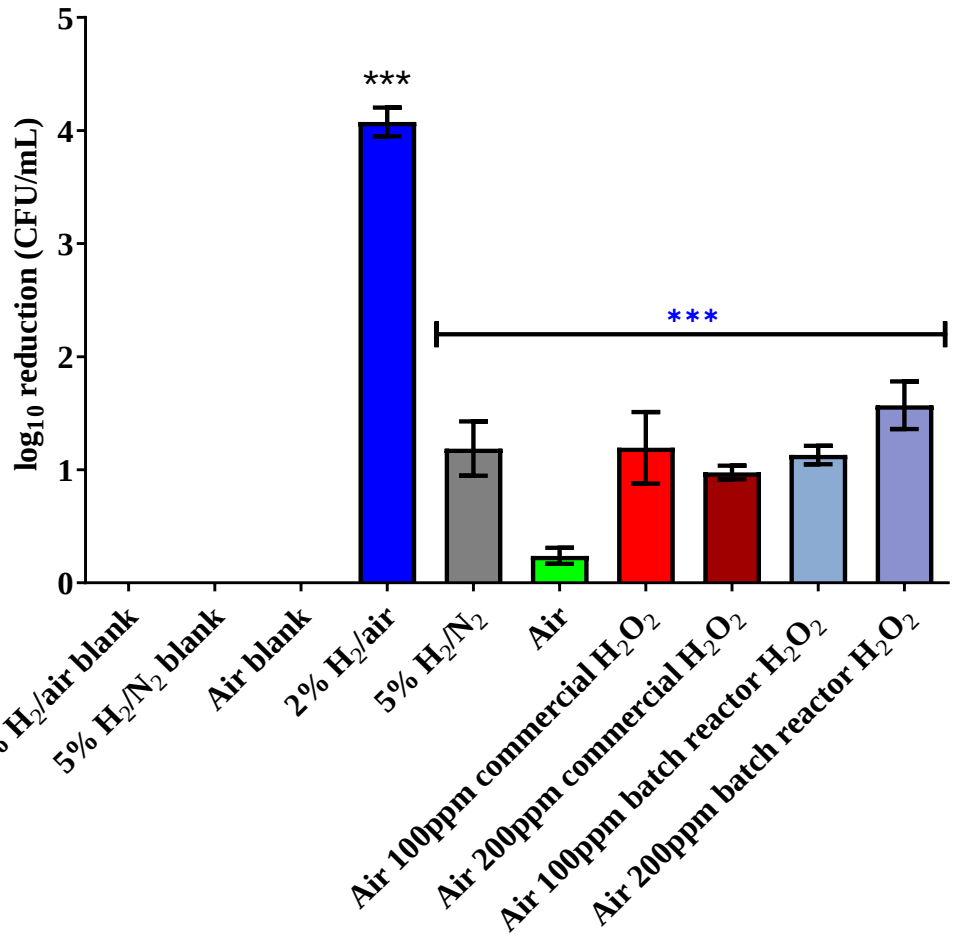


Figure 3.11 shows the efficacy of 1 w/w% $AuPd/TiO_2$ against *E. coli* K12 JM109 with different gas mixtures flowing through the reactor. Experiments with no catalyst in the catalyst bed did not show any log_{10} reduction against *E. coli* K12 JM109 in every gas mixture. The most effective gas mixture 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_2O_2 was passed through the catalyst with air. One hundred and 200 ppm of batch reactor H_2O_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).



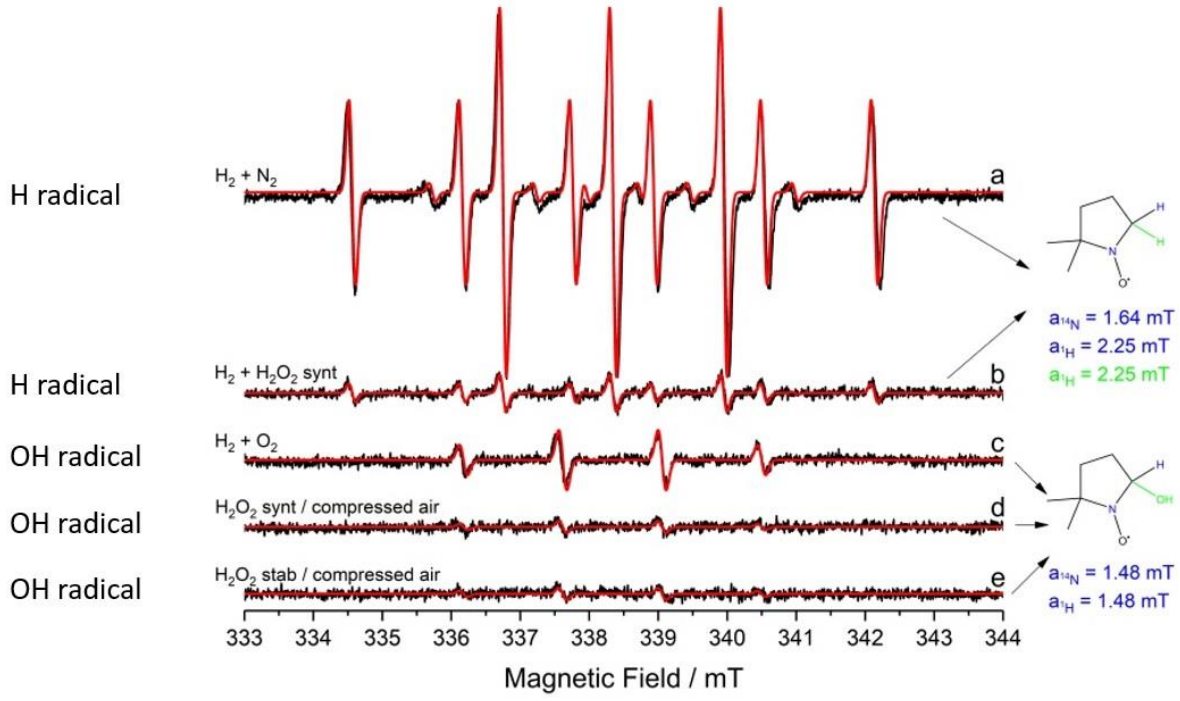
3.3.2 EPR experiments

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_2/air (gas mixture used for standard testing), 5% H_2/N_2 and compressed air. The water used for the experiments with 5% H_2/N_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_2O_2 to see the difference between the radicals synthesised on the catalyst surface and the radicals from the breakdown of H_2O_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_2/air and 5% H_2/N_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_2/N_2 which shows that the catalyst is able to produce H free radicals in the absence of O_2. A much less pronounced signal could be observed when batch reactor H_2O_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_2O_2. Experiments carried out with the 2% H_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 H_2O_2 were passed through the flow reactor in compressed air. This also indicates that the 1 w/w% AuPd/TiO_2 catalyst produces more OH free radicals than there are obtained from the breakdown of H_2O_2.

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% H_2/air or 5% H_2/N_2 or compressed air, solvent flow rate = 0.2 mL/min, 120 mg of 1 w/w% AuPd/TiO_2 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% H_2/air and that there was a strong H free radical signal when glutathione passed through the flow reactor in the 5% H_2/N_2 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^{o}C, 42 mL/min gas flow of either 2% H_2/air or 5% H_2/N_2, solvent flow rate = 0.2 mL/min, 120 mg of 1 w/w% AuPd/TiO_2 catalyst. DMPO was used as a spin trap in these experiments (Dr A. Folli; Cardiff University, School of Chemistry).

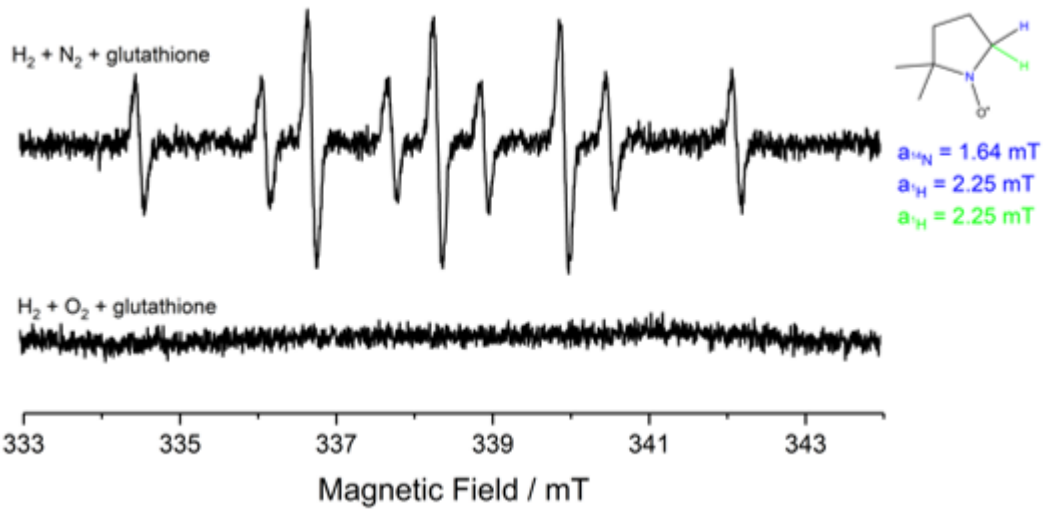
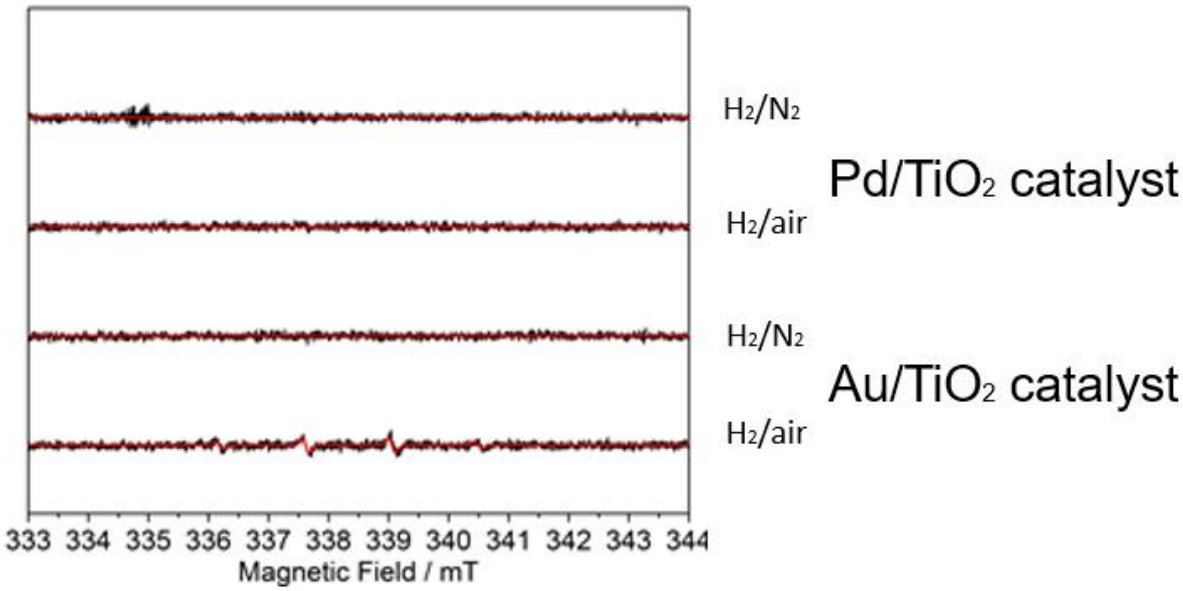


Figure 3.14 shows EPR spectra obtained from experiments carried out on 1 w/w% Au/TiO_2 and 1 w/w% Pd/TiO_2. As can be observed from the spectra, there were flat lines for experiments carried out on 1 w/w% Pd/TiO_2 with both 5% H_2/N_2 and 2%

H_2/air gas mixtures. There was also a flat line on a spectrum obtained from the experiment on 1 w/w% Au/TiO_2 with a 5% H_2/N_2 mixture, however, there was a signal indicating OH free radicals when 2% H_2/air gas mixture was passed through the catalyst.

Figure 3.14 Comparison of EPR spectra obtained from experiments on 1 w/w% Au/TiO_2 and 1 w/w% Pd/TiO_2. Reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of either 2% H_2/air or 5% H_2/N_2, solvent flow rate = 0.2 mL/min, 120 mg of 1 w/w% $AuPd/TiO_2$ 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_2O_2 from O_2 and H_2 (106 mol_{H2O2} $kg^{-1}cat$ h^{-1}) than such catalysts made using a conventional impregnation (23 mol_{H2O2} kg $cat^{-1}h^{-1}$) or sol immobilisation (32 mol_{H2O2} kg $cat^{-1}h^{-1}$) 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).

