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# The effects of oxygen and reactive oxygen species on antibiotic resistance and microbial communities in chronic wounds

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**THE EFFECTS OF OXYGEN AND REACTIVE OXYGEN SPECIES ON  
ANTIBIOTIC RESISTANCE AND MICROBIAL COMMUNITIES IN CHRONIC  
WOUNDS**

by

**LINDSEY GLEW**

A thesis submitted to Plymouth University  
in partial fulfilment for the degree of

**DOCTOR OF PHILOSOPHY**

School of Biomedical and Biological Sciences

In collaboration with  
The Diving Diseases Research Centre

## **Abstract**

Infection is one of the factors that may contribute to non-healing of chronic wounds; the presence of antibiotic resistant bacteria serves to exacerbate the problem due to limited treatment options. Bacteria utilise several mechanisms to survive exposure to antibiotics, including synthesis of deactivating enzymes, target modification or substitution, changes to membrane permeability, upregulation of efflux pumps and the formation of a biofilm. Quorum sensing is a density-dependent mechanism of bacterial cell to cell communication that can be instrumental in co-ordinating biofilm initiation. Hyperbaric oxygen therapy (HBOT) is an option offered to some patients with chronic wounds, including diabetic foot ulcers. Evidence suggests that HBOT can reduce the incidence of major amputation in these patients. As well as the direct toxicity of increased tissue oxygenation on anaerobic bacteria HBOT may also increase levels of reactive oxygen and nitrogen species in the wound environment. This study aimed to investigate the effects of hyperoxia and oxidative damage on three specific mechanisms of antibiotic resistance: the activity of penicillinase, an antibiotic deactivating enzyme synthesised by bacteria; the activity of quorum sensing signalling molecules (AHLs); and biofilms and their associated bacteria. It also analysed the population dynamics of, primarily, bacteria in diabetic foot ulcers during HBOT, by the use of molecular analysis tools such as PCR-DGGE. The presence of fungal species was investigated in wounds prior to HBOT and in two wounds at two points during HBOT. This study found that hydrogen peroxide, hypochlorous acid and peroxyxynitrite reduced the activity of penicillinase *in vitro*. Hypochlorous acid reduced the activity of a range of AHLs *in vitro* but not *in vivo*. Oxygen concentration did not have any impact on biofilm mass, nor did it significantly affect the ability of an oxidant-generating enzyme to reduce live bacterial cells within a biofilm. The population dynamics of bacterial species identified in all the wounds were complex and did not undergo identifiable changes during HBOT. Fungal species were identified in all wounds prior to HBOT, though different profiles were observed in the two wounds investigated during HBOT. These results suggest that oxidants could play a role in the attenuation of antibiotic resistance in chronic wound bacteria. It is unclear whether HBOT alters the population dynamics of non-healing wound microflora.

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## Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

Work submitted for this research degree at the Plymouth University has not formed part of any other degree either at Plymouth University or at another establishment.

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## Abbreviations and Definitions

ABCs	ATP-binding cassette transporters
AGE	advanced glycation end product
AHL	acyl-homoserine lactone
AIP	autoinducing peptide
ADP	adenosine diphosphate
APS	ammonium persulfate
ATA	atmospheres absolute
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
CFU	colony forming unit
CLSM	confocal laser scanning microscopy
CVU	chronic venous ulcer
Da	Daltons
DDRC	Diving Diseases Research Centre
DFC	diabetic foot clinic
DFU	diabetic foot ulcers
DGGE	denaturing gradient gel electrophoresis
DHFR	dihydrofolate reductase
dH <sub>2</sub> O	distilled water
DMF	N,N-dimethylformamide
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetic acid
EPS	extracellular polymeric substances
ESBLs	extended spectrum $\beta$ -lactamases

FISH	fluorescence in-situ hybridization
GFP	green fluorescent protein
GSH	reduced glutathione
HBO	hyperbaric oxygen
HBOT	hyperbaric oxygen therapy
HCl	hydrochloric acid
HHQ	4-hydroxy-2-heptylquinoline
HO <sup>•</sup>	hydroxyl radical
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
HOBr	hypobromous acid
HOCl	hypochlorous acid
HSL	homoserine lactone
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny Broth (Luria Broth)
LPO	lactoperoxidase
MASP	membrane associated sensor protein
MDR	multi-drug resistant
MDS	multidimensional scaling
MHB	Mueller Hinton II broth
MIC	minimum inhibitory concentration
MPO	myeloperoxidase
MRSA	methicillin resistant <i>Staphylococcus aureus</i>
MS-MS	tandem mass spectroscopy
MV	membrane vesicles
MW	molecular weight
<i>m/z</i>	mass to charge ratio

NLF	non-lactose fermenting
NO <sup>•</sup>	nitric oxide
NOS2	nitric oxide synthase 2
O <sub>2</sub> <sup>•-</sup>	superoxide radical
ONOO <sup>-</sup>	peroxynitrite
ONOOH	peroxynitrous acid
PBPs	penicillin binding protein
PCR	polymerase chain reaction
PNA	peptide nucleic acid
PNAG	poly- <i>N</i> -acetylglucosamine
pO <sub>2</sub>	partial pressure of oxygen
PQS	<i>Pseudomonas</i> quinolone signal
PU	pressure ulcer
QS	quorum sensing
RLU	Relative Luminescence Unit
RNS	reactive nitrogen species
ROS	reactive oxygen species
RS	reactive species
SCN <sup>-</sup>	thiocyanate
SOD	superoxide dismutase
TCOM	transcutaneous oxygen tension monitoring
tcpO <sub>2</sub>	transcutaneous partial pressure of oxygen
TEMED	<i>N,N,N',N'</i> - tetramethylethylenediamine
TMB	3,3',5,5'-tetramethylbenzidine
Tris	tris(hydroxymethyl)aminomethane
TSA	tryptone soya agar

TSB	tryptone soya broth
Ultrapure water	MilliQ, resistivity = $18 \text{ M}\Omega\cdot\text{cm}^{-1}$
VBNC	viable but non-culturable
VEGF	vascular endothelial growth factor
X-Gal	5-bromo-4-chloro-indolyl- $\beta$ -D-galactopyranoside

## Chapter 1

### Introduction

#### 1.1. Introduction

Antibiotic resistance has become a regular feature of news reports. Government targets to reduce the incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) in our hospitals are not just constructed to quell public fears; nosocomial infections also carry a high financial burden. Antibiotic resistance is not restricted to *Staphylococcus aureus* (*S. aureus*), other organisms of clinical relevance include *Enterococcus faecium* (*E. faecium*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and several *Acinetobacter* species (Arias & Murray, 2009). To illustrate the extent of this problem, in 2006, 25-50% of *S. aureus* isolates were found to be resistant to oxacillin in much of Europe, and in Britain, 10-25% of *E. faecium* isolates were found to be vancomycin resistant (Gould, 2008).

Bacteria utilize several mechanisms to resist antibiotics, an arsenal that is now considered to include living within a biofilm. A biofilm is a heterogeneous community of organisms within a matrix of extracellular polymeric substances (EPS). Biofilm bacteria have been shown to be more resistant to antibiotics than their planktonic counterparts, a problem which is now recognised in many clinical areas. Recalcitrant infections associated with medical implants (e.g. hip prostheses and cardiac pacemakers), urinary catheters, endotracheal tubes, and some chronic conditions (e.g. otitis media, sinusitis and chronic wounds) are all thought to involve biofilms (Costerton *et al.* 2003). At present, the failure of antibiotic therapy to resolve infection may result in removal of the prosthesis (Fernandes and Dias. 2013) or, in the case of diabetic chronic wounds, limb amputation (Ramsey *et al.* 1999).

A consensus on the exact definition of chronic wounds is still to be determined, but it has been suggested that they are wounds that have not healed within 3 months (Werdin *et al.* 2009) and include venous, pressure and diabetic foot ulcers (DFU). Many factors contribute to non-healing: hypoxia; ischemia; underlying pathology (e.g. diabetes) and the presence of infection (Demidova-Rice *et al.* 2012). A therapeutic option for some patients is the delivery of hyperbaric oxygen (HBO); the inhalation of 100% oxygen at a pressure greater than that at sea level. The clinical benefits of HBO include increased angiogenesis, increased fibroblast proliferation, facilitated phagocytic response and direct killing of obligate anaerobes (Mader *et al.* 1987). Though HBO has been shown to enhance the effects of some antibiotics (Bornside, 1967), it is so far unknown whether it can modify, either positively or negatively, mechanisms of antibiotic resistance.

### *1.2. Antibiotic resistance*

Antibiotics are compounds that are either bacteriocidal or bacteriostatic and are produced naturally by microorganisms, though it is common practice also to use this name for synthesised chemotherapeutic agents, and semi-synthetic agents (modified natural compounds). The main mechanisms of action against bacteria (Table 1.1) are inhibition of cell wall synthesis; disruption of membrane structure; inhibition of nucleic acid synthesis; and inhibition of protein synthesis (Neal, 1997, Page, 1997).

An intrinsic ability to resist antibiotics, released into their immediate environment by co-habitants, has been an evolutionary process for bacteria, necessary for their survival. However, the extensive use of antibiotics in medicine and agriculture, coupled with the acquisition of genes carrying resistance, has resulted in strains that are resistant to multiple drugs, reducing the therapeutic

arsenal at the disposal of clinicians (Davies and Davies. 2010). Bacteria may use several mechanisms to resist antibiotics; the production of de-activating enzymes, target modification/substitution, altered membrane permeability, induction of efflux pumps and biofilm lifestyle (Section 1.4). The phenomenon of persister cells, cells that are neither inhibited nor killed by bactericidal concentrations of antibiotics, will be discussed in the section on biofilms (Section1.4).

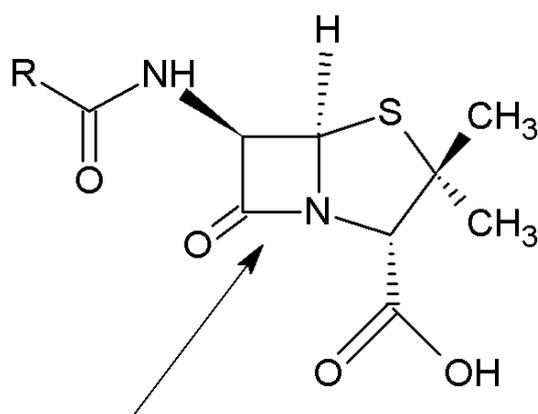
Table 1.1. Antibiotic modes of action (Neal, 1997, Page, 1997).

Mode of Action	Antibiotic Group	Examples
<b>Inhibition of cell wall synthesis</b>		
(a) Prevention of cross linking between peptidoglycan chains	Penicillins	Amoxicillin
	Cephalosporins	Cefuroxime
	Carbapenems	Imipenem
	Monobactams	Aztreonam
(b) Prevention of elongation of peptidoglycan backbone	Glycopeptides	Vancomycin
<b>Disruption of membrane structure</b>		
Selectivity towards lipopolysaccharide of outer membrane	Polymixins	Polymixin B
<b>Inhibition of nucleic acid synthesis</b>		
(a) Inhibition of DNA gyrase	Quinolones	Levofloxacin
(b) Inhibition of dihydropteroate synthetase	Sulphonamides	Sulphadiazine
(c) Inhibition of dihydrofolate reductase		Trimethoprim
(d) Inhibition of DNA supercoiling	Nitroimidazoles	Metronidazole
(e) Inhibition of DNA-dependent RNA polymerase	Rifamycins	Rifampin
<b>Inhibition of protein synthesis</b>		
(a) Blocks the binding of transfer RNA to messenger RNA-ribosome complex	Tetracyclines	Tetracycline
	Aminoglycosides	Gentamycin
(b) Incorrect reading of messenger RNA		
(c) Inhibition of ribosome translocation		
(d) Inhibition of transpeptidation	Macrolides	Erythromycin

### 1.2.1. De-activating enzymes

De-activating enzymes can act in one of three ways, catalysing hydrolysis, group transfer and redox processes. The classic example of hydrolytic enzymes is the  $\beta$ -lactamase family of enzymes, found in both Gram-positive and Gram-negative bacteria, which catalyse the cleavage of the  $\beta$ -lactam ring, a common structural feature of penicillins, cephalosporins, carbapenems and

monobactams (Fig. 1.1). The classification and nomenclature of  $\beta$ -lactamases have undergone various forms; the simplest is that based on sequence (Ambler, 1980) and consists of four classes, A, B, C and D. Class A includes extended spectrum  $\beta$ -lactamases (ESBLs), globally widespread enzymes that are able to hydrolyse a wider spectrum of drugs, due to amino acid mutations. A second classification system, based on function (Bush *et al.* 1995) organises the enzymes according to substrate and susceptibility to inhibition with drugs such as clavulanic acid, a serine  $\beta$ -lactamase inhibitor (Table 1.2).



$\beta$ -lactam ring

*Figure 1.1.* Chemical structure of a penicillin-type antibiotic. The arrow indicates the  $\beta$ -lactam ring.

*Table 1.2.* Interrelatedness between two  $\beta$ -lactamase classification systems. Adapted from Table1, page 10 (Helfand & Bonomo, 2003).

Ambler (sequence)	Bush (function)
Class A – serine $\beta$ -lactamases penicillinases and ESBLs	Group 2a, 2b, 2be, 2br, 2c, 2e penicillinases; clavulanic acid susceptible
Class B – metallo- $\beta$ -lactamases	Group 3a, 3b, 3c - metallo- $\beta$ -lactamases
Class C – serine $\beta$ -lactamases cephalosporinases	Group 1 – cephalosporinases clavulanic acid resistant
Class D – serine $\beta$ -lactamases oxacillinases	Group 2f- oxacillinases
	Group 4 – miscellaneous

The mode of action of  $\beta$ -lactamases depends on the constituents at the active site. Classes A, C and D enzymes utilize a serine ester at the active site which disrupts the  $\beta$ -lactam ring at the amide bond to form a covalent acyl enzyme complex, which catalyses the hydrolysis of the ring liberating the active enzyme and the inactivated drug (Matagne *et al.* 1998). Class B enzymes are known as metallo-enzymes and utilize divalent cations (usually zinc) at the active site (de Seny *et al.* 2001). These enzymes catalyse the cleavage of the amide bond of the  $\beta$ -lactam ring (Bounaga *et al.* 1998).