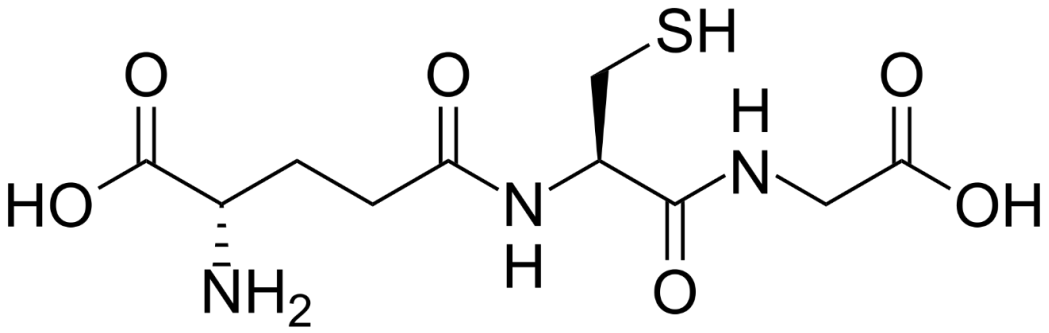
Flow reactor standard reaction conditions were optimised by (Freakley *et al.*, 2013). Just like in the batch reactor, the 1 w/w% $AuPd/TiO_2$ catalyst produced more H_2O_2 than the monometallic 1 w/w% Au/TiO_2 and 1 w/w% Pd/TiO_2. This also correlated with the higher efficacy of 1 w/w% $AuPd/TiO_2$ 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_2O_2 productivity (259 and 220 ppm respectively). This provides an indication that

H_2O_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% H_2/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 $\log_{10}$ reductions were observed from the flow reactions with 2% H_2/air which corroborates with the highest production of OH free radicals. The flow reaction with 5% H_2/N_2 which correlates with H free radicals production caused a much lower $\log_{10}$ reduction of *E. coli* K12 JM109. This further solidifies that OH• is the main killing factor. What is more, the flow reaction with 2% H_2/air which provided a much stronger signal for OH• than the flow reactions with both commercial and batch reactor H_2O_2 in air, caused a much higher $\log_{10}$ reduction of *E. coli* K12 JM109. This shows that the free radicals are more important than H_2O_2 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/TiO_2 and 1 w/w% Pd/TiO_2 showed a small signal for OH• production by 1 w/w% Au/TiO_2 and flat line spectra for 1 w/w% Pd/TiO_2. It has been shown that Pd is able to directly catalyse production of H_2O_2, 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 H_2O_2 formation as Pd, therefore, it produces a low amount of free radicals. However, the fact that 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_2O_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_2O_2 in suspension and flow reactor. (n=3).

4.2.3 H_2O_2 synthesis

Batch reactor H_2O_2 synthesis was described in Chapter 2. Section 2.2.3. H_2O_2 was synthesised in a batch reactor in order to compare its activity with commercial H_2O_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% 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. 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% H_2/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 H_2O_2 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 10^7 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 H_2O_2 over a 6 hours period i.e. the inoculum for the 200 ppm H_2O_2 (commercial, batch reactor and flow reactor) was adjusted to 5 x 10^5 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_2O_2. The volume of liquid in every well was 200 µL. The plate was incubated in a shaking incubator at 120 rpm, 37^{o}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_2O_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_2O_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^{o}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^{o}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.

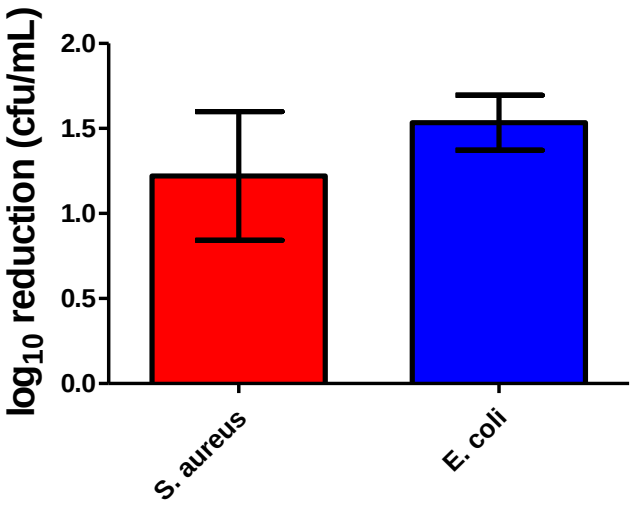
4.3 Results

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 H_2O_2 against Gram-positive and Gram-negative bacteria was not significantly different (P value = 0.49). There was a 1.22 ± 0.65 $\log_{10}$ reduction in *S. aureus* and 1.53 ± 0.28 $\log_{10}$ reduction in *E. coli* (Figure 4.1).

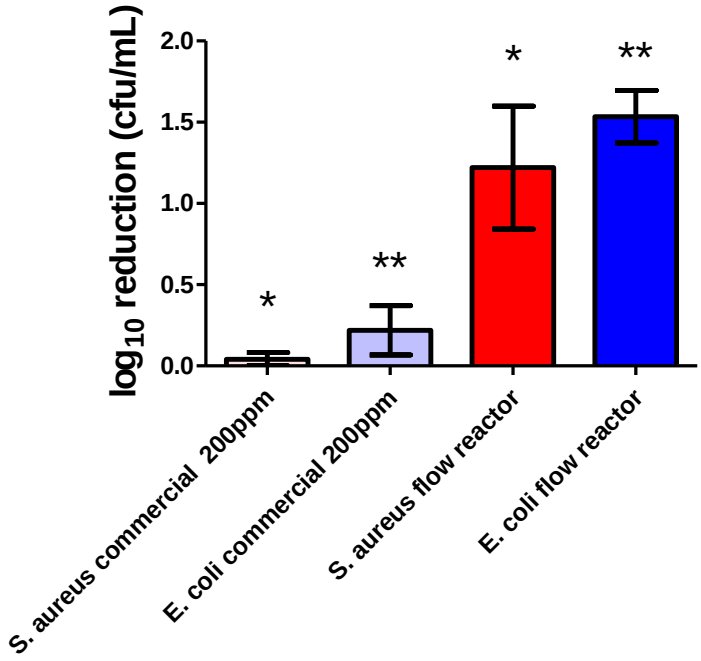
Figure 4.1 Efficacy of flow reactor against *S. aureus* NCTC10788 and *E. coli* NCTC10418 under standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H_2/air, solvent flow rate = 0.2 mL/min, 120mg of the catalyst. Data were analysed using an unpaired t-test using GraphPad Prism software. (n=3)



4.3.1.1.1 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 H_2O_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 H_2O_2 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).

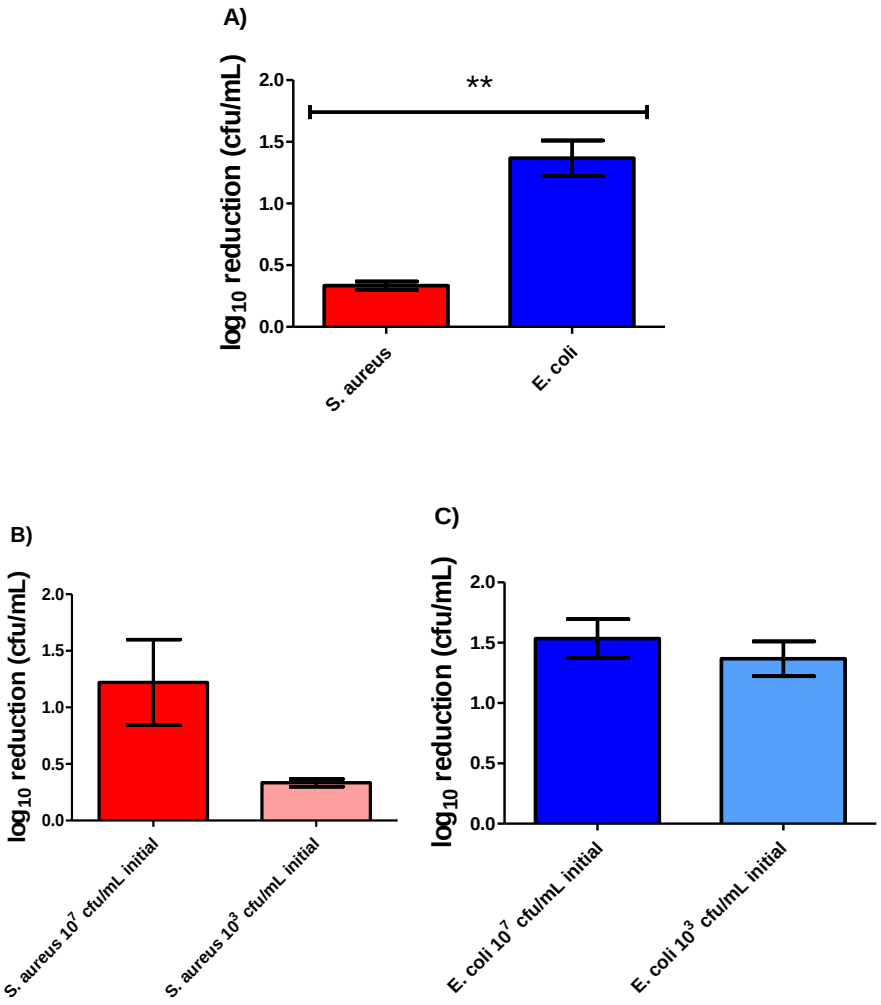


4.3.1.2 Flow reactor efficacy tests in different reaction conditions

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 ± 0.06 $\log_{10}$ reduction was observed with *S. aureus* NCTC10788 and a 1.37 ± 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 ± 0.28 and 1.37 ± 0.25 $\log_{10}$ reductions for 10^7 and 10^3 cfu/mL initial inoculum, respectively).

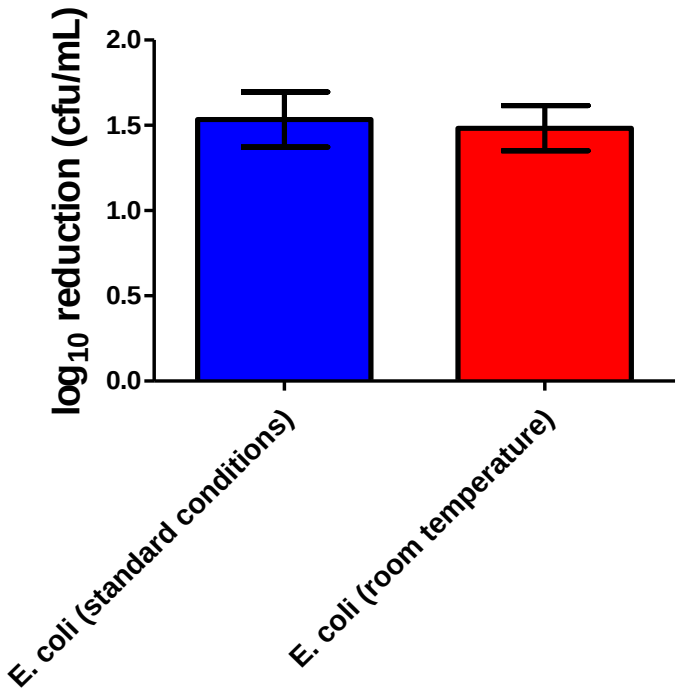
Figure 4.3 Efficacy of flow reactor with 10^3 and 10^7 cfu/mL initial inocula of *S. aureus* NCTC10788 and *E. coli* NCTC10418 under standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H_2/air, solvent flow rate = 0.2 mL/min, 120mg of the catalyst. A) Comparison of results with 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 *E. coli* NCTC10418 to determine whether temperature affects catalyst efficacy. Figure 4.4 shows that *E. coli* NCTC10418 at room temperature gave a 1.48 ± 0.23 $\log_{10}$ reduction which is virtually identical (P = 0.82) to the results obtained in 2°C (standard reaction condition temperature) (1.53 ± 0.28 $\log_{10}$ reduction).

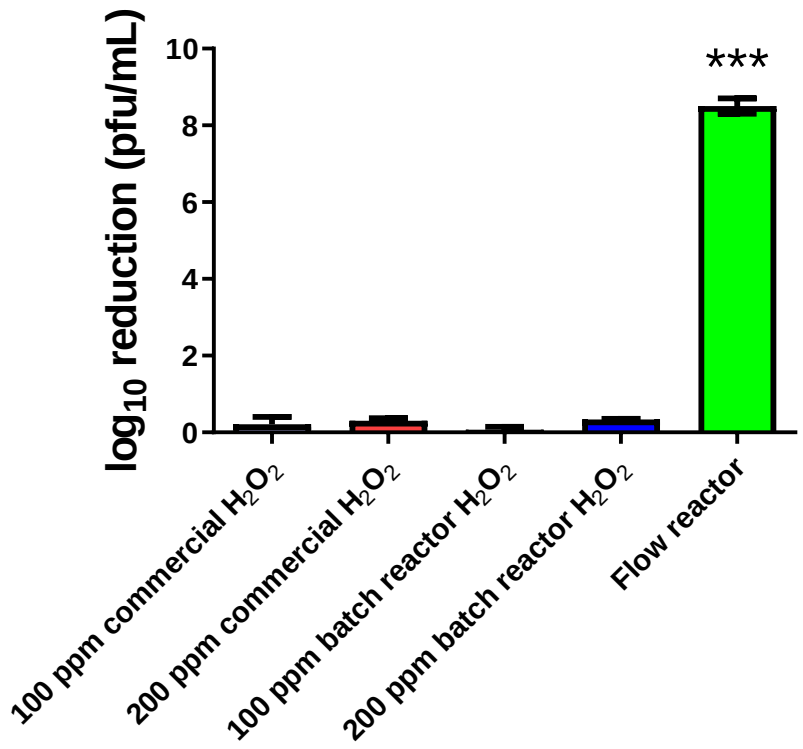
Figure 4.4 Efficacy of flow reactor against *E. coli* NCTC 10418 10^7 cfu/mL initial inoculum at 2°C (standard reaction conditions temperature) and room temperature (20°C). Reaction conditions were otherwise standard i.e. 10 bar pressure, 42 mL/min gas flow of 2% H_2/air, solvent flow rate = 0.2 mL/min, 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_2O_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_2O_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_2O_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_2O_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_2O_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 ± 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.