Resistance to the macrolide erythromycin can also include hydrolysis; this has been observed in *E. coli* (Arthur *et al.* 1987) and *Pseudomonas* sp. GD100 (Kim *et al.* 2002); an esterase is synthesized which catalyses the hydrolysis of the  $\beta$ -lactam ring of the antibiotic. Antibiotics that lack a hydrolytically vulnerable ring

can still be inactivated by covalent group transfer, such as that observed in resistance to chloramphenicol. Acetyl-CoA is necessary for this process, acting as donor of an acetyl group to the primary hydroxyl of chloramphenicol, via the action of chloramphenicol acetyltransferase (Leslie *et al.* 1988).

Group transfer is also involved in the ability of organisms such as *P. aeruginosa* and *Mycobacterium tuberculosis* to resist aminoglycosides (gentamycin, kanamycin, streptomycin). The transfer of functional groups, resulting in loss of inhibition of protein synthesis; is facilitated by a diverse group of enzymes which constitute three main families, aminoglycoside phosphotransferases (Doi *et al.* 1968), aminoglycoside acetyltransferases (Brzezinska *et al.* 1972), and aminoglycoside adenylyltransferases (Angelatou *et al.* 1982). The modified aminoglycosides have reduced affinity for their binding target, bacterial RNA.

Tetracycline is an inhibitor of bacterial protein synthesis; resistance is usually achieved by ribosomal protection and efflux of the antibiotic from the bacterial cell, processes facilitated by the *tet* family of genes, widely distributed throughout Gram-positive and Gram-negative bacteria (Chopra & Roberts, 2001). However, *tetX*, found on two transposons isolated from *Bacteroides fragilis* codes for a product that is involved in cellular redox processes. TetX is an oxygen requiring, flavin-dependent monooxygenase, shown to hydroxylate tetracycline rendering the antibiotic unstable (Yang *et al.* 2004). Though not yet identified in any clinically resistant bacterial strains, of cause for concern is destabilization of tigecycline, a new generation tetracycline that is unaffected by other resistance mechanisms (Moore *et al.* 2005).

### 1.2.2. Target modification or substitution

Several mechanisms of target modification have been identified which reduce susceptibility to antibiotic drugs yet appear to maintain sufficient cell function. One such modification is the methylation of an adenine residue in the bacterial 23S rRNA. The methylase is encoded by inducible *erm* genes, and is thought to initiate a conformational change in the ribosome which results in resistance to erythromycin (Lai & Weisblum, 1971).

Type II bacterial topoisomerases, which include DNA gyrase and topoisomerase IV, are necessary for DNA replication in bacteria, and are targets of quinolone (nalidixic acid and ciprofloxacin) and coumarin (novobiocin) antibiotics. Mutations to the *gyr* and *gri* genes which code for DNA gyrase subunits GyrA and GyrB, and topoisomerase IV subunits GrlA and GrlB respectively, have been found to lead to resistance to these drugs, with limited fitness costs (Vickers *et al.* 2007).

Dihydrofolate reductase (DHFR) is an essential component in purine and pyrimidine biosynthesis and is inhibited by the antibiotic trimethoprim. Resistance to this drug in *S. aureus* is conferred by a single amino acid substitution in DHFR (Dale *et al.* 1997); decreased trimethoprim binding occurs though binding of enzyme substrates do not differ significantly between wild-type and mutant DHFR, suggesting again that fitness costs to the bacterium are minimal (Vickers *et al.* 2009).

Inhibition of bacterial cell wall synthesis is a function of a broad range of antibiotics, including the penicillins, cephalosporins and glycopeptides; two resistance mechanisms come into play here, structural changes to the target and the synthesis of a new protein with the same function but which is unrecognizable to the antibiotic. A structural change to the peptidoglycan

molecule results in a 1000-fold reduction in binding efficiency of vancomycin (Bugg *et al.* 1991). This drug normally binds to the C-terminus of the peptidoglycan precursor containing an acyl-D-alanyl-D-alanine, however, in resistant bacteria this terminus is substituted with either D-alanyl-D-lactate or D-alanyl-D-serine (Walsh *et al.* 1996), with no reduction in the efficiency of cross-linking between peptidoglycan polymer chains.

Amino acid changes in penicillin-binding proteins (PBPs) can result in lower drug binding affinities (Nagai *et al.* 2002) and confer resistance to several  $\beta$ -lactam antibiotics. However, of greater concern is a second resistance mechanism, the inducible synthesis of a novel PBP (PBP2a, encoded by *mecA*, a gene acquired via a mobile genetic element) which is responsible for resistance in MRSA (Ubukata *et al.* 1989). Despite carrying the fitness cost of having to activate supplementary genes MRSA continues to be a substantial clinical problem.

### 1.2.3. Altered membrane permeability

The outer membrane of Gram-negative bacteria provides a selective barrier to molecules, including antibiotics, conferring an intrinsic resistance. However, some drugs, including  $\beta$ -lactams, can enter the bacterium through non-specific porins, membrane-bound protein channels. Substitutions at strategic positions within the structure of a porin has been found to reduce conductance, lowering the permeability of the outer membrane to  $\beta$ -lactam antibiotics (De *et al.* 2001); porin-deficient phenotypes of *Enterobacter* spp. are associated with resistant strains (Charrel *et al.* 1996).

#### 1.2.4. Induction of efflux pumps

Working synergistically with selective membrane permeability are efflux pumps, maintaining a high level of intrinsic antibiotic resistance by actively pumping drugs out of the organism. Several types of pump have now been identified in Gram-negative and Gram-positive bacteria (Piddock, 2006), and may be specific or broad-ranging in their substrates. However, resistance can be increased due to over expression of the pumps; higher minimum inhibitory concentrations (MICs) of antibiotics for post-therapy *P. aeruginosa* clinical isolates than pre-therapy isolates are associated with the over expression of OprM, a protein component of a multidrug efflux pump (Ziha-Zarifi *et al.* 1999). The efflux pump structure in this organism is encoded by an operon consisting of *mexA-mexB-OprM* and the multi-drug resistant (MDR) mutant OCR1 over expresses this operon. Evidence indicates that this may be due to a point mutation in the regulator gene *mexR* (Poole *et al.* 1996), whose normal function is to repress the operon. OCR1 exhibits increased antibiotic resistance.

The clinical importance of efflux pump over expression is a cause for concern; it has been estimated that up to 46% of *P. aeruginosa* isolates are MexA-MexB-OprM overproducing strains (Hocquet *et al.* 2007). Coupled with regulated membrane permeability control and enzymatic degradation of  $\beta$ -lactams, these strains carry a high level of multiple drug resistance.

#### 1.3. Chronic wounds

The financial, physical and emotional consequences of non-healing wounds are considerable; despite many months, or even years, of treatment aggressive surgical debridement or amputation of a limb is a common outcome (Ramsey *et al.* 1999) Normal healing is a complex and dynamic process that usually follows three phases; inflammation, proliferation and remodelling (Table 1.3.).

Table. 1.3. The stages of normal wound healing (Broughton *et al*, 2006).

Stage	Process	Events
<b>Inflammation</b>	Haemostasis	Clotting cascade
	Initiation of inflammation	Platelets release pro-inflammatory mediators, vasoconstriction is followed by vasodilation and increased blood vessel porosity
	Removal of bacteria and debris	Neutrophils and macrophages phagocytose bacteria, debris and damaged tissue. Macrophages release factors that stimulate angiogenesis and cells that reepithelialise the wound
	Decline in inflammation	Levels of pro-inflammatory mediators falls
<b>Proliferation</b>	New tissue formation	Angiogenesis Entry of fibroblasts for production of collagen and extracellular matrix Formation of granulation tissue. Epithelialization. Contraction and wound closure
<b>Remodelling</b>	Maturation	Type III collagen replaced by stronger type I. Collagen fibres realigned and cross-linked

Any factor that disrupts or prolongs the processes outlined in Table 1.3 may result in a chronic wound. Most chronic wounds fall into one of three categories: venous leg ulcers, pressure ulcers and diabetic foot ulcers (Mustoe *et al*. 2006). There are thought to be many potential contributors to non-healing, e.g. hypoxia, ischaemia, underlying pathology and the presence of infection (Demidova-Rice *et al*. 2012).

Hypoxia occurs when oxygen delivery is less than that necessary for normal tissue function (Sen, 2009). A particular problem for chronic wounds is their increased demand for oxygen: (i) healing tissue has a high demand for the cellular fuel adenosine triphosphate (ATP) (Im & Hoopes, 1970), (ii) molecular oxygen is necessary for the hydroxylation of pro-collagen peptides (Kivirikko *et al*. 1989), a required step for collagen deposition, and (iii) oxygen is a substrate

for NADPH oxidase during the respiratory burst of neutrophils. Hypoxia has been shown to reduce neutrophil bactericidal effects (Allen *et al.* 1997).

The effect of oxygen concentration on angiogenesis is complex and the exact molecular mechanisms involved are still not fully explained. On the one hand hypoxia is known to stimulate the production of hypoxia-inducible factor (HIF-1) which binds to hypoxia-inducible genes, resulting in the transcription of pro-angiogenic growth factors, including vascular endothelial growth factor (VEGF), a key regulator of angiogenesis (Karamysheva, 2008). However, the ability of hyperoxia to increase levels of VEGF in wounds has also been demonstrated (Sheikh *et al.* 2000) and the supposition that this leads to angiogenesis is supported in a study involving a murine wound model (Hopf *et al.* 2005); angiogenesis was significantly lower in mice breathing 21% oxygen compared to those breathing 100% oxygen. It is accepted clinically that hypoxic wounds form granulation tissue<sup>1</sup> poorly; fibroblasts, the cells that form the basis of this tissue, require oxygen to function effectively and vascularization of tissue is essential for this process, so despite stimulation of VEGF production in hypoxic wounds, and the fact that hypoxia indirectly stimulates increased expression of VEGF receptors on endothelial cells (Takagi *et al.* 1996), neovascularization is still impaired.

Considering the role of oxygen in the formation of collagen and the requirement for collagen for the structural integrity of new blood vessels it has been proposed that this is the key reason for increased angiogenesis in well oxygenated wounds (Hopf *et al.* 2005). Hopf suggests that reactive oxygen species (ROS) may also have a role to play in the initiation of angiogenesis;

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<sup>1</sup> Perfused fibrous connective tissue.

hydrogen peroxide stimulates VEGF production and collagen synthesis, and ROS production in immune cells is oxygen dependent. The significance of ROS as beneficial cellular signalling molecules, at low concentrations, is now fairly well accepted (Miki and Funato, 2012). It seems that hypoxia in normal, healthy tissue results in cellular and tissue responses that can maintain function if the hypoxic episode is transient and/or not too severe. The situation in damaged tissue is very different due to the inherent higher demand for oxygen to facilitate tissue healing.

Ischaemia is defined as a reduction in blood perfusion to tissue and it has been estimated that at least 80% of patients with diabetic foot syndrome<sup>2</sup> have peripheral arterial disease (Weck *et al.* 2011). Reduced perfusion results in hypoxic tissue, with all the associated problems outlined previously. However, concurrent to this is a reduction in the delivery of nutrients, immune cells and specific molecules that are necessary for wound healing.

Underlying pathology can have an enormous impact on both the type of wound that develops and the state of healing. For diabetic patients non-healing foot ulcers may be initiated and exacerbated by several disease conditions, including peripheral neuropathy, peripheral oedema, vascular dysfunction and foot deformities. It is beyond the scope of this chapter to elucidate the many pathological processes associated with diabetes, however three biochemical mechanisms in particular are thought to be involved in generating the above diabetic complications; the formation of advanced glycation end products<sup>3</sup> (Goh & Cooper, 2008), oxidative damage (Brownlee, 2005) and impaired leukocyte

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<sup>2</sup> An umbrella term for diabetic patients that have evidence of several pathologies associated with their feet.

<sup>3</sup> Advanced glycation end products (AGEs) are formed following interactions between sugars and proteins, lipids or nucleic acids.

function (Delamaire *et al.* 1997). The common ground of diabetic pathology, and other chronic wound related pathologies, is that of reduced tissue oxygenation, either due to micro or macrovascular disease (Jeffcoate & Harding, 2003).

### 1.3.1. *Infected chronic wounds*

Though no wound is sterile, the presence of clinical infection has been determined to be a contributory factor to non-healing (Gjodsbol *et al.* 2006, Robson & Heggors, 1969). Wounds containing bacterial population growth to  $10^5$  colony forming unit (CFU)  $\text{ml}^{-1}$  of fluid/tissue ( $10^5$  rule) are considered to be particularly susceptible to non-healing (Bowler, 2003).

The microflora of chronic wounds has been extensively studied, though the choice of sampling techniques and analysis tools determine the range of bacteria found. Commonly used techniques include swabs, tissue biopsy, curettage and fine-needle aspiration. Debate continues over the benefits of taking tissue biopsies rather than swabs; it is argued that swabs do not reflect species that invade deeper tissue (Stephens *et al.* 2003), and biopsies may be considered to be too localized and not representative of the whole wound (Bowler, 2003). As in other environmental niches, bacterial species in chronic wounds do not exist in isolation; synergistic or antagonistic relationships result in complex communities, demonstrating temporal adaptations.

For many years the identification of wound microflora has depended on standard culture techniques in hospital laboratories; samples are incubated on agar or in liquid medium for 16 to 48 hours (or for up to five days if clinically indicated), and are usually identified to species level by following various protocols which may include (i) isolation media, (ii) Gram stains, (iii) oxidase test, (iv) catalase test and (v) coagulase test (Agency 2007). Antibiotic sensitivities are given for prominent identified species. The main drawback of

this method is the inability to identify viable but non-culturable (VBNC) organisms and those with fastidious growth requirements. Anaerobic bacteria may be a group particularly under-represented (Bowler and Davies, 1999). A more realistic picture of wound microorganism diversity has been achieved with the use of molecular techniques; the polymerase chain reaction (PCR) followed by gel electrophoresis e.g. denaturing gradient gel electrophoresis (DGGE) and usually sequencing. A study examining the microbial diversity of chronic wounds compared results from routine culture methods and PCR, followed by sequencing. Of 18 microorganisms detected by sequencing only 5 were detected by routine culture (Frank *et al.* 2009). Another study using high-throughput pyrosequencing identified an average of 17 bacterial genera in each sample compared to three species identified by standard culture (Han *et al.* 2011). Molecular analysis requires careful consideration of the choice of primer sets to reduce bias, but can also present evidence of novel bacteria in wounds (Hill *et al.* 2003, this study, Chapter 3).

This leads us back to the role of individual species in the pathogenicity of wound infection. The  $10^5$  rule is often deemed to be too simplistic a guideline, particularly in relation to the synergistic interaction between aerobes and anaerobes. For example, aerobes may consume oxygen in a localized environment facilitating the survival of anaerobes. In a guinea pig wound model a challenge inoculation of *E. coli* at  $9 \times 10^5$  CFU ml<sup>-1</sup> did not interfere with wound healing; a separate challenge with *Bacteroides fragilis* (*B. fragilis*) at  $9.3 \times 10^5$  CFU ml<sup>-1</sup> also did not affect healing, however when a challenge consisting of equal amounts of each organism (total  $9.2 \times 10^5$  CFU ml<sup>-1</sup>) was used the wounds became inflamed and exuded pus (Kelly, 1980). Despite the overall bacterial load remaining the same the interaction between the two

species had a detrimental effect on the wounds. A more recent study (Pastar *et al.* 2013) demonstrated that a wound model inoculated with both *S. aureus* and *P. aeruginosa* incurred delayed healing compared to single species inoculation, and that this was due to the induction of virulence factors in *S. aureus* by *P. aeruginosa*.

The above examples indicated that just two species of bacteria were sufficient to delay healing in a wound, but is this specific to these particular organisms or does the species diversity determine the outcome in a wider context? In a clinical study involving chronic leg ulcers the presence of four or more bacterial groups resulted in a failure to heal of 42%, a significantly higher rate than for wounds containing less than four bacterial groups (Tregrove *et al.* 1996). The authors did not see any correlations between individual groups and failure to heal, though it is not clear how they determined this as the analysis was qualitative and did not explore quantitatively whether a particular species was more abundant in a particular wound, just that a bacterial group was present. Unfortunately, some groups were too broadly defined; anaerobes, for example, were categorized as one group. However, the study did determine that there were temporal changes in the bacterial community composition (median number of swabs taken per patient was 9 over a 6 month period); in 82% of patients at least one new group was identified in subsequent swabs.

### 1.3.2. Prevalent bacteria found in chronic wounds

Several studies have examined the bacterial diversity of chronic wounds, using cultural and molecular methods, and the list of organisms found often reflects the type of wound, i.e. venous, diabetic or pressure ulcer; however some commonalities do occur (Table 1.4). *S. aureus* is a notable inclusion in most of the studies and was identified as the most prevalent organism in diabetic chronic wounds when three different molecular methods were compared (Dowd *et al.* 2008). However, a causal relationship between presence of a species and non-healing cannot be assumed. It does not necessarily follow that because an organism is prevalent in chronic wounds it is the reason that they do not heal; it may be thriving in an ideal growth environment but have no significant pathological role.

Table 1.4. Most prevalent organisms found in chronic wounds. DFU, diabetic foot ulcer; CVU, chronic venous ulcer; PU, pressure ulcer; C, culture; M, molecular analysis; N/S, not specified.

Study	Aerobe	Anaerobe	Type	Method
Goldstein <i>et al.</i> 1996	<i>S. aureus</i>	Individual species not specified	DFU	C
Chincholikar & Pal, 2002	<i>S. aureus</i> <i>P. aeruginosa</i>	<i>Bacteroides melaninogenicus</i> <i>Bacteroides fragilis</i>	DFU	C
Raja, 2007	<i>S. aureus</i>		DFU	C
Gerding, 1995		<i>Peptostreptococcus</i> sp. <i>Bacteroides</i> sp.	DFU	C
Abdulrazak <i>et al.</i> 2005	<i>S. aureus</i> <i>P. aeruginosa</i>	<i>Bacteroides fragilis</i>	DFU	C
Gjødsbøl <i>et al.</i> 2006	<i>S. aureus</i>	Individual species not specified	CVU	C
Citron <i>et al.</i> 2007	<i>S. aureus</i>	Gram positive cocci	DFU	C
Ng <i>et al.</i> 2008		<i>Peptostreptococcus</i> sp.	DFU	C
Dowd <i>et al.</i> 2008	<i>Stenotrophomonas</i> sp. <i>S. aureus</i> <i>Corynebacterium</i> sp.	<i>Enterobacter</i> sp. <i>Peptoniphilus</i> sp. <i>Peptoniphilus</i> sp.	CVU DFU PU	M M M
Frank <i>et al.</i> 2009	<i>S. aureus</i>	<i>E. faecalis</i>	N/S	M
Daltrey <i>et al.</i> 1981	<i>S. aureus</i>	<i>Bacteroides fragilis</i>	PU	C

The pathological role of *P. aeruginosa* in chronic wounds is still to be resolved; a study using peptide nucleic acid (PNA) and fluorescence in-situ hybridization (FISH) on wound biopsy tissue found that of the samples containing large bacterial aggregates, approximately 70% of them contained *P. aeruginosa*, compared to only 15% containing *S. aureus* (Kirketerp-Moller *et al.* 2008). An accurate interpretation of this data is problematic as the analysis of the microscopy was not statistically quantitated. Again the variability of sampling techniques could be of relevance here; *P. aeruginosa* may be associated with

deep wound tissue rather than superficially on the wound surface. If swabs are used they may not collect organisms embedded deeper in the tissue. A study using cultural techniques identified *S. aureus* in 93.5% of chronic leg ulcers, 71.7% contained *E. faecalis*, and 52.2% contained *P. aeruginosa*. However, further interpretation of this work regarding wound demography is difficult as even though samples were taken in the form of swabs and biopsies, the origin of the material in relation to the results was not presented.

A study comparing the microbial diversity of infected and non-infected wounds, determined by the presence of clinical signs (exudate, malodour, erythema<sup>4</sup>) found that all wounds were colonized by aerobic bacteria, the most prevalent in both types of wound being *S. aureus*, whereas *P. aeruginosa* had low isolation rates; 82% of infected wounds contained anaerobes compared to 73% of non-infected (Bowler and Davies, 1999). The mean number of anaerobes in infected wounds was significantly higher than the mean number in non-infected wounds (Bowler and Davies, 1999), the most prevalent being *Peptostreptococcus* sp. (now known as GPAC, Gram-positive anaerobic cocci) and *Prevotella* sp. The isolation of these two anaerobes has been duplicated by several studies (Wall *et al.*, 2002). Another interesting finding of this study is that two *Candida* spp. (*Candida albicans* and *Candida parapsilosis*) were isolated in infected wounds, but were not isolated in non-infected wounds.

The complex nature of the interaction between a microorganism and its host needs to be considered when examining how a particular bacterial species may delay wound healing. Returning to *P. aeruginosa*, a small study of ten chronic venous leg ulcers found that the two patients with ulcers that rapidly enlarged, compared to the other wounds, had positive antibody titres against exotoxin A

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4 Redness of the skin.

antibody (Danielsen *et al.* 1996); the remaining eight patients tested negative. These two patients were colonized with the same strain of *P. aeruginosa*, a strain not isolated in the other patients. This study supports earlier work that demonstrated delayed closure, in a rat wound model, when wounds were treated topically with exotoxin A; the application of antiexotoxin to these wounds healed at the same rate as the controls (Heggens *et al.* 1992).

A temporal component may also be of importance; a study monitoring bacterial community changes in chronic venous leg ulcers over 8 weeks defined resident bacteria as those bacteria that were isolated at all swabbing events and observed significantly larger wounds in those that had resident *P. aeruginosa* (Gjodsbol *et al.* 2006). Bacteria secrete many virulence factors and it is beyond the scope of this chapter to discuss all of those that may play a role in delayed healing. However, to illustrate the point a few examples will be presented. Secreted factors may impede neutrophil activity; a *B. fragilis* culture filtrate inhibited phagocytic and killing activity of neutrophils, with evidence suggesting that short-chain fatty acids may be the factors responsible (Rotstein *et al.* 1989); extracellular polysaccharides synthesized by *Streptococcus mutans* appear to reduce oxygen radical production in neutrophils and provide protection against phagocytosis (Steinberg *et al.* 1999). If the presence of a critical bacterial load in a wound is detrimental to healing then mechanisms to disrupt immune cell mediated clearance will be an exacerbating factor. Some anaerobic bacteria are known to excrete hydrolytic enzymes, such as collagenase (Steffen & Hentges, 1981), which may delay healing by inhibiting the formation of new tissue, a process reliant on collagen formation. If the delay in wound healing is caused by a secreted factor it is possible that the concentration of the factor in the wound environment, and/or a critical exposure time, is a key determinant of its

pathological role. The effect seen with the *B. fragilis* filtrate occurred only when the bacterial population had reached stationary phase of growth (Rotstein *et al.* 1989) so bacterial population density may be an important factor.

The key message appears to be that an effort to reduce the burden of pathological organisms in a wound is an important component of chronic wound treatment plans. Herein lies a conundrum; a Health Technology Assessment review of chronic wound care management strategies concluded that there were “no beneficial effects” of topical or systemic antibiotics in the treatment of diabetic foot ulcers (O'Meara *et al.* 2000). A later review (Nelson *et al.* 2006) concluded that the evidence for specific diagnostic testing for infection and antibiotic treatment of diabetic foot ulcers was “weak” and incapable of guiding clinical practice. It is unfortunate that only a small number of trials satisfied the review criteria (only two studies in the first review); the heterogeneous nature of diabetic foot ulcers, and the variability of diagnosis of infection and treatment options make correlations between infection and wound healing problematic. An infected wound is never treated with antibiotics alone; debridement, hydrocolloid/antiseptic dressings, patient education, corrective vascular surgery and glycaemic control are frequently included in a multidisciplinary approach (Edmonds. 2006).

A consideration of one component of infection, that appears to be lacking in most of the studies included so far, is the incidence and implication of antibiotic resistant bacteria in wounds. A retrospective analysis of the incidence of MRSA in diabetic foot ulcers found that 30.2% of the patients had MRSA isolated from their wounds (Dang *et al.* 2003); in an earlier analysis 40% of *S. aureus* isolated from wounds was MRSA, and not surprisingly MRSA was more prevalent in the wounds of patients who were already being treated with antibiotics.

Interestingly, the wounds containing MRSA were slower to heal than wounds containing Methicillin-sensitive *S. aureus* (Tentolouris *et al.* 1999). The presence of MRSA in a DFU is also associated with greater treatment failure (35.3%) compared to that for DFUs not containing MRSA (23%) (Vardakas *et al.* 2008).

### 1.3.3. *The presence of fungi in chronic wounds*

Studies reporting the presence and diversity of fungi in chronic wounds are few. An investigation of fungi in DFUs, using microscopy and culture methods as means of identification (Heald *et al.* 2001), found *Candida* species to be the most prevalent with *Candida parapsilosis* (*C. parapsilosis*) found in 9 out of 17 wounds and *Candida albicans* (*C. albicans*) found in 5 wounds. *Candida humicola*, *Candida glabrata* and *Candida tropicalis* (*C. tropicalis*) were also identified. Leake *et al.* (2009) in an investigation of three chronic wounds using molecular methods, identified three fungal species: *C. albicans*, *C. parapsilosis* and *C. tropicalis*. All three wounds contained *C. tropicalis*, though in smaller quantities than either *C. albicans* or *C. parapsilosis*. A later, larger study by the same group (Dowd *et al.* 2010), identified 48 fungal species in 208 chronic wounds. Again, the most abundant genus found was *Candida* and the five most prevalent species, in terms of the number of wounds in which they were found, were *C. albicans*, *C. parapsilosis*, *Curvularia lunata*, *Candida orthopsilosis* and *Ulocladium botrytis*.

### 1.4. *Biofilms*

In natural and synthetic environments microorganisms can be found in either planktonic or sessile form, the latter comprising a surface-associated community of organisms, which, when encased within a matrix of extracellular polymeric substances (EPS), is known as a biofilm. The components and dynamics of the biofilm microorganism community are as variable as the

environments in which they are found, from marine ecosystems to the plaque on our teeth. The first stage of biofilm development is the attachment of planktonic cells to a surface, a process that is influenced by the presence of a conditioning film. This is followed by a steady accumulation of cells which start to aggregate together. Cells then excrete EPS and become enclosed within a matrix (Fig. 1.2).