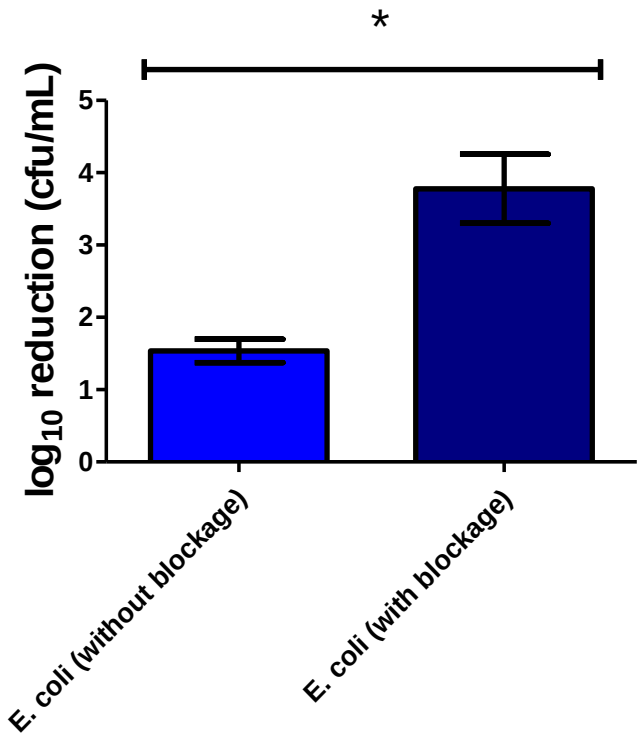
Table 4.1 Log_{10} reduction in *E. coli* NCTC10418 under standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H_2/air, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst. Results highlighted in yellow are from experiments performed under increased pressure due to a blockage in the catalyst bed. (n=5)

E. coli					
Experiment	Mean log before treatment	Mean log after treatment	Mean log reduction	Catalyst	Run on the catalyst
1	7.90	6.37	1.53	GS 2	1st
2	7.81	6.56	1.25	GS 2	2nd
3	7.96	6.15	1.82	GS 2	3rd
4	7.26	3	4.26	GS 5	1st
5	7.26	3.96	3.30	GS 6	1st

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).

S. aureus					
Experiment	Mean log before treatment	Mean log after treatment	Mean log reduction	Catalyst	Run on the catalyst
1	7.59	6.50	1.09	GS 1	1st
2	7.24	5.31	1.93	GS 1	2nd
3	7.77	7.13	0.64	GS 2	4th

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_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.

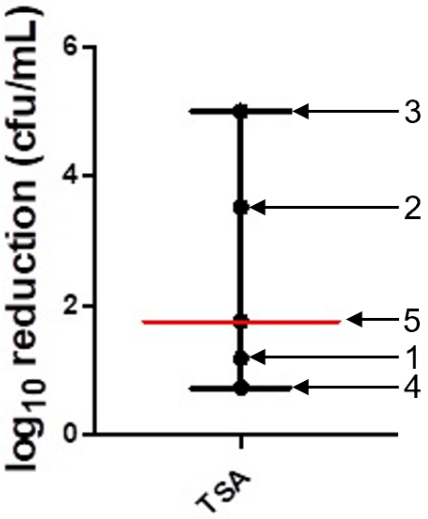


4.3.3.2 Efficacy of flow reactor against *E. coli* K12 JM109

Five independent experiments were carried out. The first three in one day and the 4th and 5th 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 4th repeat carried out on the next day gave a much lower log_{10} reduction (0.74), however, the 5th repeat carried out on that day (1.76 log_{10} reduction) was an improvement from the 4th. 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.

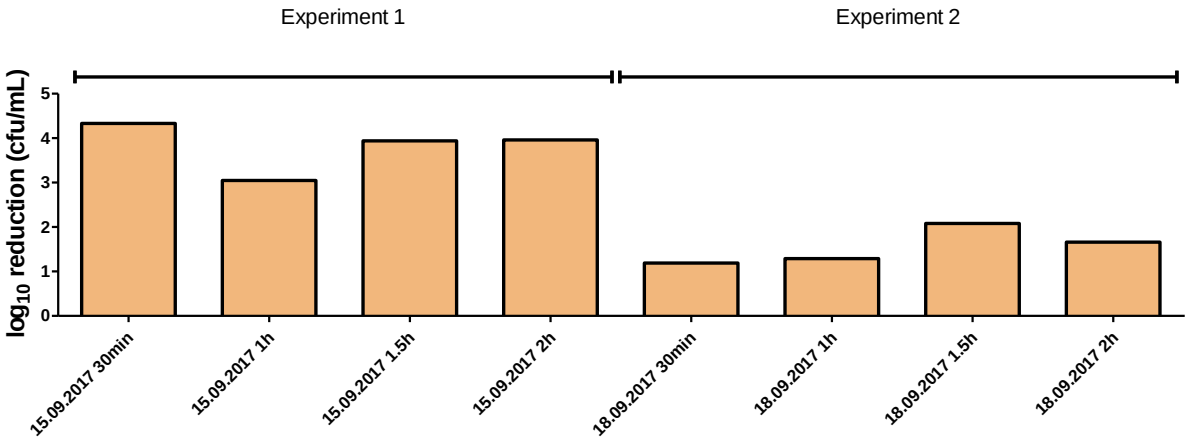
Figure 4.7 Scatter plot of 5 independent flow reactor experiments on *E. coli* K12 JM109 under standard reaction conditions: 10 bar pressure, 2^{o}C, 42 mL/min gas flow of 2% H_2/air, solvent flow rate = 0.2 mL/min, 120mg of the catalyst. Median is presented as a red line. Numbers by the arrows represent 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).

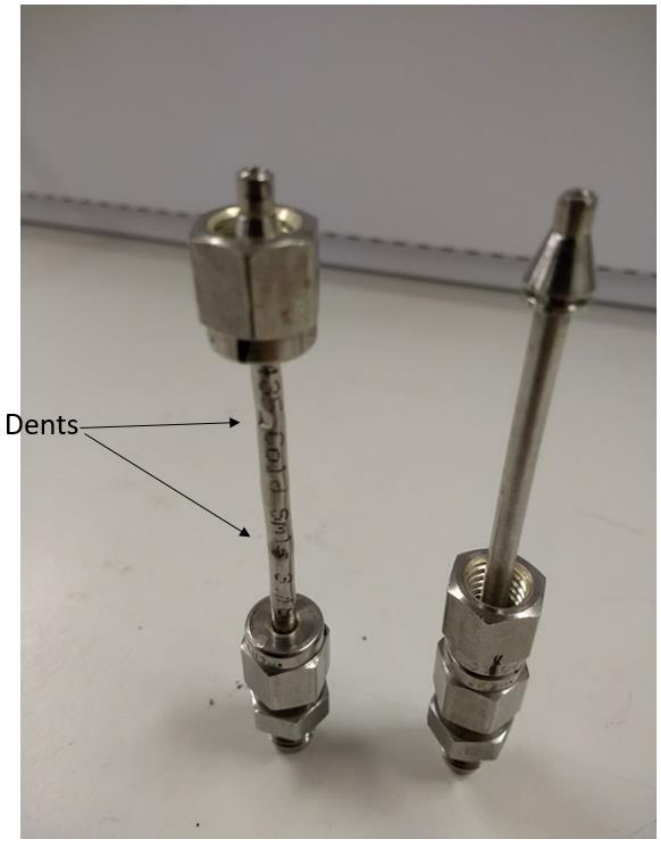
Figure 4.8 Efficacy of the flow reactor against *E. coli* K12 JM109 over a 2 hours period with 30 min time points under standard reaction conditions: 10 bar pressure, 2°C, 42 mL/min gas flow of 2% H_2/air, solvent flow rate = 0.2 mL/min, 120 mg of the catalyst. (n=2).



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.

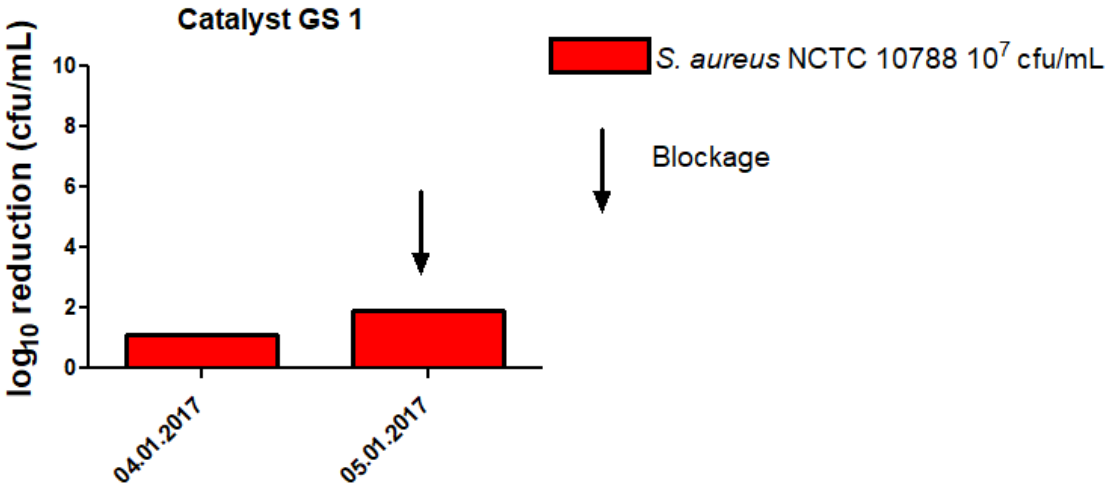
Figure 4.9 Artificial dents were introduced to the catalyst bed using a crimping tool.



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/TiO_2) timelines with microbicidal efficacies of every run on these catalyst beds. Figure legends are present by each timeline.



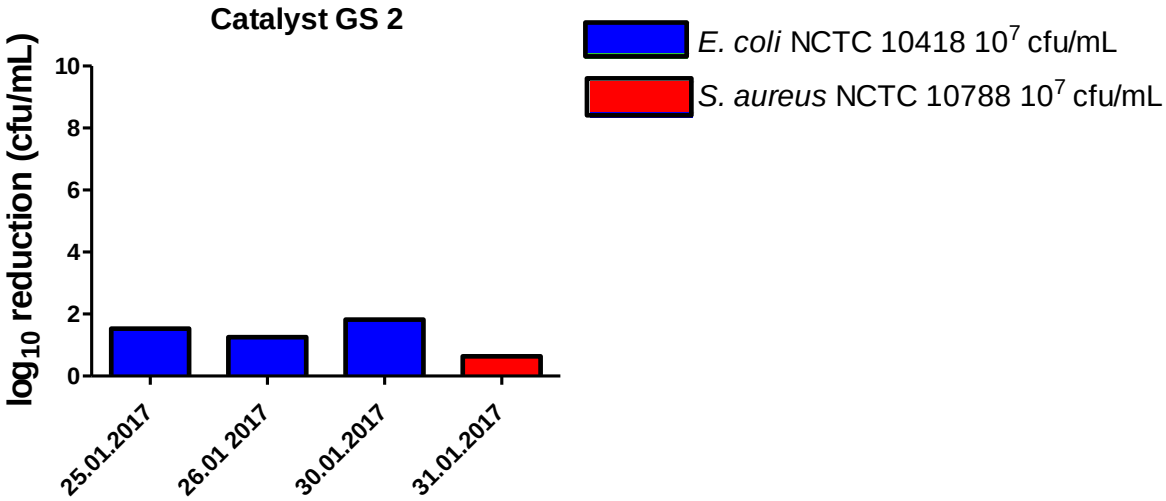
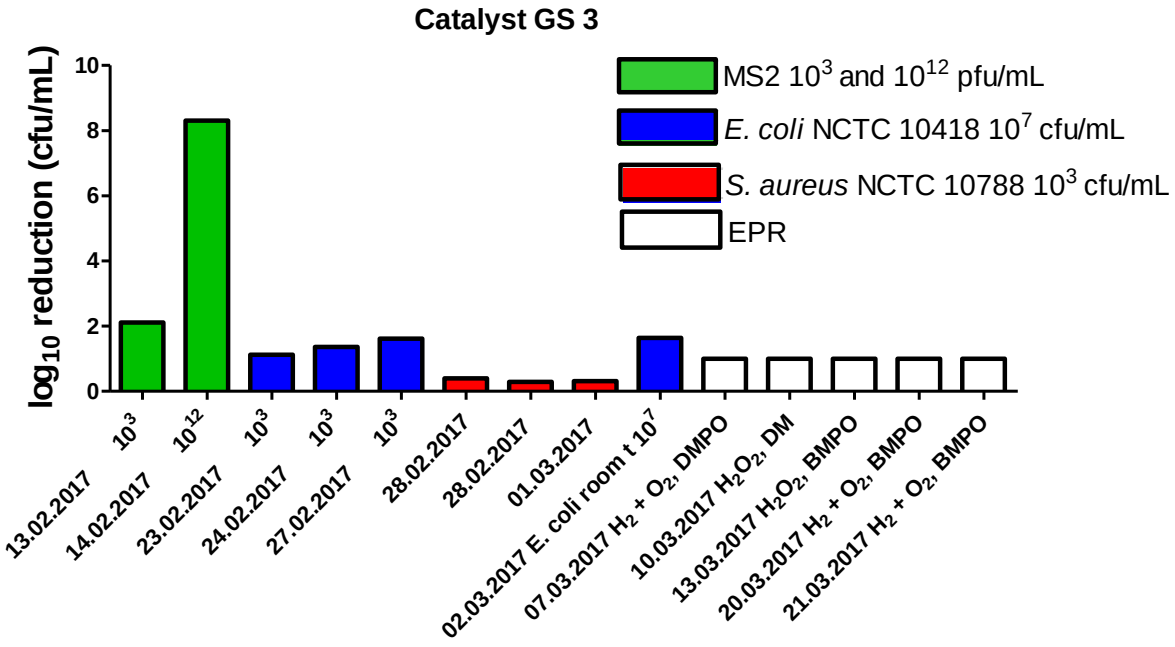