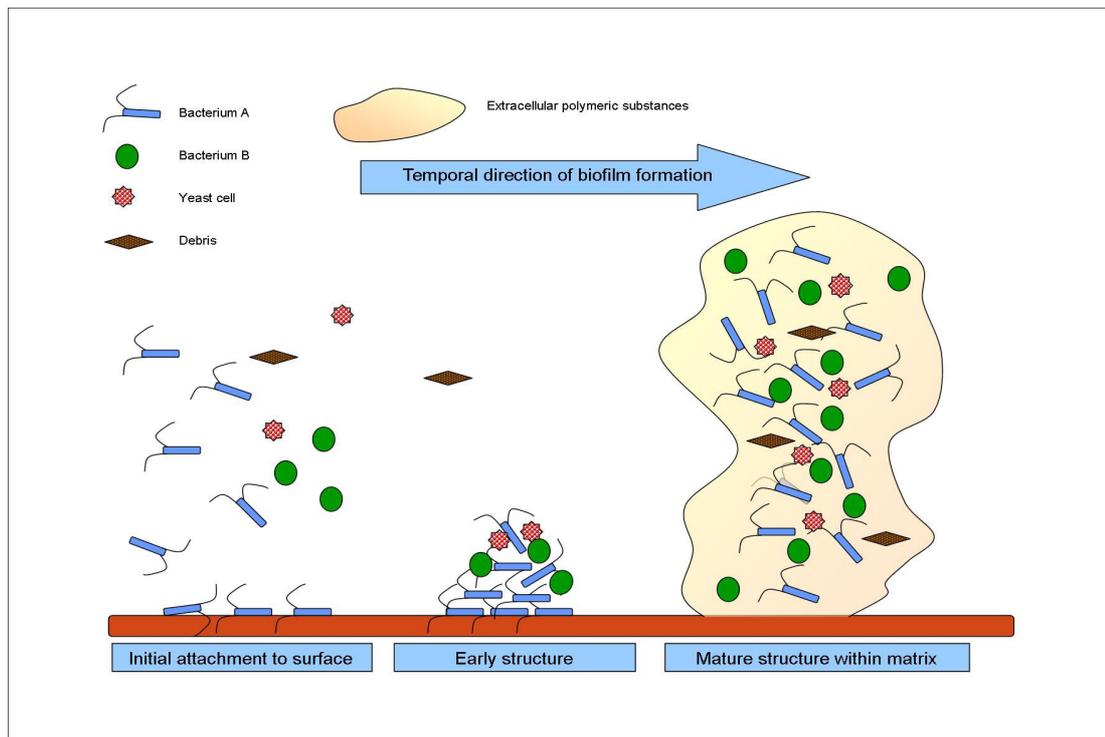


Figure 1.2. Formation of a biofilm. Bacterial and fungal cells attach to a surface and then aggregate together. The cells secrete extracellular polymeric substances (EPS) which encompasses the microbial community and eventually forms the mature matrix structure.

#### 1.4.1. Biofilm structure

Before discussing mature biofilm structure it is necessary to consider the process of cell adhesion to a substratum; this may have particular significance to the design of medical devices and to the treatment of chronic wounds. The ability of planktonic cells to adhere to a surface depends on the properties of both the bacterial strain and the substratum, for example, most bacteria carry a net negative charge, the magnitude of which is strain dependent; hydrophobicity will also determine whether an organism will readily adhere to a surface (Dickson & Koochmaraie, 1989).

The adsorption of a conditioning film to a substratum alters the surface properties and therefore adhesion capabilities of bacterial cells. Studies examining biofilms in marine environments have found that conditioning films can consist of polysaccharides, proteins, lipids, nucleic acids and amino acids,

proteins usually being the first molecules to appear (Bakker *et al.* 2004, Poleunis *et al.* 2002). The films can be temporally heterogeneous, changing composition according to the time of year, films formed during spring contain more protein and organic carbon than winter films, with consequent differences in marine organism adherence (Bakker *et al.* 2003).

Silicone urinary catheters have been shown to develop conditioning films during the early stages of placement within human bladders (Tieszer *et al.* 1998) though the effects of films on pathogens in various *in vitro* situations has been complex. A conditioning film containing fibrinogen and fibronectin in silicone catheter lumens decreased the number of *S. epidermidis* adhering, but increased the number of *S. aureus* (Espersen *et al.* 1990). In an oral biofilm model conditioning films formed in the presence of a saliva medium increased the adherence of some oral bacterial species but slowed down the adherence of some *Staphylococcus* and *Streptococcus* species (Busscher *et al.* 1997). Selectivity of adherence could occur due to the presence of receptors for specific strains on salivary glycoproteins (Murray *et al.* 1992). These were identified for 8 out of 16 strains of oral *streptococci*. The evolution of specific interactions between oral bacteria and salivary components is logical; there is a need for organisms to adhere rapidly and strongly to a surface in an environment of high shear pressure.

Non-specific interactions should not be discounted however, blood preconditioning of central venous catheters resulted in increased adherence of *P. aeruginosa*, *P. agglomerans* and *Enterobacter cloacae* (Murga *et al.* 2001) and bacterial adherence on a plastic substratum was increased when mussel juice conditioning film was present (Herrera *et al.* 2007). A common conclusion from the studies mentioned is that early surface colonisers may utilise the

conditioning film as a temporary nutrient source though the bacterial cells themselves may contribute to the film components; a conditioning film on glass slides *in situ* during the stationary phase of *Bacillus cereus* (*B. cereus*) population growth contained DNA, which in turn appeared to contribute to adherence (Vilain *et al.* 2009). Damaged tissue present in chronic wounds may contain a cocktail of conditioning film components increasing the likelihood of bacterial adherence at a very early stage of colonisation.

The extracellular matrix has been estimated to be at least 97% water when fully hydrated (Zhang *et al.* 1998), other components include bacterial metabolites, nutrients, polysaccharides, proteins, DNA and debris from the surrounding environment (Sutherland, 2001). One of the functions of the biofilm matrix appears to be a protective one. It maintains the structure of the biofilm, and protects cells from dehydration (Ophir & Gutnick, 1994) and ultraviolet radiation (Elasri & Miller, 1999). Microbial cells usually form a small percentage of the total mass of the biofilm which is highly heterogeneous regarding pH, density, components and structure, which are influenced by factors such as nutrient and oxygen availability, cell-to-cell signalling, gene expression and environmental stressors (such as shear stress) (Puevdorj-Gage *et al.* 2002, Hall-Stoodley *et al.* 2004). Biofilms of *P. aeruginosa* grown with citrate as a carbon source are flat, whereas those grown with glucose have a mushroom-like structure with water filled channels; further to this is evidence that microcolonies within the structures are phenotypically different. Non-motile cells have been found in the stalks of the mushrooms, and motile cells of the same species were localized in the cap of the mushroom (Klausen *et al.* 2003).

Physiological activity in *P. aeruginosa* biofilms has been found to be dependent on oxygen availability; active cells were visualized around the edge of the

biofilm, a layer which becomes thicker in the presence of 100% oxygen compared to 21% (Xu *et al.* 1998). Gradients for gases and other substances such as nutrients are logical and probably account, in part, for the heterogeneous nature of the biofilm. However, each biofilm will develop its own unique physical properties which will influence the interaction between solutes and matrix; a consensus on diffusion coefficients for molecules within the biofilm remains elusive. The low diffusivity of biofilms is often postulated as a reason for increased antibiotic resistance of biofilm cells, however, by fluorescently tagging solutes of various molecular weights (MW) from 3,000 to 150,000 Da, and tracking their penetration into a model oral biofilm it was observed that solutes reached the centre of cell aggregates within 3 minutes (Takenaka *et al.* 2009). Most antibiotics have a MW well below 3,000 Da so if their transport through the biofilm matrix is impaired it is likely that another factor is involved (Section 1.4.3). Membrane vesicles (MVs) have also been found in biofilm matrices. They are derived from the outer membranes of bacteria and the contents will depend on the parent organism. Isolated MVs from *P. aeruginosa* biofilms demonstrated proteolytic activity, and, interestingly, also bound the antibiotic gentamycin (Schooling & Beveridge, 2006).

#### *1.4.2 Planktonic and sessile cells are phenotypically different*

The observation of different phenotypes among the same bacterial species within a biofilm prompted work to explore the genetic behaviour of these cells. Multiple stages were uncovered for some species where the extent of protein expression changes according to the developmental stage of the biofilm. For example, when *P. aeruginosa* cells were grown as a biofilm and as planktonic cells the correlation coefficient for protein expression decreased until day 6 of biofilm growth (Sauer *et al.* 2002), during this time more than 50% of proteins

were upregulated in the biofilm cells. These proteins included membrane proteins, oxidative stress response proteins and those necessary for biosynthesis. How changes in protein expression affect cell behaviour in biofilms was demonstrated, again with *P. aeruginosa*; bacteria from 5-day old biofilms showed significant diversity in swimming capability whereas planktonically grown cells showed no swimming variability at all (Boles *et al.* 2004). The degree of variability indicated that multiple genetic changes had been initiated rather than a single mutation event.

These phenotypic changes may contribute to the heterogeneity of biofilms and the formation of environmental niches within the biofilm structure, facilitating survival and mutual co-operation within and between species. The example of mushroom formation by *P. aeruginosa* and the generation of water channels facilitate the dissemination of oxygen and nutrients deeper into the biofilm, benefitting other members of the biofilm community. This structural feature of biofilms is not confined to *P. aeruginosa*; mushroom morphology has been observed in the biofilms of *Caulobacter crescentus* (Kirkpatrick and Viollier. 2010).

#### *1.4.3. Biofilms and antibiotic resistance*

Numerous studies have now demonstrated an inherent resistance of biofilms to antibiotics; minimum inhibitory concentrations (MICs) of tobramycin against *P. aeruginosa* biofilms are 1000 fold higher than those for planktonic cells (Nickel *et al.* 1985), ciprofloxacin fared better against *P. aeruginosa* biofilms with eradication occurring at 16 fold higher than the MIC for planktonic cells (Abdi-Ali *et al.* 2006). Different antibiotics have different responses and there are many factors that influence this: (i) the ability of the drug to diffuse through the matrix and actually reach the bacterial cells; (ii) the extent of binding and/or antibiotic

deactivation within the matrix; (iii) target cells may be phenotypically different to planktonic cells; and (iv) nutrient and oxygen gradients in the matrix may result in a heterogeneous physiological response. An important point to note, before discussing these mechanisms of biofilm resistance, is that the methodology chosen to quantify bacterial inhibition/killing and any reduction in biofilm mass needs to be scrutinized whilst interpreting the data as this will influence the analysis. For example, measuring viable cell counts may give a significantly different result compared to measuring bacterial respiratory activity; studies need to differentiate between biofilm matrix mass and cellular mass when quantifying reduction, or make it clear that they are measuring the whole biofilm mass.

It has long been hypothesized that the gelatinous nature of the biofilm matrix will impede the diffusion of antibiotics, either via physical properties such as the presence of macromolecules and charge interactions, or by directly binding to a matrix component, but what is the evidence for this? The antibiotic ciprofloxacin penetrated *Klebsiella pneumonia* (*K. pneumonia*) biofilm to MIC concentrations within 20 minutes, demonstrating no significant impairment of diffusion. However, ampicillin was able to penetrate a  $\beta$ -lactamase negative mutant *K. pneumonia*, but not the  $\beta$ -lactamase positive wild-type strain (Anderl *et al.* 2000), providing evidence that extracellular deactivating enzymes may play a role in resistance. An interesting observation from this particular study was that the increased resistance noted for this organism (including the  $\beta$ -lactamase positive strain) to both antibiotics, indicates that a separate mechanism of resistance is occurring here. Penetration of rifampin through *S. epidermidis* biofilms (Zheng & Stewart, 2002), vancomycin through *S. aureus* biofilms (Jefferson *et al.* 2005) and tetracycline through *E. coli* biofilms (Stone *et al.*

2002) have all been observed. In some cases penetration was slow but target concentrations were achieved. However, despite this, killing was still impaired. Slow penetration of drugs may allow time for biofilm cell mass to increase before a cidal or inhibitory concentration is reached, but this is probably not sufficient to explain the levels of resistance seen.

The addition of slime, removed from a biofilm-positive strain of *S. epidermidis*, to a MIC assay, decreased killing of clinical *Staphylococcal* isolates by vancomycin; the slime had no effect on the MICs of rifampicin (Mathur *et al.* 2005). This study illustrates the heterogeneous nature of antibiotic resistance; both drugs are glycopeptides but have a differential response in the presence of biofilm slime. Vancomycin is hydrophilic; rifampicin is lipophilic, properties which may influence the nature of their interactions with components of the biofilm matrix. A criticism of this study is that they performed MIC assays on planktonic cells, to which they then added slime; evidence of differences in phenotype between planktonic and biofilm cells has been discussed (Section 1.4.2) and needs to be considered; upregulated proteins during biofilm growth may modify the level of resistance to either antibiotic.

The presence of genetic material in the biofilm matrix has been considered to be a non-significant by-product of cell lysis; however, evidence is now accumulating that supports a more active role for nucleic acids. Apart from a structural role (Allesen-Holm *et al.* 2006) extracellular DNA has been shown to chelate cations in *P. aeruginosa* biofilms, resulting in the induction of an antibiotic resistance operon which increased MICs for cationic antimicrobial peptides and aminoglycosides (Mulcahy *et al.* 2008). In support of this Tetz *et al.* (2009) observed that DNase treated biofilms were structurally altered and allowed increased antibiotic penetration. There is evidence to support a

regulatory role of QS in DNA release into the biofilm matrix (Nakamura *et al.* 2008) supported by the observation that a *P.aeruginosa* QS mutant that has reduced extracellular DNA was more susceptible to aminoglycoside antibiotics than the wild-type organism (Chiang *et al.* 2013).

Evidence for the presence of antibiotic deactivating enzymes in biofilm matrices was discussed previously in this section (Anderl *et al.* 2000), however a picture of heterogeneous distribution of the induced enzyme is emerging. A reporter plasmid carrying an unstable green fluorescent protein (GFP) was used to discern the induction pattern of  $\beta$ -lactamase within *P. aeruginosa* biofilms when sub-MICs of two  $\beta$ -lactams were introduced; induction occurred primarily around the periphery of the biofilm, with no induction occurring in microcolonies in the centre of the biofilm (Bagge *et al.* 2004). The authors of the study admit that their method of detection may not be sensitive enough to detect low levels of induction in the central colonies; however a differential in induction is still evident. A suggestion that the central cells response is due to a slower growth rate due to nutrient and oxygen limitation is postulated, a theory supported by other studies (see later in this Section), though it is also possible that extracellular  $\beta$ -lactamase (perhaps circulated and stored in MVs) is sufficient to reduce the concentration of antibiotic reaching the centre of the biofilm to sub-induction levels.