Figure 4.10 continued



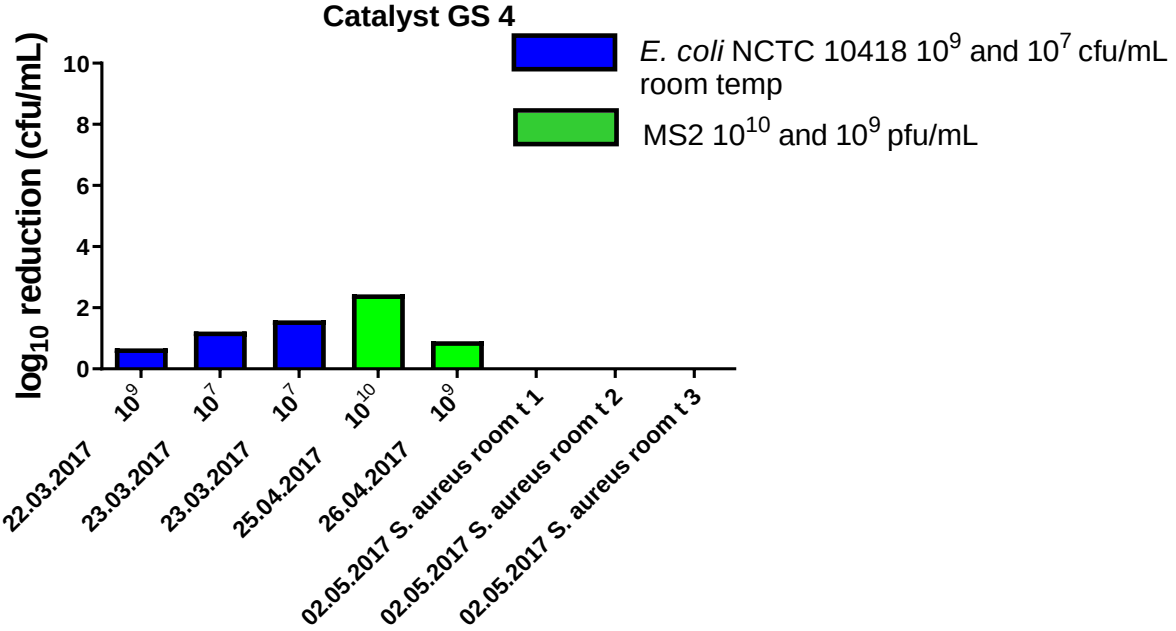
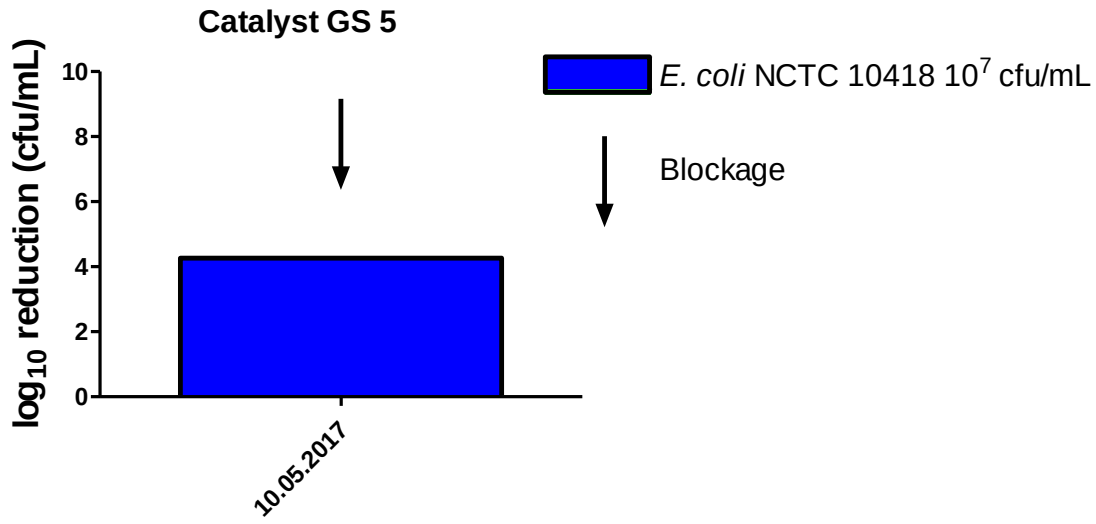
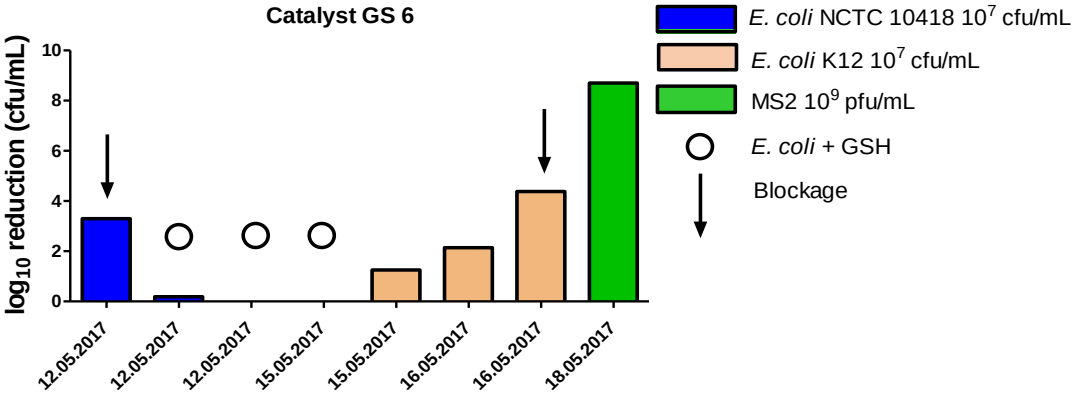
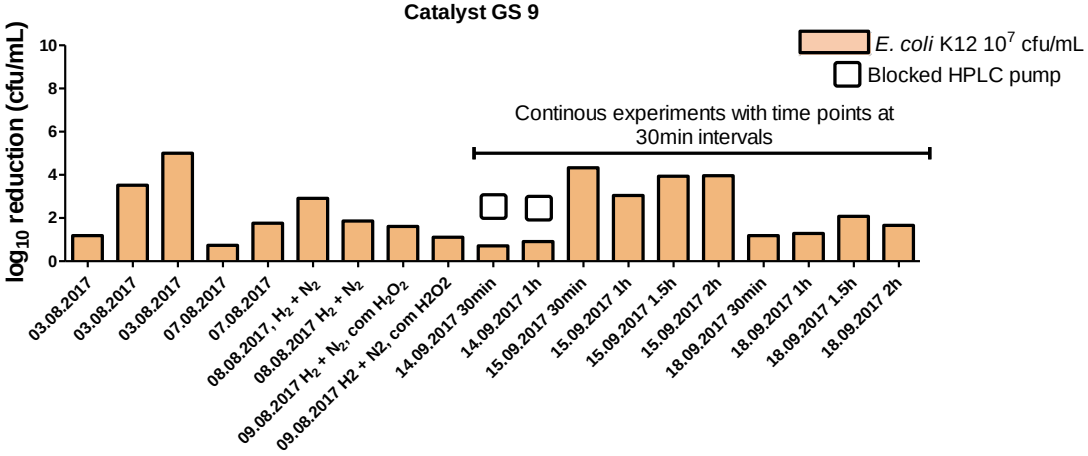


Figure 4.10 continued







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 $\log_{10}$ 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 $\log_{10}$) carried out on catalyst GS3 provided an 8.31 $\log_{10}$ reduction, whereas, the two experiments in high initial inoculum of MS2 (10.3 and 9.6 $\log_{10}$, respectively) carried out on catalyst GS4 provided much lower $\log_{10}$ 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 $\log_{10}$) on catalyst GS6 provided another high $\log_{10}$ 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 $\log_{10}$ reductions.

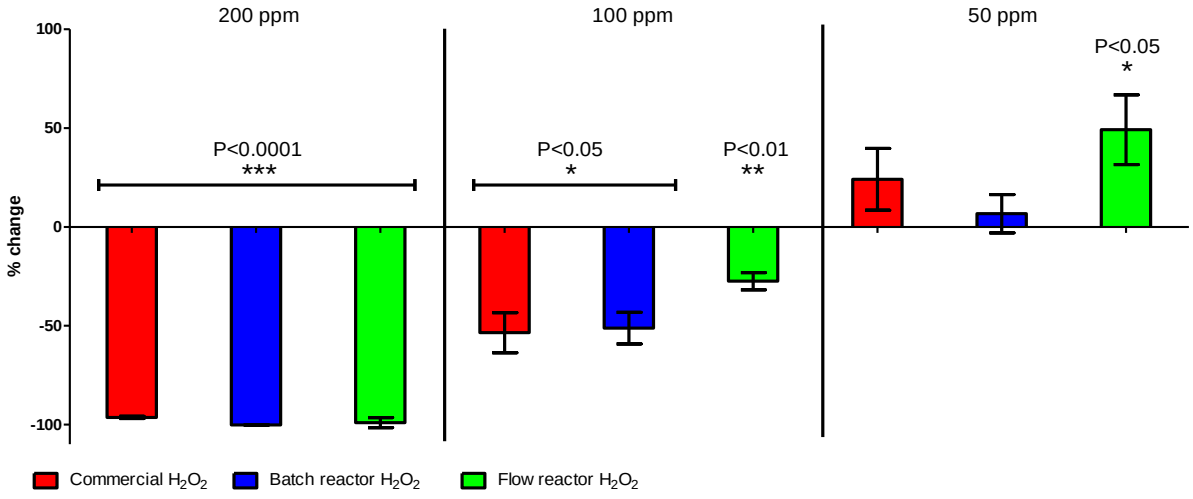
Another example of inconsistencies in results were results obtained in experiments carried out on catalyst bed GS9. The inconsistencies in the first five experiments were the $\log_{10}$ reductions obtained during the experiments carried out on the same day kept on increasing with each repeat. Another example of inconsistencies from the catalyst GS9 was when 2 hour experiments with 30 minutes time points provided large differences between the average $\log_{10}$ 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_2O_2 caused a 96% decrease of biofilm adhesion compared to the negative control biofilm and the batch reactor H_2O_2 caused a 100% decrease (all $P< 0.0001$). The 100 ppm concentration of commercial and batch reactor H_2O_2 caused a 54% and 51% decrease of biofilm adhesion respectively (both $P< 0.05$) and the flow reactor H_2O_2 caused a 27% biofilm adhesion decrease ($P< 0.01$). The 50 ppm concentration of commercial and batch reactor H_2O_2 caused a 24% and 7% increase in biofilm adhesion and a flow reactor H_2O_2 caused a 49% increase ($P< 0.05$).

Figure 4.11 Efficacy of H_2O_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_2O_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).

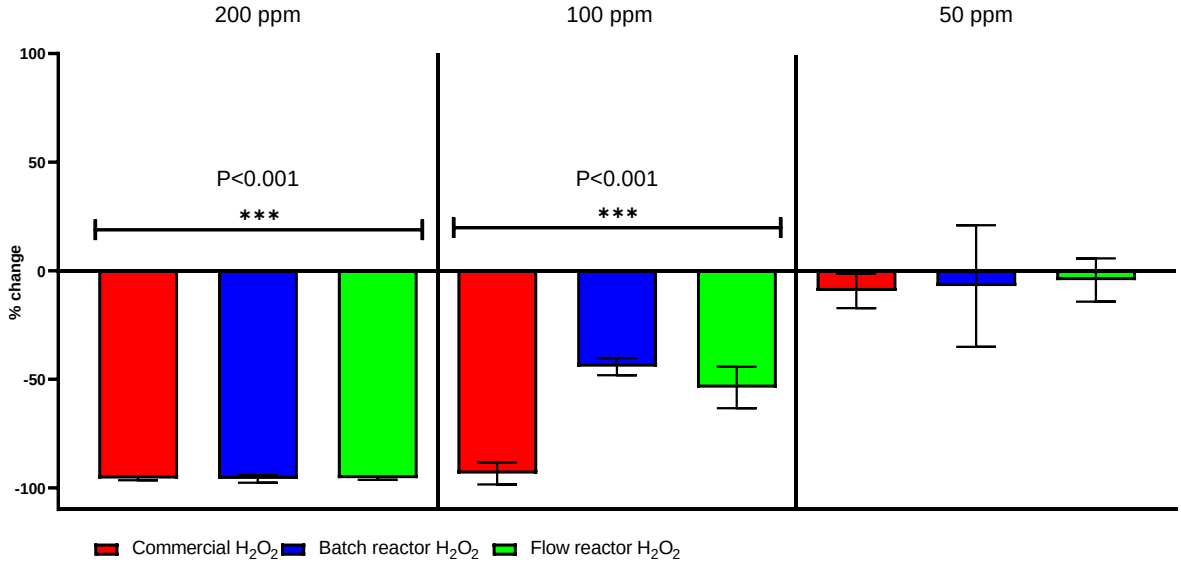


4.3.4.2 *B. subtilis* ATCC6633 biofilm prevention on a PVC surface

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_2O_2 all caused a 96% decrease in biofilm adhesion (P< 0.001). The 100 ppm concentration of commercial H_2O_2 caused a 93% decrease in biofilm adhesion (P< 0.001). Batch reactor H_2O_2 of 100 ppm concentration caused a 44% decrease in biofilm adhesion (P< 0.001). The 100 ppm concentration of flow reactor H_2O_2 caused

a 54% decrease in biofilm adhesion (P< 0.001). The 50 ppm concentration of commercial, batch reactor and flow reactor H_2O_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_2O_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_2O_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).