Many antibiotics, including the  $\beta$ -lactams are only effective against actively growing cells, and this is as true for biofilm cells as it is for planktonic cells. Gradients for nutrients and oxygen develop due to the physical structure of the biofilm in relation to the environmental interface (de Beer *et al.* 1994), and to local depletion by microcolonies and accretion due to cell lysis. This phenomenon has been modelled mathematically (Roberts & Stewart, 2004) and

demonstrated *in vitro*; only cells in an oxygenated region adjacent to a *P. aeruginosa* biofilm interface were able to express a GFP, cells near the centre did not (Borriello *et al.* 2004). Oxygen measurements in the biofilm demonstrated a rapid fall in oxygen concentration from the biofilm surface to a depth of 50  $\mu\text{m}$  so it was suggested that the core of the biofilm was anaerobic and therefore cell growth was minimal. The use of GFP in biofilm studies is very common but it does require oxygen in order to produce the fluorophore<sup>5</sup>, it is also sensitive to low pH, being undetectable at pH 4.5 and below (Hansen *et al.* 2001). Cells in anaerobic, acidic conditions in localised pockets of a biofilm may be non-growing but if these conditions are suspected then an alternative means of measuring physiological activity may be preferential.

Comparisons between biofilm cells and stationary phase planktonic cells have been made to illustrate the similarities between limited nutrient availability and the organism's resistance to antibiotics; resistance is increased in both situations, and again is linked to slow growth (Anderl *et al.* 2003). One study confirmed the link by exposing both types of cells to a fluoroquinolone antibiotic (which does not depend on cell growth to exert its bactericidal action) and  $\beta$ -lactams; the *P. aeruginosa* stationary phase and biofilm cells were susceptible to killing by the fluoroquinolone but not the  $\beta$ -lactam antibiotic, logarithmic phase cells were vulnerable to killing by both antibiotics (Spoering & Lewis, 2001).

The effect of biofilm age on antibiotic resistance also has implications for wound treatment protocols. Mature biofilms have been shown to produce more  $\beta$ -lactamase than young biofilms when exposed to antibiotics (Bowler *et al.* 2012), though even biofilms not exposed to antibiotics can evolve increased resistance

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<sup>5</sup> A component of a molecule that is fluorescent.

with the maturation of the biofilm (Tyerman *et al.* 2013). Evidence suggests that increasing oxidative stress in a maturing biofilm results in increased mutation conferring greater antibiotic resistance (Ryder *et al.* 2012, Bernier *et al.* 2013). The characterisation of the cells that survive cidal concentrations of antibiotics is an area of research that has led to a theory that was, for a long time ignored but is now much debated; the cells, which have been labelled “persisters”, do not demonstrate antibiotic resistance, but rather tolerance, they “neither grow nor die” (Keren *et al.* 2004). It has been argued that these cells are senescent (Klapper *et al.* 2007) possibly as a result of asymmetric binary fission which, over time, leads to the altered phenotype of an aged cell (Lindner *et al.* 2008, Stewart *et al.* 2005). This is a fascinating area of research and there is not the space to discuss the topic fully here, but factors other than aging do seem to be involved; the identification of a persister related gene *hipB/hipA* which is a toxin/antitoxin module may only be part of the story and the reader is directed to a review for more details (Jayaraman, 2008). The main weakness of the senescence argument is that any genetic component conferring antibiotic resistance should be passed on to daughter cells, however, daughter cells of persisters are susceptible to antibiotics (Bigger, 1944).

Whatever the nature of persister cells the important property of relevance to this work is their multidrug tolerance. Whichever antibiotic is administered it will only be effective if it corrupts a target within the bacterium, the unique property of persister cells seem to be their dormancy-like state where all cellular functions shut down conferring on them a physiological ambivalence to antimicrobials. The fact that these cells are found in biofilms (Brooun *et al.* 2000), where they benefit from the protection of the matrix, is a cause for concern and may account for the multidrug resistance and virtual incurability of certain biofilm

related infections, e.g. *P. aeruginosa* in the lungs of cystic fibrosis patients. All of the above studies have investigated mono-species biofilms, a situation that rarely occurs in natural environments, including wounds (Section 1.3). It is of concern then that multispecies biofilms show increased biofilm mass and an increase in antibiotic resistance compared to mono-species biofilms (Burmølle *et al.* 2006).

#### 1.4.4. Biofilms and the immune response

It is probably fair to say that the mechanisms of interaction between biofilms and immune cells have come predominantly from work with *P. aeruginosa*, and the overall consensus seems to be that the exopolysaccharide component of the biofilm EPS is responsible for a degree of protection for biofilm enclosed bacterial cells. Using confocal laser scanning microscopy (CLSM) to monitor the interaction between *P. aeruginosa* biofilms and neutrophils, on contact with the biofilm neutrophils appeared to become immobile and retain an unstimulated rounded morphology; despite evidence of the presence of oxidative burst products and phagocytosis of bacterial cells their immobility resulted in only localised clearance (Jesaitis *et al.* 2003). Evidence suggests that alginate production is a key factor in protection; alginate negative strains of *P. aeruginosa* were susceptible to leukocyte killing, a situation that was reversed on the addition of exogenous alginate to the culture (Leid *et al.* 2005). Despite the observation that some biofilm cells are phagocytosed and an oxidative burst occurs, evidence suggests that immune cell function may still be compromised. Slime producing *Enterococcus* sp. survived for 48 hours within rat peritoneal macrophages compared to 24 hours for non-slime producing strains (Baldassarri *et al.* 2001). Antibody-mediated phagocytic killing of cells may also be compromised in a biofilm environment; the

exopolysaccharide poly-*N*-acetylglucosamine (PNAG) normally elicits an opsonic antibody response when secreted by *S. aureus* and *S. epidermidis*, however, in a biofilm model bacterial cells were more resistant to this mode of killing than planktonic cells (Cerca *et al.* 2006). The results indicated that large amounts of PNAG had accumulated in the biofilm environment which overwhelmed the antibody response conferring a degree of protection to bacterial cells.

#### 1.4.5. Biofilms in wounds

Chronic wounds, particularly before cleaning, are often covered in a sloughy material that has the appearance of a biofilm, and it has long been suspected that this is the case, but what is the evidence that biofilms do actually develop in wounds? Bacterial cell aggregations surrounded by EPS were visualised in tissue biopsies from 60% of chronic wounds included in a study (James *et al.* 2008), this is fairly diagnostic of biofilm presence. Only 6% of acute wounds contained evidence of biofilms which suggests that the environment in a chronic wound may be particularly conducive to biofilm production. The presence of biofilm producing strains in clinical isolates has been demonstrated (Sanchez *et al.* 2013); and an isolate from a burn wound developed an EPS coating within 5 hours of *in vitro* growth, and had grown into a mature biofilm by 10 hours (Harrison-Balestra *et al.* 2003). Many bacteria certainly express the virulence factors necessary for attachment to a wound surface, e.g. *S. aureus* expresses cell-wall bound adhesions which are able to bind to collagen, fibronectin and fibrinogen (Foster & Hook, 1998). A wound provides substrates for biofilm growth in the form of plasma and lysed cells; poorly controlled diabetic patients are likely to have glucose rich fluids in a wound environment though no evidence has been found to support this hypothesis.

The presence of vascular disease in diabetic patients results in poor tissue perfusion and a consequence of this could be a reduction in the local concentration of antibiotics in a wound environment. Apart from inadequate inhibitory or cidal effects, low levels of antibiotics may induce DNA release. This has been observed, concurrent with increased biofilm formation, in *S. aureus* (Kaplan *et al.* 2012), a prominent organism found in non-healing wounds.

One aspect of the polymicrobial character of biofilms, that may have an impact on wound pathology, is the interaction between bacterial and fungal species. The presence of fungi in dual-species biofilms can result in enhanced biofilm growth; this has been seen for *C. albicans* and *S. epidermidis* (Adam *et al.* 2002) and *Candida lusitanae* and *E. faecalis* (van Merode *et al.* 2007). Of equal concern is the evidence that *S. aureus* is able to physically associate itself with the hyphae of *C. albicans* in dual-species biofilms (Peters *et al.* 2010), potentially giving the bacteria a means of colonising deeper tissue. Any increased pathology, however, may also be due to a differential in *S. aureus* protein expression between single and dual-species biofilm cells which could increase survival and virulence (Peters *et al.* 2010). The relationship between species in a biofilm may also be antagonistic; the *P. aeruginosa* QS signalling molecule, O-C12-HSL, has been shown to inhibit filamentous growth of *C. albicans* (Hogan *et al.* 2004).

Despite the paucity of *in vivo* evidence for the presence and significance of biofilms in chronic wounds there is much support for the theory that biofilms, particularly multi-species biofilms (Seth *et al.* 2012) contribute to the non-healing pathology. If chronic wounds are colonised exclusively by antibiotic sensitive planktonic cells then antibiotics should reduce their number sufficiently to a level that can be cleared by the hosts' immune response, though this may

not be the case if they are predominantly persister cells. It could be argued that patients who develop chronic wounds often have impaired immune systems, and this may be a factor but not all patients do, yet they still may have a non-healing pathology and recalcitrant infection. Biofilms provide a constant source of inoculating bacterial cells, and coupled with increased antibiotic resistance and various strategies to evade or impair an immune response it is now accepted that procedures to facilitate the removal of biofilms in a chronic wound treatment regimen is crucial for a successful outcome (Wolcott & Rhoads, 2008).

### *1.5. Quorum sensing*

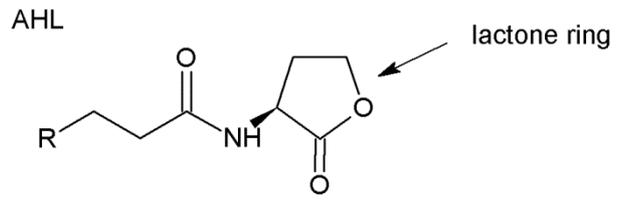
First described in 1970, in *Vibrio fischeri* (*V. fischeri*) (Nealson *et al.* 1970), quorum sensing (QS) is a complex and diverse system of genetic expression co-ordination. Utilised by multiple bacterial species, the common components of QS are small secreted molecules, receptor proteins and down-stream effectors. The signalling molecules, initially designated autoinducers, broadly fall into two groups: acyl-homoserine lactones (AHLs) secreted by Gram-negative bacteria (Eberhard *et al.* 1981) and small peptides, secreted by Gram-positive bacteria (Camilli & Bassler, 2006). Quorum sensing is a density-dependent mechanism of bacterial cell to cell communication. A wide range of functions are regulated by QS, perhaps the most important of which, in the context of pathogenesis, are virulence and biofilm formation (Camara *et al.* 2002). Biofilm formation and maturation have been shown to be regulated by QS (Sakuragi & Kolter, 2007) along with other virulence mechanisms that may contribute to non-healing of chronic wounds. QS is seen as a potential target for interference, triggering the search for viable therapeutic options.

### 1.5.1. Quorum sensing in Gram-negative bacteria

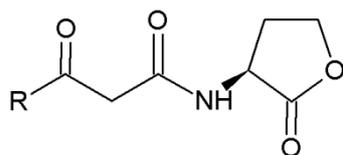
The ability of bacterial cells to communicate with each other in order to modify the expression of bioluminescence was demonstrated in *V. fischeri* in 1970 (Nealson *et al.* 1970), when it was proposed that this mechanism of *lux* gene autoinduction was density-dependent. It soon became clear that this form of coordinated genetic expression was neither confined to luminescence, nor *V. fischeri*. The biosynthesis of the antibiotic carbapenem, in *Erwinia carotovora*, was also found to be regulated in this manner (Bainton *et al.* 1992). Homologues of the *lux* gene have now been documented for numerous Gram-negative bacteria, including *SdiA* in *E. coli* (Sitnikov *et al.* 1996), and *lasR/lasI* in *P. aeruginosa* (Gambello & Iglewski, 1991, Passador *et al.* 1993), two organisms found in chronic wounds.

Structural analyses of autoinducers from various Gram-negative bacteria sources uncovered a group of related small molecules, the acyl-homoserine lactones (Eberhard *et al.* 1981, Pearson *et al.* 1994). The core structure of AHLs consists of a homoserine lactone (HSL) ring with acyl side chains of between 4 to 18 carbons long attached via an amide bond (Fig. 1.3.).

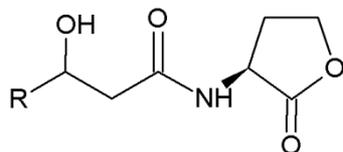
The third carbon in the chain may carry an oxo or hydroxyl substitution. The side chain length and substitution determine the specificity of the AHL, the different types of which are directed by different *lux* homologues (Taga & Bassler, 2003). The majority of AHLs identified are products of fatty acid biosynthesis, thus the acyl side chains, (Schaefer *et al.* 1996), however, a soil bacterium *Rhodopseudomonas palustris* utilises environmental *p*-coumaric acid to synthesise *p*-coumaroyl-HSL (Schaefer *et al.* 2008).



3-oxo-AHL



3-hydroxy-AHL



3-oxo-C8-AHL

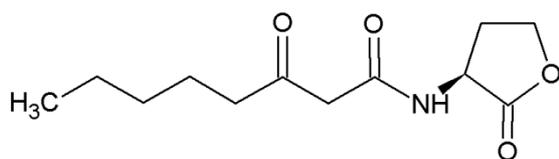


Figure 1.3. Structures of representative AHLs. AHL, acyl-homoserine lactone. The homoserine lactone ring (indicated) is a common feature of AHLs. The chain length can be between 4 and 18 carbons and the third carbon in the chain may carry an oxygen or a hydroxyl substitute.