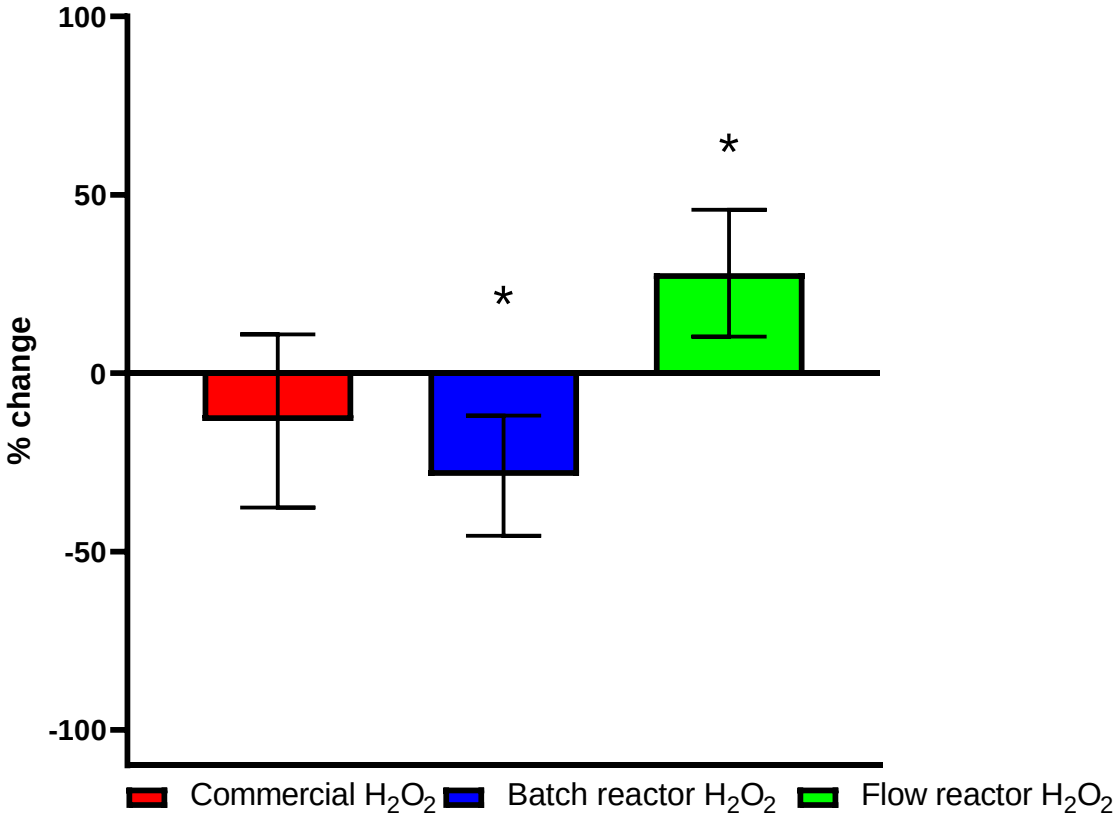


4.3.4.3 *B. subtilis* AEWD isolate biofilm prevention on a PVC surface

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 H_2O_2. Commercial H_2O_2 caused a 13% decrease in biofilm adhesion which was statistically insignificant. Batch reactor H_2O_2 caused a 29% decrease in biofilm adhesion ($P< 0.05$). Flow reactor H_2O_2 caused a 28% increase in biofilm adhesion ($P< 0.05$).

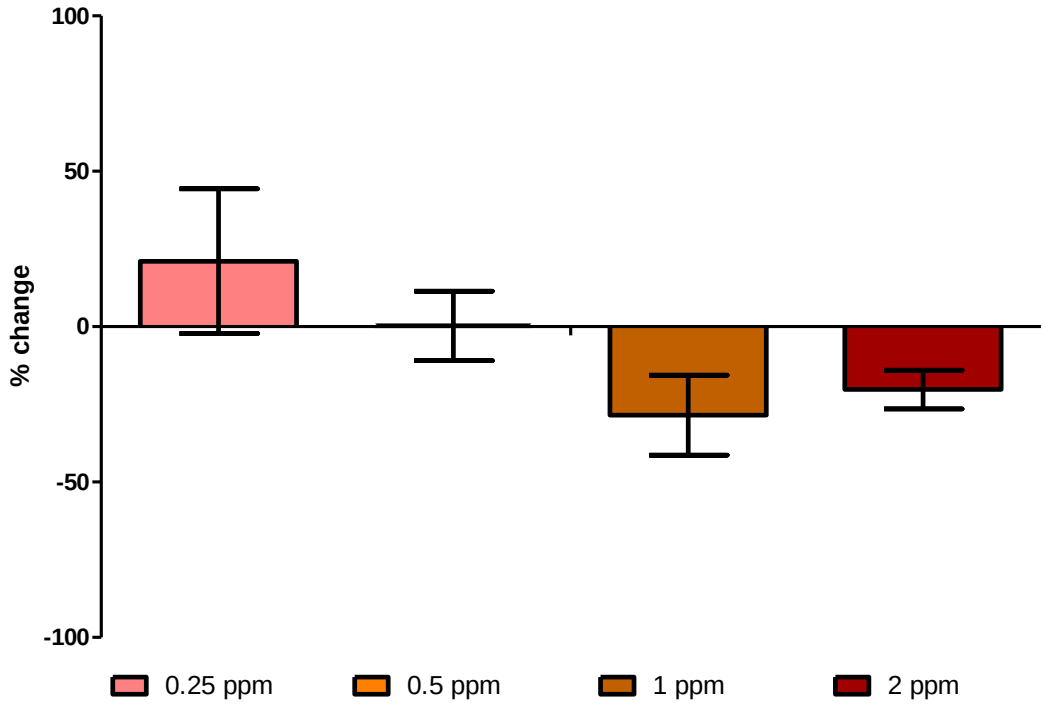
Figure 4.13 Efficacy of H_2O_2 on biofilm adhesion prevention experiments against *B. subtilis* AEWD isolate using 200 ppm concentration of commercial, batch reactor and flow reactor H_2O_2. Data were analysed using an unpaired t-test using GraphPad Prism software. The significance of results is shown as asterisks above the bars * = $P< 0.05$. (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).

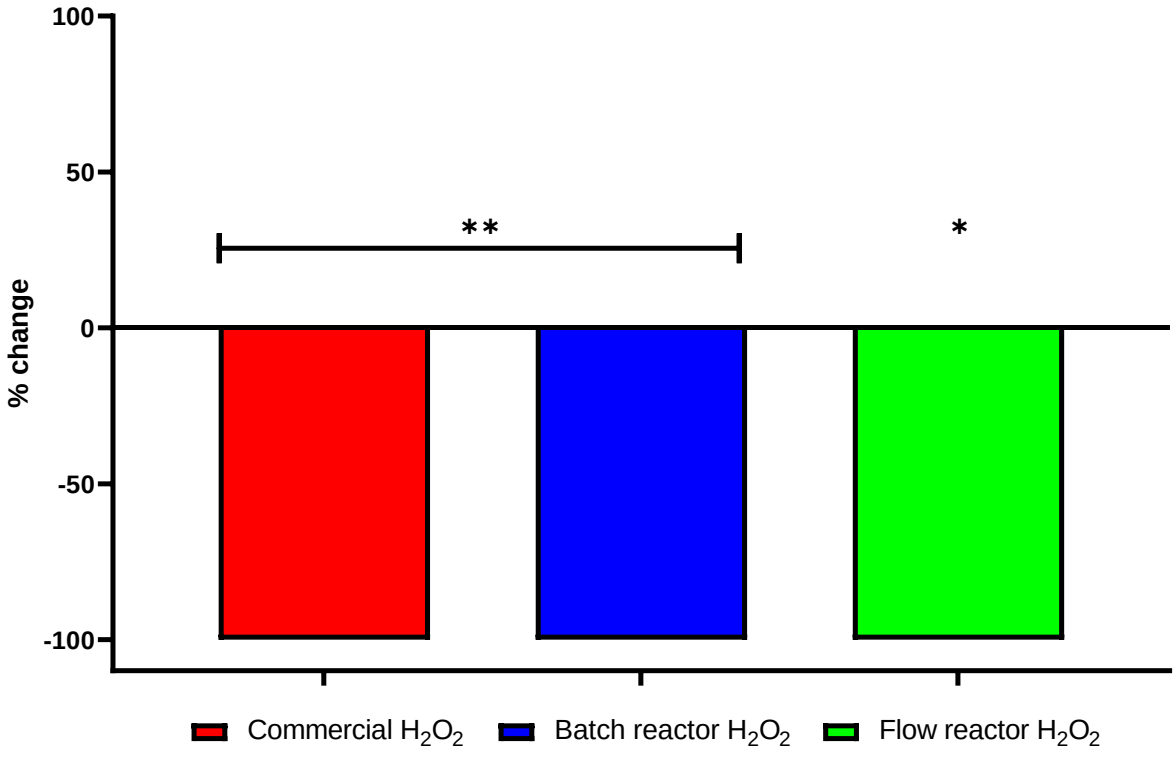
Figure 4.14 Efficacy of H_2O_2 on biofilm adhesion prevention experiments against *E. coli* K12 JM109 using NaOCl at 0.25 ppm, 0.5 ppm, 1 ppm and 2 ppm of active chlorine. Data were analysed using an unpaired t-test using GraphPad Prism software. (n=3).



4.3.4.5 *E. coli* K12 JM109 biofilm prevention on a stainless steel surface

200 ppm of commercial, batch reactor and flow reactor H_2O_2 caused a 100% decrease in adhesion to the stainless steel surface ($P< 0.01$ for commercial and batch reactor H_2O_2 and $P< 0.05$ for flow reactor H_2O_2) (Fig. 4.15).

Figure 4.15 Efficacy of H_2O_2 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 H_2O_2. Data were analysed using an unpaired t-test using GraphPad Prism software. The significance of results is shown as asterisks above the bars * = $P< 0.05$, ** = $P< 0.01$. (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_2O_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_2O_2. Such a marked difference in results between these two treatments indicates that H_2O_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_2O_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^{o}C. The temperature of 2^{o}C is used under standard reaction conditions as it maximises the concentration of H_2O_2 produced, therefore, when the temperature of the reaction is higher, it will produce less H_2O_2. A study conducted by Freakley *et al*. (2013) showed that increasing the reaction temperature from 2^{o}C to 20^{o}C decreased to production of H_2O_2 by about 30%. This observation adds to the argument that H_2O_2 plays a lesser part in killing bacteria in the flow reactor. H_2O_2 is continuously produced by the catalyst indicating that bacteria should always be in contact with H_2O_2. Furthermore, differences in efficacy between 100 and 200 ppm of H_2O_2 measured in a suspension test were not statistically significant. This reinforces the point that H_2O_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_2O_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*.,

2013). 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 theses 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_2O_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 H_2O_2, 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; Rossmoore 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_2O_2, superoxide (O_2•-), hydroxyl radical (OH•) and singlet oxygen ($^1O_2$). The effects of H_2O_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_2O_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

catalyst in the flow reactor used in this project catalyses the production of OH• 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 H_2O_2. This is to see the importance of free radicals and H_2O_2 for the mechanisms of microbicidal action.
2. Test flow reactor and H_2O_2 efficacy against *Deinococcus radiodurans* (*D. radiodurans*), a bacterium that is highly resistant against oxidative damage, to see the importance of free radicals and H_2O_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 H_2O_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^{o}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_2O_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_2O_2 synthesis

H_2O_2 synthesis was considered in detail in Chapter 2 Section 2.2.3. H_2O_2 was synthesised in a batch reactor in order to compare its activity with commercial H_2O_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^oC, 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_2O_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_2O_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.

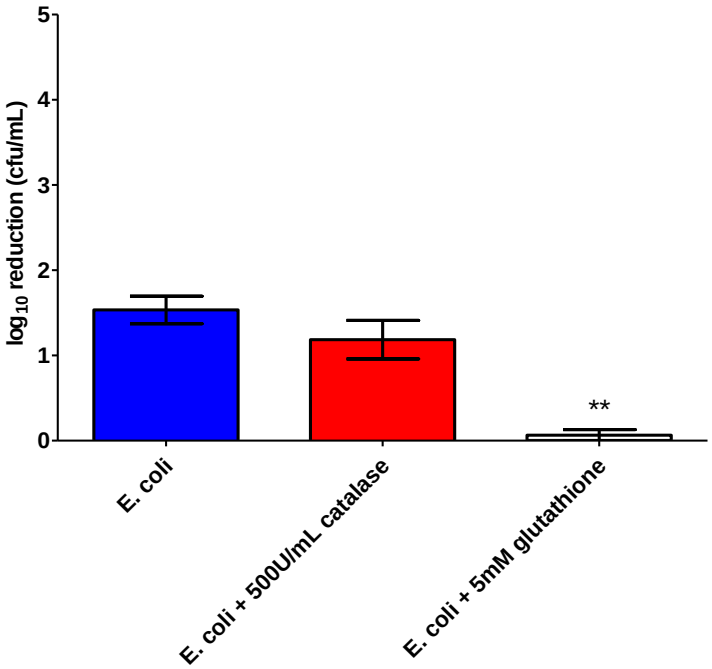
5.3 Results

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_2O_2 into oxygen and water (Alfonso-Prieto *et al*., 2009).

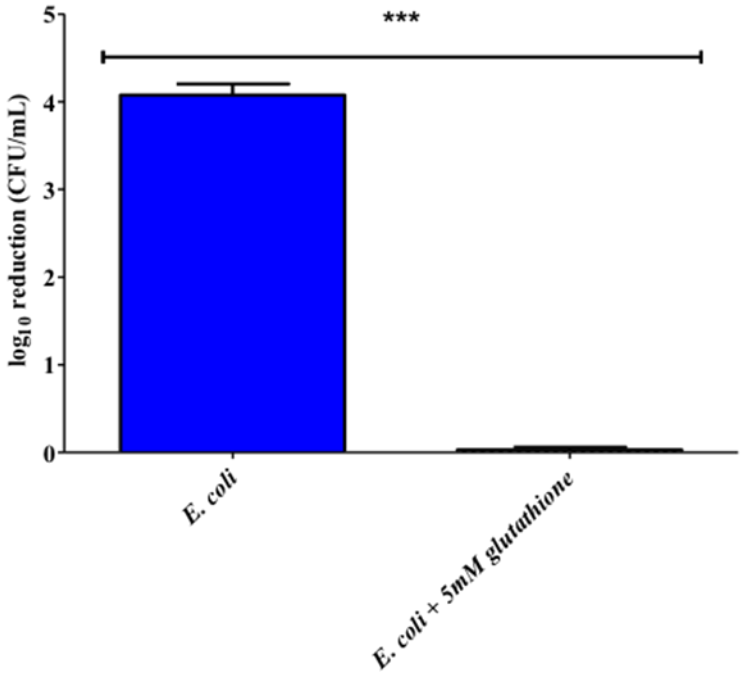
Results of the flow reactor experiments against *E. coli* NCTC10418 was 1.53 ± 0.28 $\log_{10}$ reduction. Results of the flow reactor experiments with 5 mM glutathione and 500 U/mL catalase were 0.06 ± 0.11 and 1.19 ± 0.39 $\log_{10}$ reductions respectively. A statistically significant difference in 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_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).

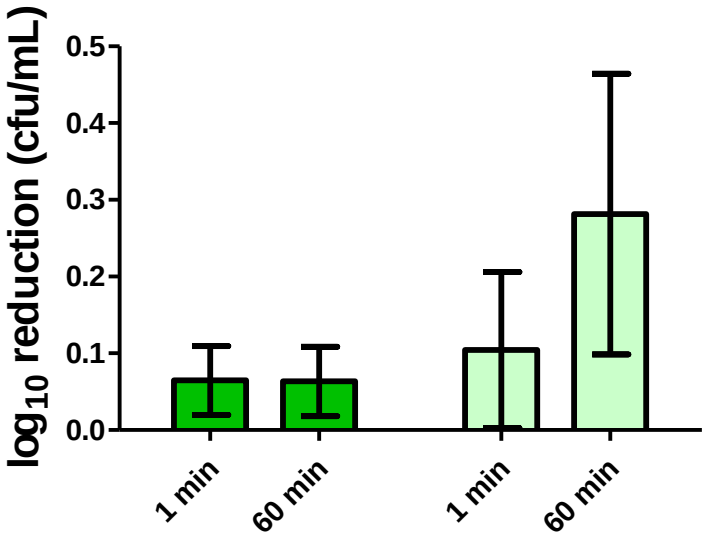


5.3.2 Flow reactor efficacy tests against *Deinococcus radiodurans*

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_2O_2 in suspension.

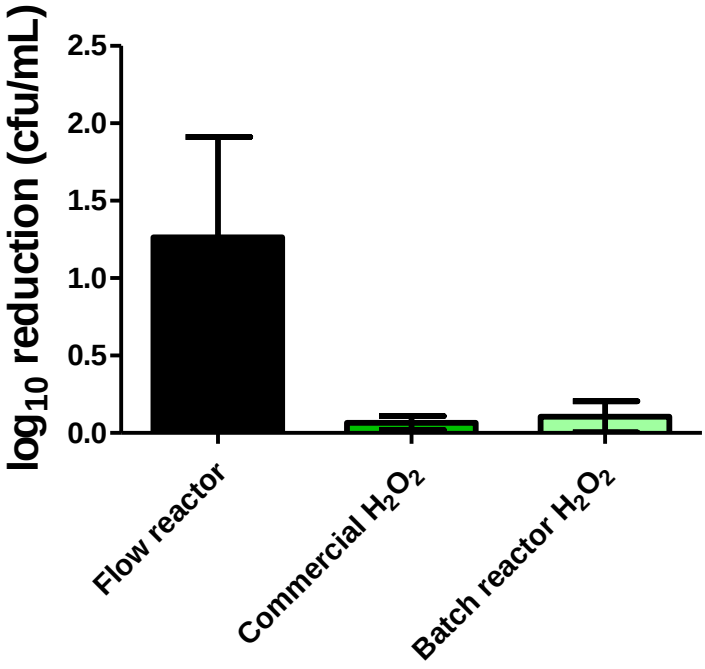
Figure 5.3 shows results of suspension tests against *D. radiodurans* at 1 minute and 1 hour contact times with commercial and batch reactor H_2O_2. Log_{10} reductions of the commercial H_2O_2 experiments were 0.06 ± 0.08 for both 1 min and 60 min time points. For experiments with the batch reactor H_2O_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_2O_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_2O_2 and □ 200ppm batch reactor H_2O_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_2O_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).

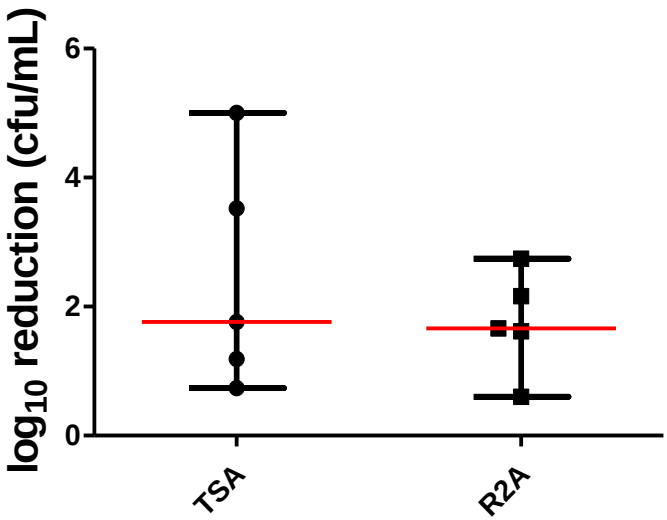


5.3.3 Observing bacterial ability to repair after treatment

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^{o}C, 42 mL/min gas flow of 2% H_2/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 H_2O_2 exposure. Figure 5.6 shows the effects of using 200 ppm of commercial and batch reactor H_2O_2 on *E. coli* K12 JM109 on SEM pictures (n=3). The flow reactor treatment caused an over the limit of detection $\log_{10}$ cfu/mL reductions (>7 $\log_{10}$ 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 H_2O_2 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 $\log_{10}$ reduction. Flow reactor experiment was carried out using standard reaction conditions. The initial inoculum for the experiments was 10^9 cfu/mL. A) control sample (n=3), B) 200 ppm of commercial H_2O_2 treatment (n=3), C) 200 ppm of batch reactor H_2O_2 treatment (n=3) and D) flow reactor treatment (n=1). SEM taken with a Hitachi TM3030Plus. 25 fields were observed for each treatment.

A)

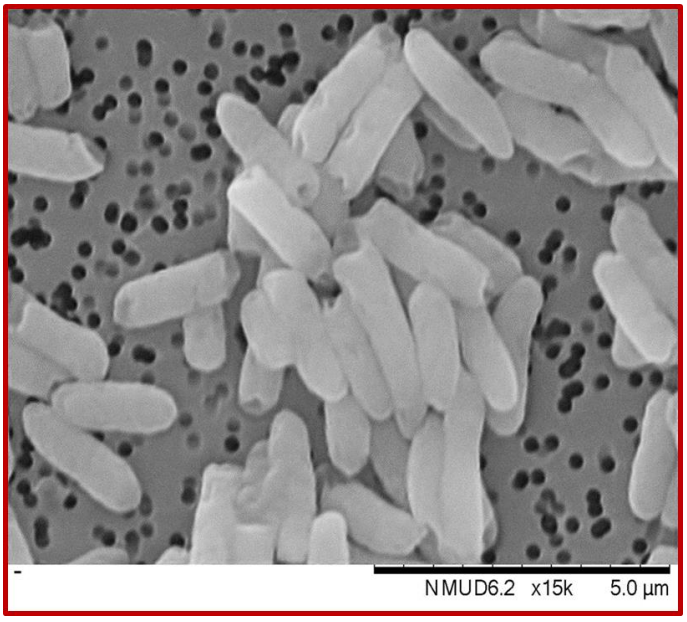
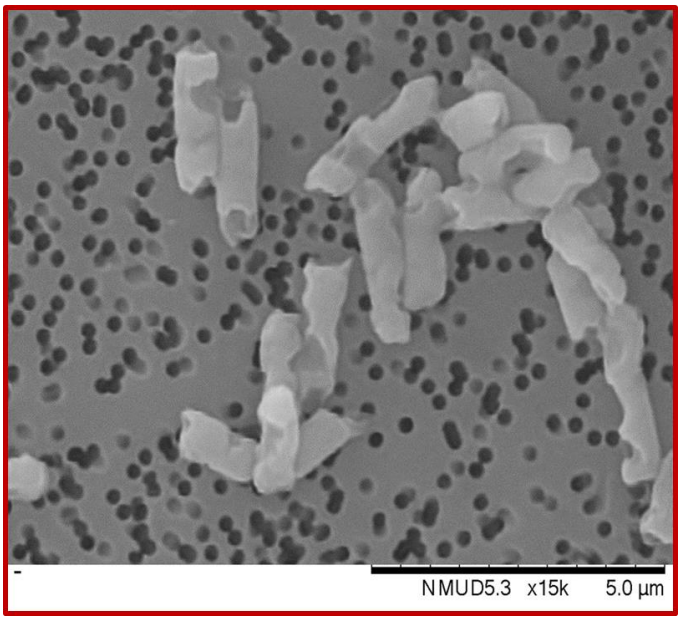


Figure 5.6 continued.

B)



C)

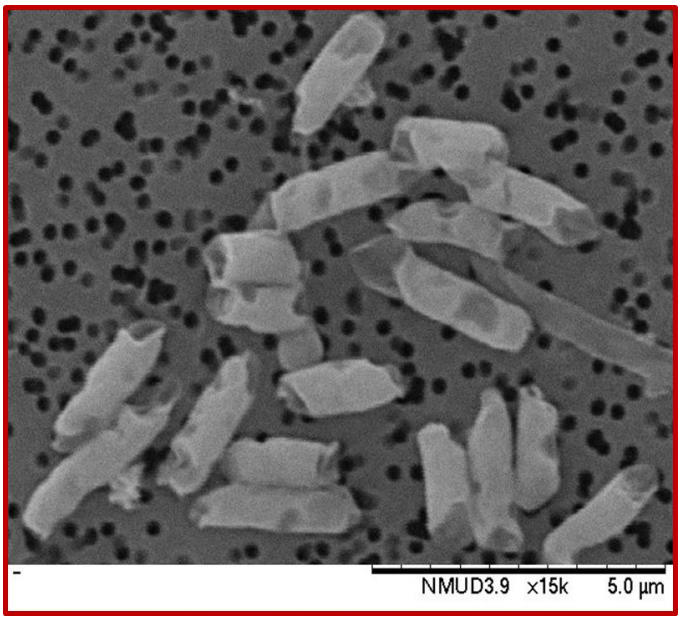
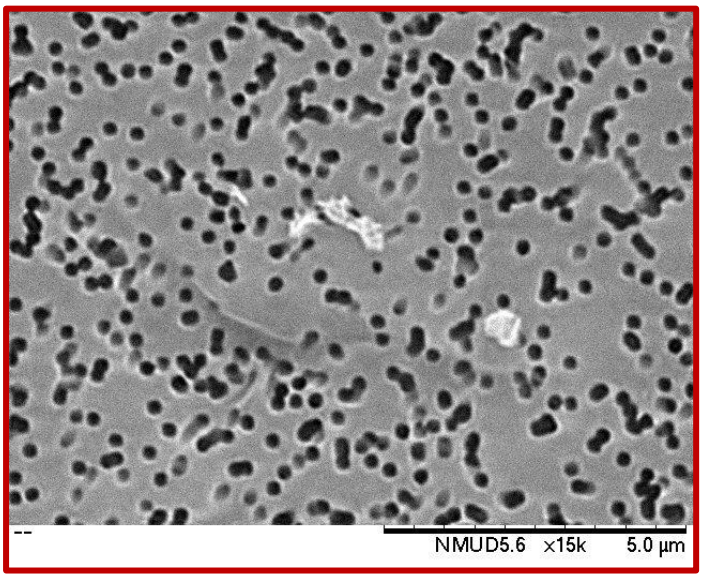


Figure 5.6 continued

D)



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_2O_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_2O_2 into H_2O 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_2O_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_2O_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_2O_2 itself. A study conducted by (Baatout *et al*., 2006) showed that a 1 hour treatment with 3% H_2O_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_2O_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_2O_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_2O_2 at around 200 ppm in the flow reactor system. The difference in H_2O_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_2O_2 in suspension (4 $\log_{10}$ reduction with the flow reactor treatment and ~ 1 $\log_{10}$ with the H_2O_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_2O_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_2O_2) was added to the suspension. SEM visualisations showed that commercial and batch reactor H_2O_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_2O_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 H_2O_2 prevented formation of *E. coli* K12JM109 and *B. subtilis* ATCC6633 biofilms.