Probably because it is such a widely studied bacterium, a second class of signalling molecule has been identified in *P. aeruginosa* (Pesci *et al.* 1999); it belongs to the 2-alkyl-4-quinolone family of compounds and is known as the *Pseudomonas* quinolone signal (PQS). It is synthesised from anthranilate and  $\beta$ -keto-(do)decanoic acids (Bredenbruch *et al.* 2005) and is involved in *rhl* regulation, a QS system which produces virulence factors such as rhamnolipid (a lipid biosurfactant), elastase, cyanide and lectins (Diggle *et al.* 2006). Further details of QS specific to *P. aeruginosa* can be found in Chapter 5, Section 5.1.1 and Figure 5.1. In some bacteria the degradation of tryptophan leads to the production of indole, which is now recognised as an interspecies signalling molecule (Lee *et al.* 2007). In *E. coli* the formation of biofilms has been shown to be regulated by the presence of indole and subsequent modification of *SdiA*,

which interestingly responds to AHLs even though *E. coli* does not synthesise these (Yao *et al.* 2006).

Within the cell LuxI or its homologues are responsible for the synthesis of autoinducers which readily diffuse out of the cell. With increasing cell population density an AHL concentration gradient, between the extra-cellular and intra-cellular environment, develops over time leading to a net movement into bacterial cells; once a threshold level is reached the signalling molecules bind to their cognate receptor, LuxR or its homologues, which show high specificity for their ligands. LuxR then binds to the promoter region of a target gene initiating gene expression (Fig 1.4.) (Schauder & Bassler, 2001).

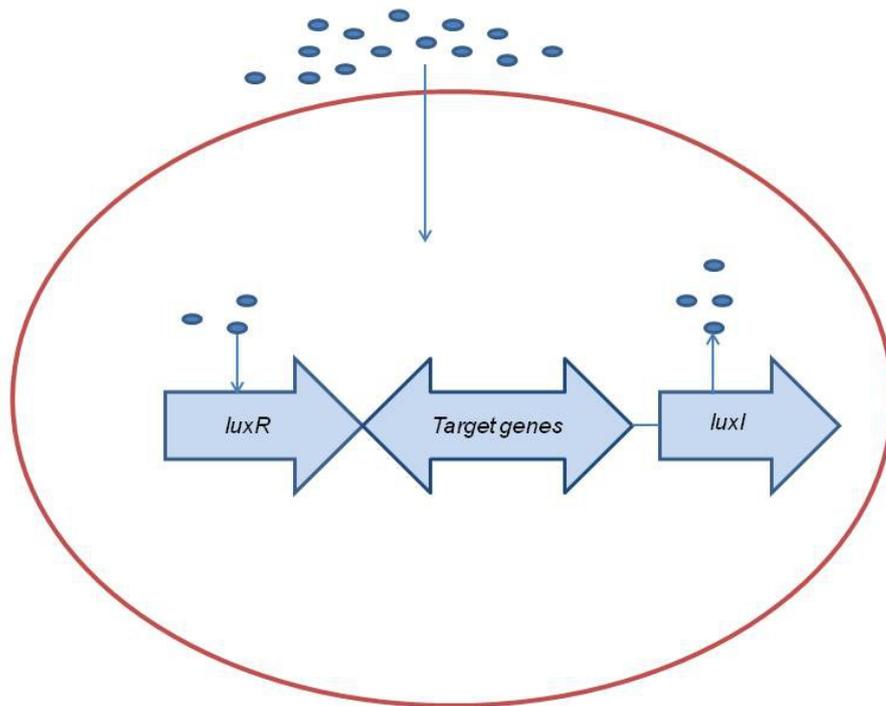


Figure 1.4. LuxI/LuxR QS system of Gram-negative bacteria. LuxI, autoinducer synthase; LuxR, autoinducer regulator, ● autoinducer. With increasing cell population density an AHL concentration gradient develops leading to a net movement into bacterial cells; once a threshold level is reached the signalling molecules bind to their cognate receptor, LuxR or its homologues. LuxR then binds to the promoter region of a target gene initiating gene expression.

The products of QS induced genetic expression vary between species (Table 1.5), though the production of virulence factors is a common theme. It must be noted that the net effect of QS is complex and different signalling molecules may have opposing effects; biofilm formation may depend on a combination of both positive and negative effects and a signalling molecule may induce a biofilm phenotype in one organism and inhibit it in another (Lee et al. 2007).

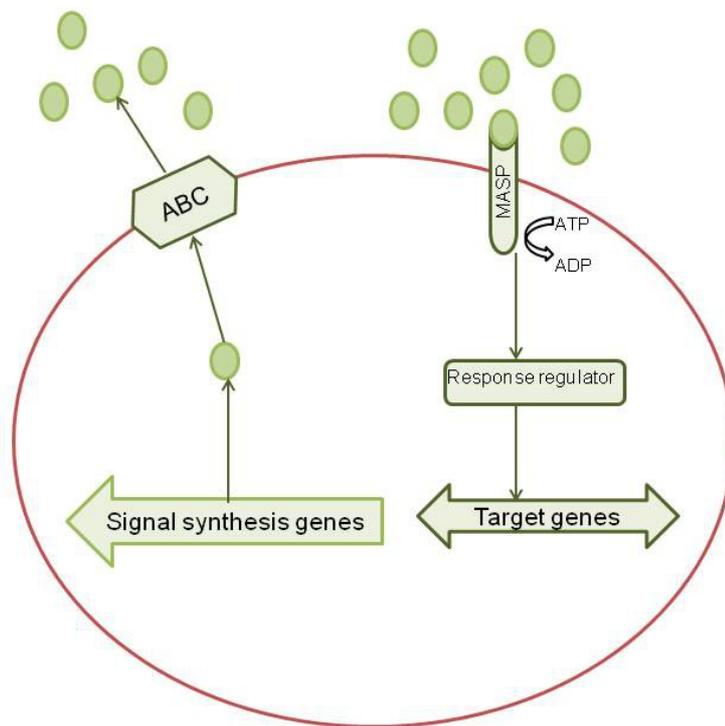
*Table 1.5.* Gram-negative bacteria and their LuxI/LuxR homologue, AHL, and phenotypic products. LuxI, autoinducer synthase; LuxR, autoinducer regulator; AHL, Acyl-homoserine lactone; HSL, homoserine lactone.

Organism	LuxI/LuxR Homologue	AHL	Phenotype	Reference
<i>Burkholderia cepacia</i>	CepI/CepR	C8-HSL	Protease Siderophore	Lewenza <i>et al.</i> 1999
<i>E. coli</i>	SdiA	Unknown Indole	Cell division Biofilm formation	Sitnikov <i>et al.</i> 1996 Lee <i>et al.</i> 2007
<i>P. aeruginosa</i>	LasI/LasR	3-oxo-C12-HSL	Exoenzymes Biofilm formation RhIR	Gambello and Iglewski, 1991
	RhII/RhIR	C4-HSL	Exoenzymes Cyanide Lectins Pyocyanin	Latifi <i>et al.</i> 1996

### 1.5.2. Quorum sensing in Gram-positive bacteria

To date, Gram-positive bacteria are not known to synthesise AHLs. Their QS signalling system is based on autoinducing peptides (AIP), cleaved from precursor peptides, which undergo further post-translational modification (Ji *et al.* 1995). The mechanism leading to gene expression is a two-component system (Fig. 1.5); a membrane bound histidine kinase-associated receptor autophosphorylates on binding of the AIP to the sensor domain. This results in the phosphorylation of a regulator molecule, which in turn, activates a specific gene locus. Signal synthesis genes initiate the synthesis of precursor proteins which are post-translationally modified to signal proteins (AIPs). These are exported via membrane bound ATP-binding cassette transporters (ABCs).

As for the Gram-negative system, QS signalling in Gram-positive bacteria is, primarily, density dependent, and involves the production of virulence factors, such as proteases, hemolysins, enterotoxins, and biofilm formation. These are under the regulation of the accessory gene regulator (*agr*) in *S. aureus* (Peng *et al.* 1988, Recsei *et al.* 1986)



*Figure 1.5.* Two-component QS system in Gram-positive bacteria. ABC, ATP-binding cassette exporter; MASP, membrane associated sensor protein; ●, autoinducer peptide (AIP). An increasing AIP concentration gradient results in binding of AIP to the membrane bound histidine kinase-associated receptor (MASP) which then autophosphorylates. This leads to the phosphorylation of a response regulator, which in turn, activates target genes. Signal synthesis genes initiate the synthesis of precursor proteins which are post-translationally modified to signal proteins (AIPs). These are exported via membrane bound ABCs.

Many QS systems in Gram-positive bacteria have now been studied; a diverse range of AIPs have been identified, and more is now known about how they

achieve specificity. To take a commonly found bacterium in chronic wounds, *S. aureus*, as an example, an interesting picture of self-induction and cross-communication emerges. In common with *S. epidermidis*, sub-groups of *S. aureus* produce unique AIPs that induce self, but inhibit non-self sub-groups; concurrently, AIPs that self-induce in one species of a genus will inhibit a different species within that same genus (Wright *et al.* 2004). Despite having a thiolactone ring with a highly conserved cysteine, AIPs have amino acid extensions of varying sequence and length, which are proposed as determining specificity.

#### *1.5.3. The role of quorum sensing in chronic wounds*

The previous two sections have touched briefly on the mechanism of QS. Signalling molecules continue to be discovered, but what role do they play in the pathogenesis and recalcitrant nature of chronic wounds? The role of QS in the development and maintenance of infection in chronic wounds is gradually being ascertained. An undisturbed wound provides a warm, moist environment, conducive for an increase in bacterial populations that would generate threshold QS signalling molecule concentration. This could result in the expression of virulence factors that prolong the inflammatory response, or reduce the efficacy of polymorphonuclear monocytes (Jensen *et al.* 2007). Exotoxin release (particularly proteases), coupled with hypoxia, may result in damaged host tissue; further enriching the environment for bacterial survival. Arguably one of the most important QS-mediated mechanisms involved in wound pathology is the formation of biofilms.

#### *1.5.4. Quorum sensing and biofilms*

The role of QS in the formation of biofilms appears to be diverse. Different studies have observed conflicting results, illustrating the complexity of bacterial signalling and the processes of biofilm formation. Some researchers, using imaging techniques such as CLSM have demonstrated differences in biofilm architecture between wild-type organisms and their QS mutant equivalents (Davies *et al.* 1998); wild-type *P. aeruginosa* developing mushroom-like structures with water channels, whereas QS mutant biofilms were flat. It has been suggested that this type of biofilm morphology may be determined by QS controlled biosurfactant production (Pamp & Tolker-Nielson, 2007). However, others have found no differences in biofilm structure between wild-type and mutant strains of *P. aeruginosa* (Heydorn *et al.* 2002). Purevdorj *et al.* (2002) demonstrated that laminar and turbulent flow shear can affect biofilm structure and that QS signalling is not essential for biofilm adhesion. The growth of biofilms has been shown to be dependent on many variables, including nutrient availability, carbon source and flow conditions (Purevdorj *et al.* 2002, Kirisits & Parsek, 2006) so it is possible that the varied results are due to protocol differences.

Despite differences in biofilm morphology there is abundant evidence that QS has a significant role to play in biofilm formation in a wide spectrum of bacterial species, including those of clinical importance, such as *P. aeruginosa* (Davies *et al.* 1998), *Acinetobacter* spp. (Anbazhagan *et al.* 2012), *Burkholderia* sp. (Inhülsen *et al.* 2012), *Streptococcus pneumonia* (Vidal *et al.* 2011), *Enterobacter cloacae* (dos Reis Ponce *et al.* 2012), *Serratia marcescens* (Bakkiyaraj *et al.* 2012) and *S. aureus* (Periasamy *et al.* 2012). The importance of this in clinical applications is of particular significance due to the finding by some studies that flat, undifferentiated biofilms are more susceptible to

antibiotics (Bjarnsholt *et al.* 2005) and biocides (Davies *et al.* 1998). Though not necessarily involving the same mechanism this is supported by the observation that the presence of a lactonase (Kiran *et al.* 2011) and QS inhibitors (Brackman *et al.* 2011) increased the antibiotic susceptibility of *P. aeruginosa* biofilms.

A complex picture of biofilm regulation, by *agr* QS in *S. aureus* is also emerging. An active *agr* system, rather than leading to enhanced biofilm formation, instead, induces the formation of biofilm channels and the dissemination of bacterial cells from the biofilm (Periasamy *et al.* 2012) by the action of *agr* regulated proteases (Boles & Horswill, 2008). This has implications for the pathogenicity of the biofilms as cell detachment could lead to the spread of colonisation within and beyond a wound environment. Populations of both wild-type and mutant *agr* genotypes have been identified in *S. aureus* biofilms (Savage *et al.* 2013), suggesting that both may be necessary to maintain infection.

#### 1.5.5. Quorum sensing interference

A means of interfering with QS signalling is beneficial for several reasons, and one way this may be achieved is via inactivation of signalling molecules. Unless secreted into conditions that favour deactivation AHLs are stable for some time (Schaefer *et al.* 2000), which will serve to maintain concentration levels when a response is no longer required, wasting valuable resources. *Agrobacterium tumefaciens* produces at least two degradative enzymes, one of which is a lactonase whose expression is suppressed during logarithmic growth and increased when stationary phase has been reached (Zhang *et al.* 2004). This enzyme catalyzes the hydrolysis of the lactone ring of the AHL. The amide bond

can be cleaved by acylases, an action sufficient to attenuate QS phenotypic changes (Lin *et al.* 2003).