It has been suggested that the direct synthesis of H_2O_2 on clusters of Pd follows a non-Langmuirian mechanism with first order kinetics with respect to H_2 and zero order kinetics with respect to O_2 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 O_2. This would be attributed to a high heat of adsorption of O_2 on Pd surfaces saturated with water (Ford *et al*., 2010). H_2O_2 formation is a sequential proton-electron transfer to OOH• and O_2 with the driving thermodynamic force being the chemical potential of H_2 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% H_2/N_2 gas mixture H• was observed in a solution. This proves that a homolytic cleavage of H_2 happens on a surface of the catalyst and a free radical diffuses into the solution. Moreover, when H_2O_2 was passed through the catalyst in a 5% H_2/N_2 gas mixture, only H• was detected. This suggests that the cleavage of H_2 does not initiate the production of reactive oxygen species (ROS) from H_2O_2. The radicals in solution during synthesis of H_2O_2 over supported AuPd catalysts have not been previously reported in the literature. It can be argued that activation of H_2 would induce a reaction with O_2 species adsorbed to the catalyst surface when both O_2 and H_2 are available. H• was not detected in solution when a reaction was carried out in a 2% H_2/air gas

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% N_2/H_2 gas mixture (1.19 ± 0.42 $\log_{10}$ reduction). The EPR spectra also show a lower amount of OH• produced when commercial or batch reactor H_2O_2 was passed through the reactor in air than when 2% H_2/air was passed through the reactor which correlates with lower microbicidal activity of H_2O_2 than the flow reactor treatment.

EPR experiments showed that ROS are able to desorb from the surface of the catalyst when H_2 and O_2 react and when H_2O_2 passes through the catalyst. This is however not the case with H_2 and H_2O_2. When H_2O_2 is synthesised in the flow reactor, the newly formed H_2O_2 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 $OOH^{\bullet}/O_2^{\bullet-}$. It was also observed that when commercial and batch reactor H_2O_2 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 O_2 and H_2 were passed through the reactor. When glutathione was passed through the reactor, H• was visible in a 5% H_2/N_2 mixture and no free radicals were seen in 2% H_2/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% H_2/air gas mixture is dependent on the initiation steps of the free radical flux. In the case when the 2% H_2/air gas mixture is passed through the reactor, the H• in the solution from the H_2 homolytic dissociation initiates a cascade of reactions by turning the O_2 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 H_2O_2 synthesised in the reactor which can support the flux of free radicals out of the surface of the catalyst. Considering the preformed H_2O_2, 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 H_2O_2. Hence, the catalyst being more microbicidal in a shorter time period.

The significantly improved microbicidal activity achieved in a catalysed reaction of H_2 and O_2 instead of using commercial H_2O_2 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 H_2 and O_2 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 H_2O_2 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 H_2O_2 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^{o}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 H_2O_2 over supported AuPd catalysts have not been previously reported in the literature. Disinfection of water while directly synthesising H_2O_2 from O_2 and H_2 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 H_2O_2. It was demonstrated that a packed bed reactor design is not effective in terms of reproducibility of microbial kill. The H_2O_2 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

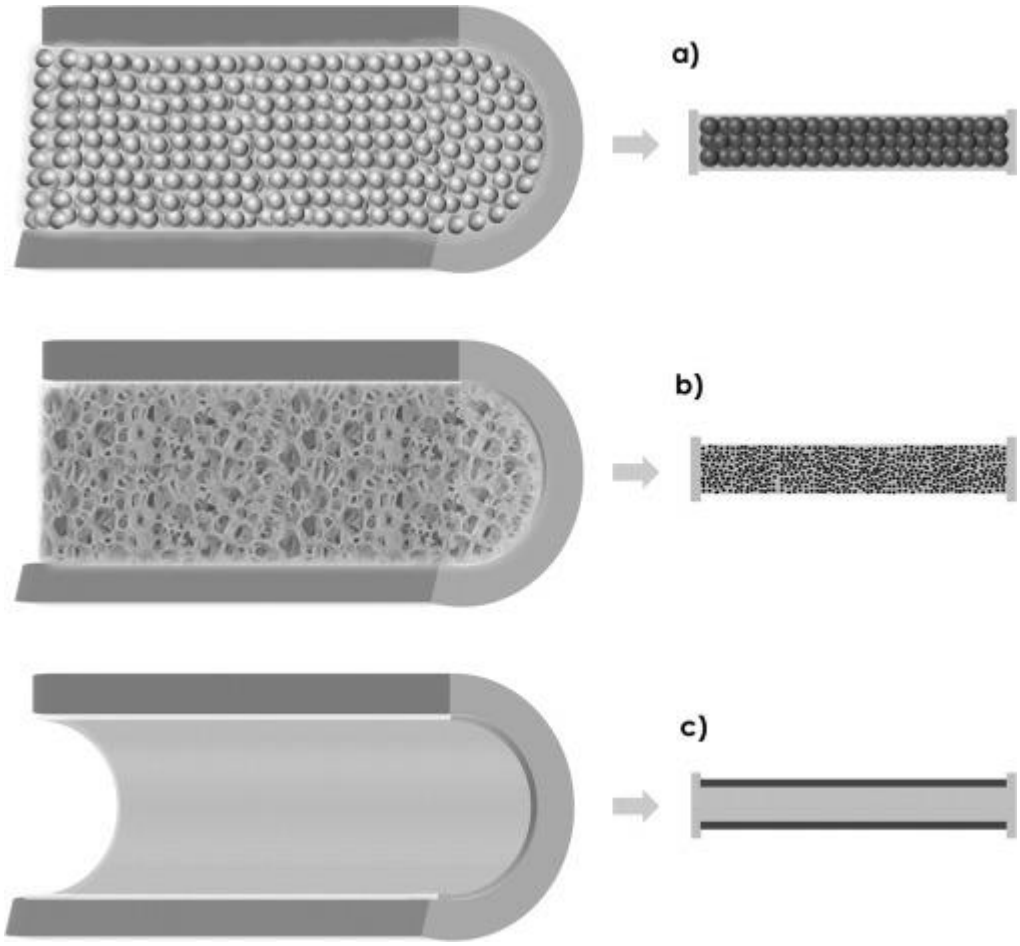
uncontrolled, significant pressure drops developing along the microchannel (which happened during this project) and limited heat transfer (Munirathinam *et al*., 2015). Therefore, exploring different designs for the catalyst bed would be an important topic of future experiments. Apart from the packed 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).

Figure 6.1 Schematic representations of microchannel cross sections a) packed-bed, b) monolithic and c) wall-coated microreactors (from Munirathinam *et al*., 2015).



Another interesting avenue to explore is producing H_2 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 O_2 and H_2 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

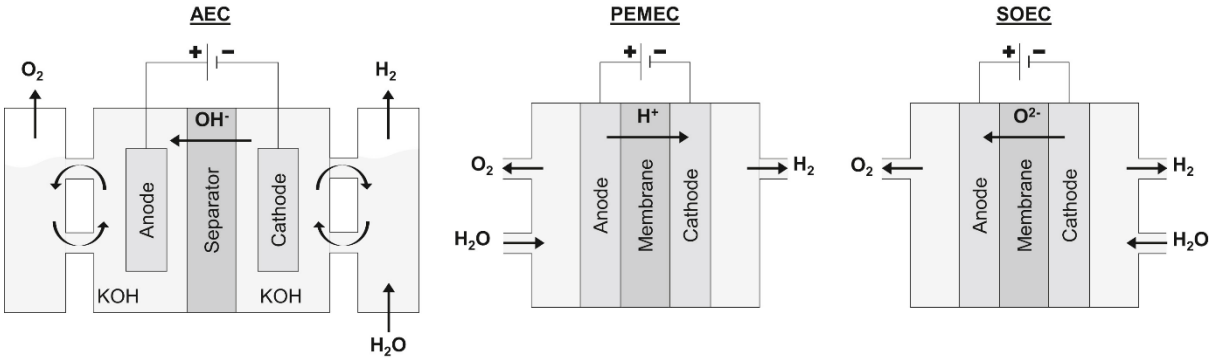
conductor. Sodium hydroxide (NaOH), potassium hydroxide (KOH) and sulphuric acid are mainly used with water in order to improve conductivity. The solution splits into negative and positive ions which readily conduct electricity in water by flowing from one electrode to another. The general principle 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^{o}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 H_2 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^{o}C caused a very similar $\log_{10}$ reduction of *E.coli* as a flow reaction in a standard 2^{o}C temperature, therefore, this should be further explored in the new optimised design.

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Appendix 1: ASM Microbe 2019 poster

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

G Suldecki[1], S Freakley[2], R Lewis[2], A Howe[2], M Perdjon[2], J Edwards[2], G Hutchings[2], J-Y Maillard[1]

1. Pharmaceutical Microbiology, School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, UK and 2. Cardiff Catalysis Institute, School of Chemistry, Cardiff University, Cardiff, UK.

Introduction & Aims

- There have been numerous outbreaks and pseudo-outbreaks associated with poorly disinfected endoscopes. [1]
- Rinse water poses an important problem during endoscope reprocessing notably with recontamination of endoscopes. [2]
- This project aims to explore the development of an integrated system that would use a novel catalytic technology to produce hydrogen peroxide (H_2O_2) to control and eliminate microbial contamination in rinse water in automated endoscope washer disinfectors.

Catalytic Reactor Design

- 1 wt. % AuPd/TiO_2 catalyst was prepared using a modified impregnation method (Fig. 1).[3]
- The catalyst bed uses Au-Pd bimetallic nanoparticles catalyst that produces H_2O_2 continuously at 100-200 ppm in water under 10 bar of 2% hydrogen/air (Fig. 2).

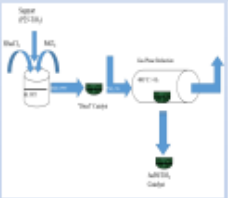


Fig. 1. Catalyst preparation process.

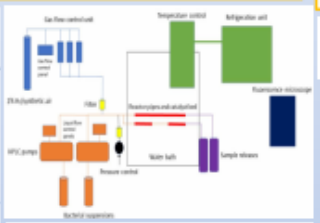


Fig. 2. Catalytic reactor set up.

Prevention of biofilm adhesion to surface

- Prevention of *E. coli* K12 JM109 attachment to PVC surface by H_2O_2 in 96 well plates measured by crystal violet assay (based on [4]).
- Plates were incubated in a shaking incubator at 120 rpm, 37ºC for 6 hours.
- ➢ H_2O_2 did not decompose significantly for the duration of the test.
- ➢ 200 and 100 ppm of H_2O_2 caused a 96-100% and 27-54% reduction in attachment of *E. coli* K12 JM109 to surface compared to untreated control (Fig. 4)
- ➢ 50 ppm of H_2O_2 treatments caused a 7-49% increase in bacterial attachment compared to untreated control (fig. 4)

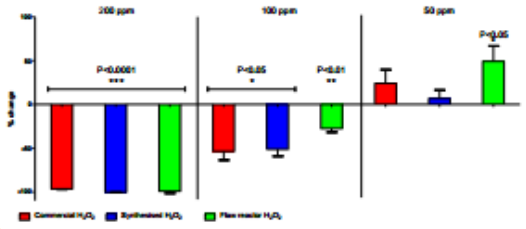


Fig. 4. Prevention of biofilm adhesion by H_2O_2. (n=3)

Catalytic Reactor & H_2O_2 Bactericidal Efficacy

- Standard reaction conditions: 10 bar pressure, temperature: 2ºC, 42 mL/min gas flow of 2% H_2/synthetic air, liquid flow rate = 0.2 mL/min, 120mg of 1wt.% AuPd/TiO_2 catalyst.
- Experiments conducted against *Escherichia coli* NCTC 10418 (10^7 cfu initial inoculum).
- Passing bacteria through the reactor gave a 1.53 log_{10} reduction for *E. coli* in 11 seconds indicating a much better activity than H_2O_2 in suspension (Fig. 3).

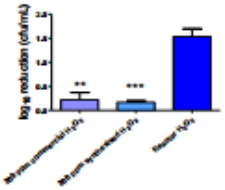
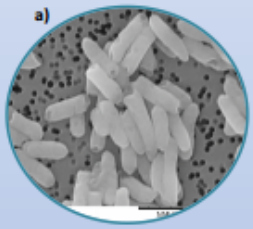
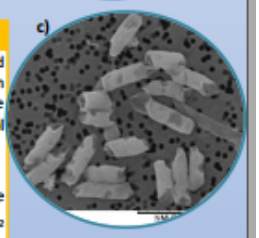


Fig. 3 Bactericidal efficacy of catalytic reactor or H_2O_2 in suspension.

Fig 5. SEM images of *E. coli* K12 JM109 a) untreated, b) treated with 200ppm of commercial stabilised H_2O_2 and c) treated with 200ppm of synthesised H_2O_2.





SEM imaging

- *E. coli* K12 JM109 samples treated with H_2O (causing 1 log_{10} reduction in viability), fixed in glutaraldehyde and dehydrated in ascending ethanol concetrations and HMDS [5]).
- SEM taken with a Hitachi TM3030Plus.
- ➢ Clear cell wall damage can be observed from 200ppm H_2O_2 regardless of the treatment (Fig 5.)

Key findings

- ➢ 1 wt. % AuPd/TiO_2 catalyst prepared using a modified impregnation method can produce 100-200ppm of H_2O_2.
- ➢ 200 ppm of H_2O_2 synthesised by the catalytic reactor prevented bacterial attachment to PVC surface.
- ➢ Low concentrations of H_2O_2 produced by catalysis produces a severe bactericidal effect linked to massive structural damages. Similar damages have been observed with other oxidising agents albeit at higher concentrations. [6]
- ➢ H_2O_2 is usually used at 75,000 ppm in sterilisation applications. [7]

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[1] Weber and Rutala (2012) *Infection Control & Hospital Epidemiology*, 33(3), pp. 230-234. [2] Joint Working Group of Hospital Infection Society and Public Health Laboratory Service (2002) *Journal of Hospital Infection*, 51, pp. 7-16. [3] Sankar *et al.* (2012) *ACS Nano*, 6(8), pp. 6600-6613. [4] Simoes *et al.* (2007) *Applied and Environmental Microbiology*, DOI: 10.1128/AEM.00837-07. [5] Hazlin-Chong and Manefield (2012) *Journal of Microbiological Methods*, 90, pp. 96-99. [6] Galeano *et al.* (2017) Disinfection by Chemical Oxidation Methods. DOI: https://doi.org/10.1007/698_2017_179. [7] Martin *et al.* (2008) *Journal of Hospital Infections*, 69, pp. 377-383.

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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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1. Pharmaceutical Microbiology, School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, UK and 2. Cardiff Catalysis Institute, School of Chemistry, Cardiff University, Cardiff, UK.

Introduction and aims

- There have been numerous outbreaks and pseudo-outbreaks associated with poorly disinfected endoscopes. [1]
- Rinse water poses an important problem during endoscope reprocessing notably with recontamination of endoscopes. [2]
- This project aims to explore the development of an integrated system that would use a novel catalytic technology to produce hydrogen peroxide (H_2O_2) to control and eliminate microbial contamination in rinse water in automated endoscope washer disinfectors.

Catalytic Reactor design

- 1 wt. % AuPd/TiO_2 catalyst was prepared using a modified impregnation method (Fig. 1).[3]
- The catalyst bed uses Au-Pd bimetallic nanoparticles catalyst that produces H_2O_2 continuously at 100-200 ppm in water under 10 bar of 2% hydrogen/air (Fig. 2).

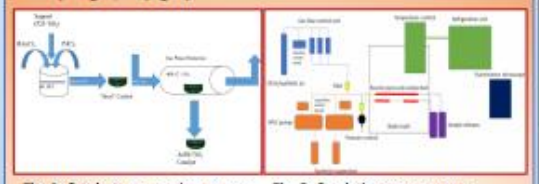

Fig. 1. Catalyst preparation process. Fig. 2. Catalytic reactor set up.

Suspension tests

- Use of a suspension test based on EN1276 to determine bactericidal efficacy of commercial formulated H_2O_2 against *Staphylococcus aureus* NCTC 10788, *Enterococcus hirae* NCTC 13359, *Escherichia coli* NCTC 10418 and *Pseudomonas aeruginosa* NCTC 13359.
- Hard (0.3g/100mL BSA, MgCl, $CaCl_2$, $NaHCO_3$) and clean (distilled water) water conditions were used with 100, 200, 1000 and 10000 ppm concentrations of commercial formulated H_2O_2.

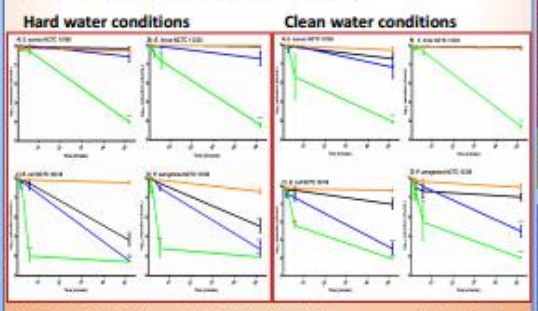

Fig 3. Bactericidal efficacy of H_2O_2 in hard and clean water conditions. (n=3)

- Gram negatives were more susceptible to stabilised H_2O_2 than Gram positives.
- It took 60 min and 10,000 ppm concentration of commercial formulated H_2O_2 to obtain a 4 log_{10} reduction for all of the bacteria.
- H_2O_2 was more effective in hard water than in clean water.

Prevention of biofilm adhesion to surface

- Prevention of *E. coli* K12 JM109 attachment to PVC surface by H_2O_2 in 96 well plates measured by crystal violet assay (based on [4]).
- Plates were incubated in a shaking incubator at 120 rpm, 37°C for 6 hours.
- H_2O_2 did not decompose significantly for the duration of the test.
- 200 and 100 ppm of synthesised H_2O_2 caused a 100% and 51% reduction in attachment of *E. coli* K12 JM109 to surface compared to untreated control (Fig. 4)
- 50 ppm of synthesised H_2O_2 treatments caused a 7% increase in bacterial attachment compared to untreated control (fig. 4)

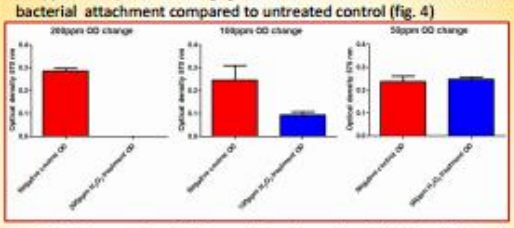

Fig. 4. Prevention of biofilm adhesion by synthesised H_2O_2. (n=3)

SEM imaging

- *E. coli* K12 JM109 samples treated with 200 ppm of commercial stabilised and synthesised H_2O_2, fixed in glutaraldehyde and dehydrated in ascending ethanol concetrations and HMDS, (based on [5]).
- SEM taken with a Hitachi TM3030Plus.
- Clear cell wall damage can be observed from 200ppm H_2O_2 regardless of the treatment (Fig 5.). Both treatments caused a 1 log_{10} reduction.

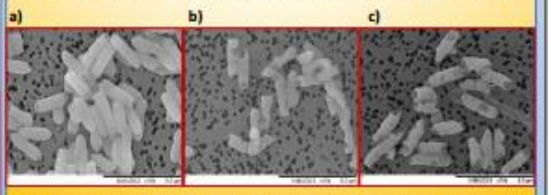

Fig 5. SEM images of *E. coli* K12 JM109 a) untreated, b) treated with 200ppm of commercial stabilised H_2O_2 and c) treated with 200ppm of synthesised H_2O_2.

Conclusions

- 1 wt. % AuPd/TiO2 catalyst prepared using a modified impregnation method can produce 100-200ppm of H_2O_2.
- 200 ppm of H_2O_2 synthesised by the catalytic reactor prevented bacterial attachment to PVC surface.
- Low concentrations of H_2O_2 produced by catalysis produces a severe bactericidal effect linked to massive structural damages and can prevent the attachment of bacteria to surfaces. Similar damages have been observed with other oxidising agents albeit at higher concentrations. [6]
- H_2O_2 is usually used at 75,000 ppm in sterilisation applications. [7]

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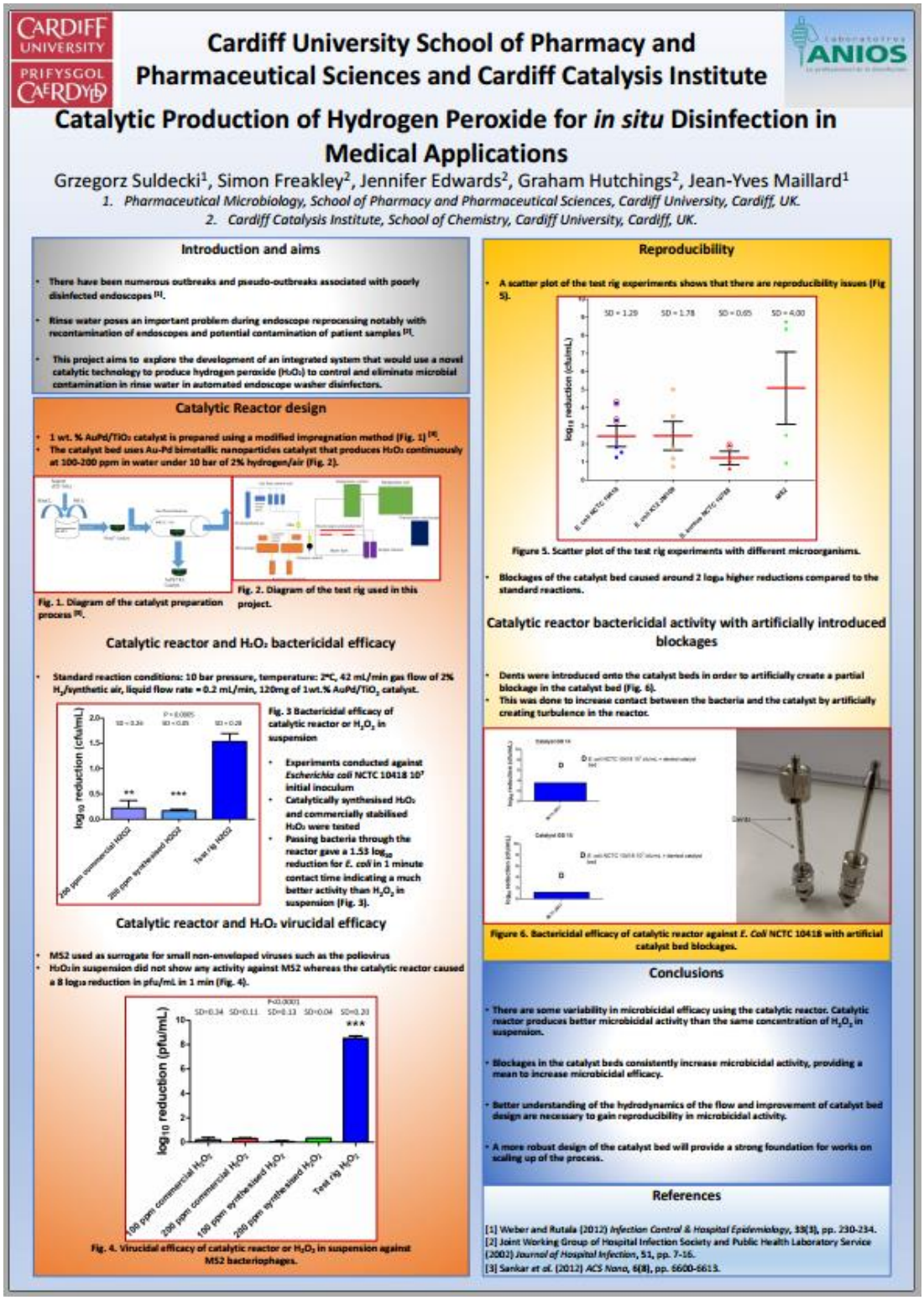
Appendix 3: Cardiff Catalysis Institute conference 2018 poster



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

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

Grzegorz Suldecki[1], Simon Freakley[2], Jennifer Edwards[2], Graham Hutchings[2], Jean-Yves Maillard[1]

1. *Pharmaceutical Microbiology, School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, UK.*
2. *Cardiff Catalysis Institute, School of Chemistry, Cardiff University, Cardiff, UK.*

Introduction and aims

- There have been numerous outbreaks and pseudo-outbreaks associated with poorly disinfected endoscopes [1].
- Rinse water poses an important problem during endoscope reprocessing notably with recontamination of endoscopes and potential contamination of patient samples [1].
- This project aims to explore the development of an integrated system that would use a novel catalytic technology to produce hydrogen peroxide (H_2O_2) to control and eliminate microbial contamination in rinse water in automated endoscope washer disinfectors.

Catalytic Reactor design

- 1 wt. % AuPd/TiO_2 catalyst is prepared using a modified impregnation method (Fig. 1) [3].
- The catalyst bed uses Au-Pd bimetallic nanoparticles catalyst that produces H_2O_2 continuously at 100-200 ppm in water under 10 bar of 2% hydrogen/air (Fig. 2).

Fig. 1. Diagram of the catalyst preparation process [3].

Fig. 2. Diagram of the test rig used in this project.

Catalytic reactor and H_2O_2 bactericidal efficacy

- Standard reaction conditions: 10 bar pressure, temperature: 2°C, 42 mL/min gas flow of 2% H_2/synthetic air, liquid flow rate = 0.2 mL/min, 120mg of 1wt.% AuPd/TiO_2 catalyst.

Fig. 3 Bactericidal efficacy of catalytic reactor or H_2O_2 in suspension

- Experiments conducted against *Escherichia coli* NCTC 10418 10^7 initial inoculum
- Catalytically synthesised H_2O_2 and commercially stabilised H_2O_2 were tested
- Passing bacteria through the reactor gave a 1.53 $\log_{10}$ reduction for *E. coli* in 1 minute contact time indicating a much better activity than H_2O_2 in suspension (Fig. 3).

Catalytic reactor and H_2O_2 virucidal efficacy

- MS2 used as surrogate for small non-enveloped viruses such as the poliovirus
- H_2O_2 in suspension did not show any activity against MS2 whereas the catalytic reactor caused a 8 $\log_{10}$ reduction in pfu/mL in 1 min (Fig. 4).

Fig. 4. Virucidal efficacy of catalytic reactor or H_2O_2 in suspension against MS2 bacteriophages.

Reproducibility

- A scatter plot of the test rig experiments shows that there are reproducibility issues (Fig 5).

Figure 5. Scatter plot of the test rig experiments with different microorganisms.

- Blockages of the catalyst bed caused around 2 $\log_{10}$ higher reductions compared to the standard reactions.

Catalytic reactor bactericidal activity with artificially introduced blockages

- Dents were introduced onto the catalyst beds in order to artificially create a partial blockage in the catalyst bed (Fig. 6).
- This was done to increase contact between the bacteria and the catalyst by artificially creating turbulence in the reactor.

Figure 6. Bactericidal efficacy of catalytic reactor against *E. Coli* NCTC 10418 with artificial catalyst bed blockages.

Conclusions

- There are some variability in microbicidal efficacy using the catalytic reactor. Catalytic reactor produces better microbicidal activity than the same concentration of H_2O_2 in suspension.
- Blockages in the catalyst beds consistently increase microbicidal activity, providing a mean to increase microbicidal efficacy.
- Better understanding of the hydrodynamics of the flow and improvement of catalyst bed design are necessary to gain reproducibility in microbicidal activity.
- A more robust design of the catalyst bed will provide a strong foundation for works on scaling up of the process.

References

[1] Weber and Rutala (2012) *Infection Control & Hospital Epidemiology*, 33(8), pp. 230-234.
[2] Joint Working Group of Hospital Infection Society and Public Health Laboratory Service (2002) *Journal of Hospital Infection*, 51, pp. 7-16.
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