AHLs with a oxo substitute on the third carbon have also been shown to be inactivated by oxidised halogens, such as hypochlorous acid, and hypobromous acid (Borchardt *et al.* 2001). This suggests that as well as an intracellular bactericidal effect, phagocyte-derived oxidants in the extracellular environment could interfere with QS.

Vulnerability to inactivation is not restricted to QS signalling molecules released by Gram-negative bacteria; a Type I AIP, from the Gram-positive bacterium *S. aureus*, has also been shown to be deactivated by phagocyte-derived oxidants (Rothfork *et al.* 2004). Rothfork also postulated that the *in vivo* attenuation of virulence was due to impaired interaction between the AIP and the receptor, rather than the oxidised molecule acting as a competitive receptor antagonist, though it cannot be concluded that this is the case for other signalling molecule inactivation.

QS inhibition is also a necessary strategy for hosts to protect themselves from the damaging effects of invading bacteria; evidence as to how they may do this comes from the Australian red alga *Delisea pulchra*. This seaweed produces halogenated furanones, molecules with some structural similarities to AHLs which have been shown to inhibit QS-induced mechanisms in *Serratia liquefaciens* (Givskov *et al.* 1996). These AHL analogues interact with LuxR-type protein, resulting in increased degradation; a concurrent reduction of a downstream virulence product supports their roles as QS inhibitors (Manefield *et al.* 2001). Evidence suggests that higher order eukaryotes may also benefit from this type of QS interference; synthetic furanones decreased QS-induced bacterial virulence in a mouse model (Wu *et al.* 2004). These are examples of

how, both natural and synthetic analogues may compete with endogenous signalling molecules for receptor binding.

The complexity of QS systems in multi-community populations is evident, making research in this area challenging. Blocking the pathway of one species may serve to facilitate the pathway in another species, shifting the balance of competition to favour one species over another, and as discussed in Section 1.5.4. inhibition of the *agr* QS system in *S. aureus* may result in the dissemination of detached biofilm cells to other parts of a wound. The dynamic of chronic wound bacterial populations is still too incomplete to predict the outcome of manipulation of this kind. However, the ability to inhibit the production of virulence factors and the formation of biofilms could augment the effects of conventional antibacterial drugs.

#### *1.6. Hyperbaric oxygen therapy*

HBO therapy (HBOT) is now accepted as a treatment option for several non-diving related disorders, including carbon monoxide poisoning, soft tissue trauma, osteomyelitis, radionecrosis, and chronic wounds (Wattel, 2004). HBO is defined as breathing oxygen at a pressure higher than local atmospheric pressure; treatment regimens vary according to clinical assessment but for chronic wounds usually involve breathing 100% oxygen at pressures of around 2.4 atmospheres absolute (ATA), for 1-2 hours daily, for several weeks (Roeckl-Wiedmann *et al.* 2005). At the Diving Diseases Research Centre (DDRC) in Plymouth the standard treatment programme lasts until the wound is healed or up to 8 weeks, at which point the wound is reassessed. HBO is administered in either a multiplace chamber, which can accommodate several patients, or in a monoplace chamber which is designed for a single person only.

HBO is still seen as a controversial treatment choice; strong evidence, based on well designed trials incorporating randomised double blind conditions with a statistically viable number of participants is rare. This is partly due to the ethical issues involved with randomising treatments; it would be ethically questionable to deny a patient HBOT. Ironically the lack of referrals to HBO centres, due to the paucity of evidence, perpetuates the limited pool of potential participants for trials. The various studies that have been published do not provide a homogeneous and comparable set of results, mainly due to: (i) the variability of ulcer types, (ii) different end-point criteria, e.g. wound fully closed, 80% closure or reduced risk of amputation, (iii) lack of suitable controls, and (iv) different treatment regimens. However, a Cochrane Database systematic review concluded that HBO significantly reduced the risk of major amputation in diabetic foot ulcers (Kranke *et al.* 2004). A second review that came to the same conclusion (Roeckl-Wiedmann *et al.* 2005) also conceded that evidence for HBO efficacy was lacking for venous or pressure ulcers.

#### *1.6.1. Tissue oxygenation*

The rationale for the effectiveness of HBOT is based on Henry's Law, which states: "The amount of gas that will dissolve into a solution is directly proportional to the partial pressure of that gas and inversely proportional to the absolute temperature" (Bove, 1997). Haemoglobin in human blood is approximately 97% saturated at normobaric pressure and blood plasma contains 0.3 ml of oxygen per 100 ml (Lambertsen *et al.* 1953); at rest tissues require approximately 6 ml of oxygen per 100 ml of plasma which is supplied primarily from that bound to haemoglobin.

The amount of oxygen dissolved in plasma is proportional to the partial pressure of oxygen ( $pO_2$ ); this increases to 1.5 ml per 100 ml when breathing

100% oxygen at normobaric pressure, and to 6 ml per 100 ml at 3 ATA (Lambertsen *et al.* 1953). This higher concentration of plasma oxygen can improve tissue oxygenation in two ways: (i) plasma can reach tissue with restricted microcirculation as it can flow through vessels too narrowed or inflexible to allow the passage of red blood cells, and (ii) an increased oxygen concentration gradient between plasma and tissue means that oxygen can diffuse further into tissue.

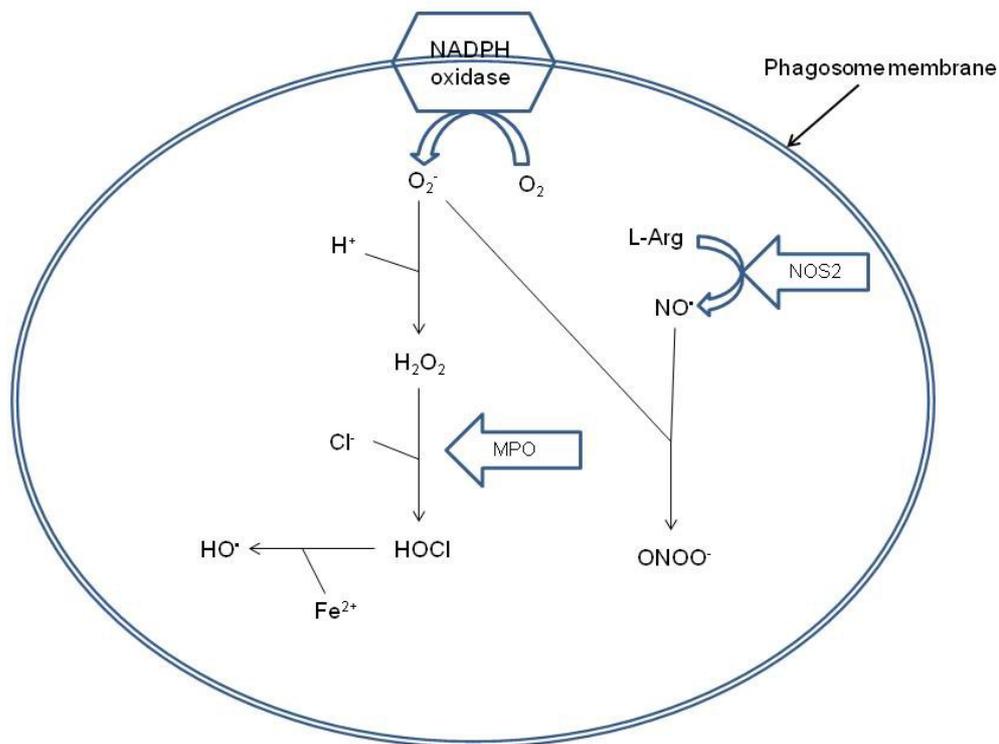
Tissue oxygen tension when breathing normobaric air is approximately 55 mm Hg (Tibbles & Edelsberg, 1996); HBO can increase this to greater than 900 mm Hg at 2.5 ATA (Fife *et al.* 2002), though this will be influenced by any underlying pathology. It is important to note that wound tissue is usually hypoxic in relation to healthy tissue; a rat wound model demonstrated tissue oxygen tension as low as 7 mm Hg in the acute stages of a wound, gradually increasing to 30 mm Hg by the 30<sup>th</sup> day of healing, however, the oxygen tension in an infected wound fell to 0 mm Hg and remained so for the 30 days of the study (Niinikoski *et al.* 1972). Hypoxia has also been measured, using transcutaneous oxygen tension monitoring (TCOM), in human chronic wounds, with levels as low as 3 mm Hg in the skin area adjacent to a wound (Fife *et al.* 2002). Significant improvement in tissue oxygenation is required in order for healing to occur; 90% of chronic wounds failed to heal if their in-chamber TCOM measurements stayed below 100 mm Hg; for wounds achieving 600-699 mm Hg during each treatment session the failure rate dropped to 14.3% (Fife *et al.* 2002). This highlights the detrimental effect of severely impaired limb vascular function on the efficacy of HBO.

TCOM has become a useful predictive tool; sea level measurements can confirm tissue hypoxia around the wound site and in-chamber measurements

with an oxygen challenge (breathing 100 % O<sub>2</sub> at 2.2 ATA) has been shown to be the best indicator as to whether a wound will respond favourably to HBO. Fife *et al.* (2002), concluded that an in-chamber TcPO<sub>2</sub> < 400 mmHg indicates that HBO will fail to result in healing, with a reliability of 75.8%.

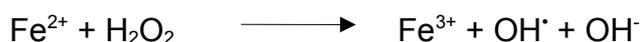
#### *1.6.2. Production of ROS/RNS*

Phagocytes require oxygen for the “respiratory burst”, an important mechanism for bacterial killing (Allen *et al.* 1997). Figure 1.6 illustrates some of the reactive oxygen species (ROS) and reactive nitrogen species (RNS) that may be produced by phagocytes during this process (Halliwell, 2006).



*Figure 1.6.* A schematic of how some ROS and RNS are generated by phagocytes. The membrane bound NADPH oxidase catalyses the production of  $O_2^{\cdot-}$  which dismutates to  $H_2O_2$ . The enzyme myeloperoxidase (MPO) catalyses the oxidation of  $Cl^-$  to produce HOCl. In the presence of free  $Fe^{2+}$ ,  $HO^\cdot$  is produced via Fenton chemistry. Inducible nitric oxide synthase 2 (NOS2) catalyses the conversion of L-arginine to  $NO^\cdot$  which reacts with  $O_2^{\cdot-}$  to produce  $ONOO^\cdot$ .

The membrane-bound NADPH oxidase catalyses the production of superoxide ( $O_2^{\cdot-}$ ) which rapidly dismutates to  $H_2O_2$ . Having relatively low reactivity,  $H_2O_2$  is able to diffuse in and out of cells and traverse some distance from its source; though capable of damaging molecules itself, in the presence of free iron the highly reactive hydroxyl radical ( $OH^\cdot$ ) is produced via Fenton chemistry.



Increased levels of iron-binding proteins have been found in chronic non-healing wounds (Yeoh-Ellerton & Stacey, 2003), though the potential for

microbial products, such as elastases, to modify their function has been demonstrated (Britigan & Edeker, 1991); OH<sup>•</sup> radicals were produced when diferrictransferrin was exposed to *Pseudomonas* elastase. It is not known whether free iron exists *in vivo* in chronic wounds or whether it contributes to Fenton chemistry to generate OH<sup>•</sup>.

Myeloperoxidase (MPO) is a mammalian haem-containing oxidoreductase and is the main component of neutrophil azurophilic granules. It uses H<sub>2</sub>O<sub>2</sub> to oxidise halides, the rate and preference of which is dependent on physiological concentrations and pH (Senthilmohan & Kettle, 2006). It has been demonstrated that at pH 5-7, 90% of H<sub>2</sub>O<sub>2</sub> oxidises chloride (Cl<sup>-</sup>) to produce hypochlorous acid (HOCl) and 10% oxidises bromide (Br<sup>-</sup>) to produce hypobromous acid (HOBr); at pH 7.8 the amount of H<sub>2</sub>O<sub>2</sub> oxidising Br<sup>-</sup> rises to 40%. Plasma concentrations of Cl<sup>-</sup> are approximately 100 mM and plasma concentrations of Br<sup>-</sup>, being in the region of 20-100 μM, are 1,000-fold lower (Harrison & Schultz, 1976) which probably accounts for the perceived preferential affinity for Cl<sup>-</sup> as a substrate for MPO. Evidence for the production of brominating intermediates *in vivo* has been demonstrated in a mouse model of polymicrobial sepsis where levels of 3-bromotyrosine were elevated in peritoneal fluid (Gaut *et al.* 2001).

MPO-generated HOCl is responsible for both oxidation and chlorination reactions (Winterbourn, 2002) and will affect a wide range of biological molecules. Chloramines, a product of amine chlorination by HOCl, is itself reactive; both HOCl and chloramines are particularly reactive towards thiols (Peskin & Winterbourn, 2001) which makes thiol-containing amino acids, such as cysteine, particularly vulnerable to modification. Methionine, histidine,

tryptophan, tyrosine and various lipid components have all been identified as potential targets (Malle *et al.* 2006).

The role of RNS in the bactericidal activity of human phagocytes remains to be elucidated, but they could also play a role in the nitration and possible modification of molecules implicated in antibiotic resistance. It is known that nitric oxide synthase 2 (NOS2 or iNOS) is expressed in tissue macrophages in infectious or inflammatory conditions, which results in a steady production of nitric oxide ( $\text{NO}^{\bullet}$ ) (Coleman, 2002). Cellular nitric oxide and oxygen concentrations are interdependent; higher concentrations of oxygen will increase NOS activity and increased nitric oxide will in turn further increase oxygen levels. Concurrently, increased oxygen also increases the rate of nitric oxide consumption (Thomas *et al.* 2008). Being an uncharged molecule,  $\text{NO}^{\bullet}$  can diffuse readily in and out of cells and though having a relatively short half-life, reacts readily with  $\text{O}_2^{\bullet-}$  to produce peroxynitrite ( $\text{ONOO}^-$ ). Peroxynitrite is a highly reactive molecule in its own right but is readily protonated at physiological pH to peroxynitrous acid ( $\text{ONOOH}$ ) which can split into two radicals.

The generation of RNS, with a metabolic product of nitric oxide, has also been demonstrated in an MPO-catalysed reaction; nitrite ( $\text{NO}_2^-$ ) when oxidised by MPO and  $\text{H}_2\text{O}_2$  went on to form nitrotyrosine (van der Vliet *et al.* 1997). Whether this system is of physiological importance is put into doubt by a study that carried out mass spectrometric analysis of bacterial proteins recovered from neutrophils; proteins were chlorinated rather than nitrated. However, they did generate nitrated proteins when the test medium contained 1 mM of nitrite (Rosen *et al.* 2002). This failed to decrease protein chlorination, a finding at odds with another study, which found a significant inhibitory effect of nitrite on the activity of MPO (van Dalen *et al.* 2000).

Accepting that various components of antibiotic resistance mechanisms may find themselves within a cocktail of reactive molecules, what evidence is there that they undergo modification? It has already been demonstrated that QS signalling molecules are vulnerable to oxidised halogens (Borchardt *et al.* 2001; Rothfork *et al.* 2004) (Section 1.5.5), so it is feasible that phagocyte-derived, and extracellular oxidants, could exert similar effects. Beta-lactamases and other antibiotic deactivating enzymes are proteins and therefore potentially vulnerable to oxidative damage, the degree of which will depend on enzyme structure and accessibility of target amino acids.

As previously discussed the heterogeneous biofilm matrix contains numerous components that could be vulnerable to oxidative damage, e.g. DNA, proteins and lipids, though this would depend on the ability of ROS and RNS to penetrate the EPS. However, quenching of reactive molecules may occur by sacrificial matrix components with limited effect on biofilm function; *P. aeruginosa* alginate has been shown to scavenge hypochlorite, conferring protection to the organism from this compound (Learn *et al.* 1987).

It is necessary to consider the role of antioxidants in an environment that is normoxic or hyperoxic; aerobic and facultative anaerobic bacteria have a range of endogenous antioxidant compounds that they may utilise as protection against oxidative damage (Hasset & Cohen, 1989). They vary from organism to organism but may include superoxide dismutase (SOD) which catalyses the dismutation of  $O_2^{\cdot-}$  to  $H_2O_2$ , and catalase, which catalyses the decomposition of  $H_2O_2$  to  $O_2$  and  $H_2O$ . Glutathione (GSH), a potent antioxidant, does not seem to be as widely distributed in bacteria, and expression is dependent on growth conditions and growth stage (Fahey *et al.* 1978); many bacteria upregulate antioxidant defences in the stationary phase of growth. Other thiol-containing

molecules such as thioredoxin may play a part in bacterial antioxidant defences. The effectiveness of GSH as a defence against HOCl and chloramines has been demonstrated in *E. coli*, both from endogenously produced GSH and when GSH was added to the extracellular environment of GSH-deficient organisms (Chesney *et al.* 1996).

It appears logical that improved tissue oxygenation and facilitated phagocyte function could contribute to the bactericidal actions of HBO. A secondary effect of phagocyte production of oxidative products is the release of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and myeloperoxidase (MPO) into the extracellular environment (Test & Weiss, 1984), a process possibly facilitated by HBO; it is within this environment that molecules secreted by bacteria to enable antibiotic resistance, e.g.  $\beta$ -lactamases, QS signalling molecules and biofilm components, could be vulnerable to oxidative damage.

### 1.6.3. HBO and antibiotics

Several studies have demonstrated a synergistic relationship between HBO and antibiotics (Bornside, 1967, Muhvich *et al.* 1989, Park *et al.* 1991); hyperoxia at atmospheric pressure achieves similar results (Brown *et al.* 1968). The main effect is seen as a reduction in MICs for a range of, but not all, antibiotics tested. It has been postulated that the effect is additive rather than synergistic; in the case of *S. aureus*, population growth was inhibited by hyperoxia alone, to the extent that a gradient of growth was dependent upon the volume of medium used, indicating that the diffusion of oxygen into the growth medium was a limiting factor for growth inhibition (Bornside, 1967).

HBO and hyperoxia have also been shown to prolong the post-antibiotic effect<sup>6</sup> of antibiotics in *P. aeruginosa* (Park *et al.* 1991), an organism which, contrary to

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<sup>6</sup> The time taken for the number of antibiotic treated bacteria to increase by 1 log<sup>10</sup> CFU ml<sup>-1</sup> after antibiotics have been removed.

*S. aureus*, showed enhanced population growth in hyperoxia. These results illustrate that the relationship between oxygen tension and antibiotics is complex and must involve more than an additive effect. Different organisms will have different responses to hyperoxia, which will be dependent on the effectiveness of their antioxidant defences and their metabolic state; these have to be considered alongside the effects of hyperoxia on the mode of action of the antibiotic and the method of entry into the bacterium.

Though various hypotheses have been expressed to explain the above results, such as an altered redox state as a result of hyperoxia or that antibiotics may inhibit induction of antioxidants such as GSH (Park *et al.* 1993), literature searches have failed to identify studies that explore further the relationship between HBO/hyperoxia and antibiotic efficacy, and arguably of more importance in present times, whether there is an interaction between HBO/hyperoxia and the mechanisms of antibiotic resistance. It is notable that all the organisms used in the above studies are antibiotic sensitive strains.

#### *1.6.4. The safety of HBO*

Provided that HBO therapy is administered in accordance with standard protocols to patients who have undergone a pre-treatment assessment it is considered a safe procedure. Mild adverse effects, mainly as a result of barotrauma, can be experienced by some patients; these can include ear pain, toothache and sinus pain (Tibbles & Edelsberg, 1996). Oxygen toxicity is minimised by inserting regular breaks in the programme where air rather than 100% oxygen is breathed. DNA damage is considered to be physiologically insignificant as damage occurring during treatment is rapidly repaired (Speit *et al.* 1998). Evidence also suggests that antioxidant defence mechanisms are upregulated during HBO (Benedetti *et al.* 2004, Gurdol *et al.* 2008). Beneficial

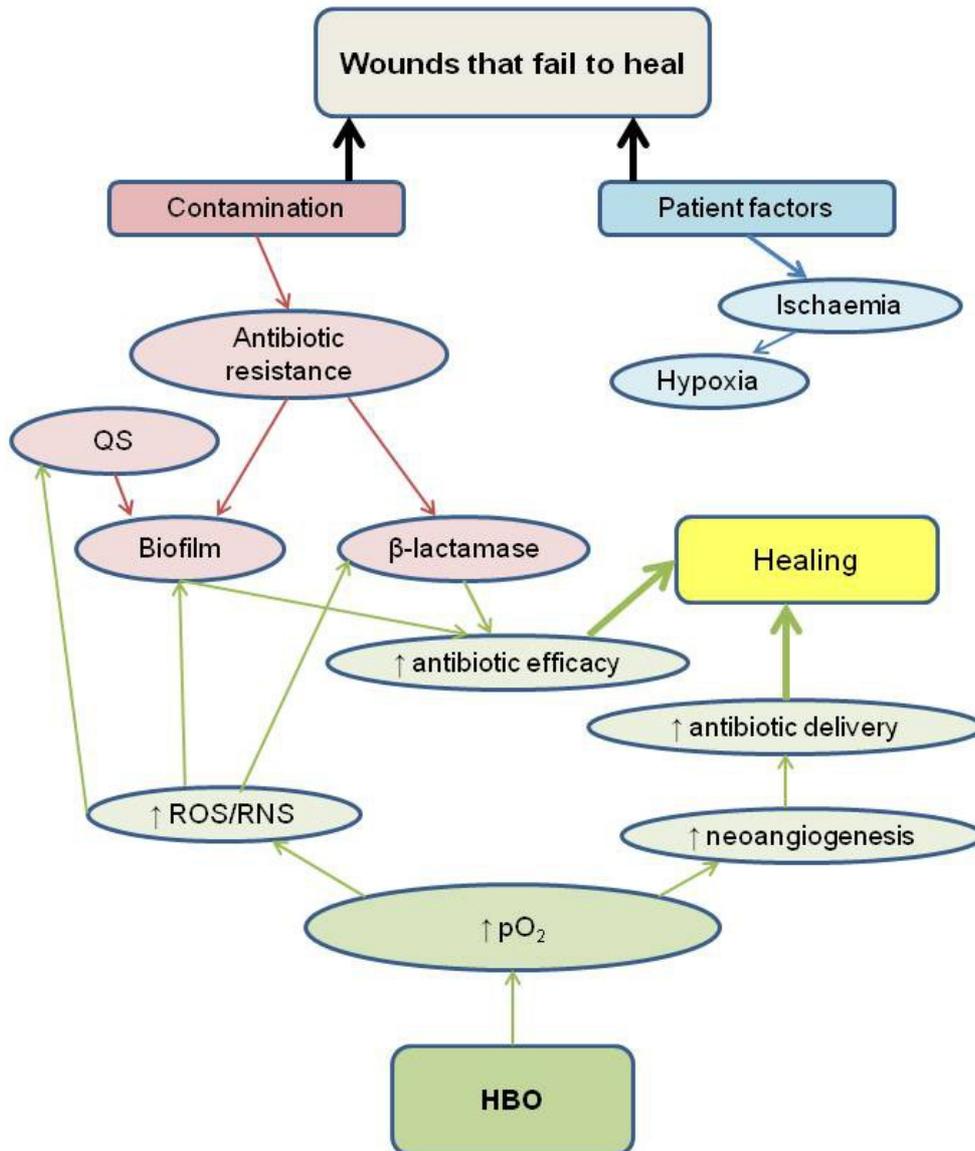
for a toxicity issue, but not for a therapeutic issue is that raised tissue oxygenation is only maintained for a short period of time following each HBO session (Siddiqui *et al.* 1997) (Fig.3.18).

### 1.7. Conclusion, hypotheses and aims

Chronic wounds carry a personal and financial burden; evidence suggests that infection plays an important role in maintaining the non-healing pathology. The ability of bacteria to resist antibiotics hinders the healing process as chemotherapeutic options to clear the wound of infection become limited. The ability of organisms to survive in a wound biofilm, co-ordinated in part by QS, is one of several mechanisms that bacteria utilise to resist antibiotics.

Oxidative damage to biological molecules may lead to modified activity, as demonstrated in the interference of QS, which offers exciting possibilities for novel methods of infection control and the attenuation of virulence. HBOT has been shown to reduce the incidence of major amputation for diabetic patients with foot ulcers which may be due to a reduction in bacterial load. In providing conditions for the potentiation of oxidative damage HBO can be considered as a treatment adjunct in the management of antibiotic resistant bacteria in chronic wounds, however, this appears to be an area as yet unexplored.

The overall aim of the *in vitro* work in this study was to investigate whether oxidants were able to modify three components related to antibiotic resistance, the action of penicillinase (Chapter 2), the signalling ability of QS molecules (Chapter 3) and the reduction in live cells within biofilms (Chapter 4). An overview of how these modifications may facilitate wound healing during a course of HBOT is presented in Figure 1.7.



*Figure 1.7.* Schematic representation of how HBO could facilitate wound healing by the modification of antibiotic delivery and efficacy. HBO increases  $pO_2$  which results in increased neoangiogenesis, facilitating improved delivery of antibiotics to the wound. Increased  $pO_2$  also facilitates ROS/RNS production by immune cells which results in damage to the QS signalling molecules that influence biofilm formation. The combination of ROS and antibiotics results in increased killing of biofilm cells and ROS/RNS damages  $\beta$ -lactamase enzymes. Together these effects increase antibiotic efficacy, which, along with improved delivery, leads to improved wound healing.

In order to gain some insight into whether an environment of increased oxidant generation (i.e. during a course of HBOT) resulted in changes to chronic wound

microflora populations, a clinical study investigating non-healing DFUs during a course of HBOT was carried out using the molecular techniques of PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) and cloning (Chapter 5).

The hypotheses for this thesis are:

- i. Oxidative damage to penicillinase results in a significant reduction of enzyme activity compared to a control.
- ii. Oxidative damage to QS signalling molecules (AHLs) results in a reduction in the QS signalling activity in bacteria, *in vitro* and *in vivo*.
- iii. An increase in oxygen concentration in growth conditions that contain an oxidant generating system results in an increase in bacterial cell death in biofilms.
- iv. HBOT results in changes to chronic wound microbiological communities that will influence wound healing.

The aims of this thesis are:

- i. to demonstrate that ROS/RNS damage to penicillinase results in a reduction in enzyme activity.
- ii. to determine the effect of oxidants on the strength of QS signalling in mono-species, planktonic, bacterial cultures
- iii. to determine the effect of oxidants on the strength of QS signalling in, planktonic, bacterial cultures of mono-species containing gene fusions.
- iv. to determine the effect of oxygen concentration on MICs in both antibiotic sensitive and antibiotic resistant strains of bacteria in planktonic culture conditions.

- v. to determine the effect of oxygen concentration on the formation of biofilms in antibiotic sensitive and resistant strains of *P. aeruginosa* and *S. aureus*.
- vi. to determine the effect of oxygen concentration on the ability of an oxidant generating system to kill bacteria in mono-species biofilms.
- vii. to complete a molecular analysis of the microbiological communities in non-healing diabetic foot ulcers during a course of HBOT.
- viii. to investigate the presence of fungi in non-healing diabetic foot ulcers before and during a course of HBOT.

## Chapter 2

### The effects of ROS/RNS on the activity of penicillinase

#### 2.1. Introduction

Penicillinase is an antibiotic deactivating enzyme (Section 1.2.1), comprised of  $\beta$ -lactamases, with specific affinity for the penicillins. It catalyses the hydrolysis of the amide bond in a  $\beta$ -lactam ring, a structural feature of all penicillins, rendering the drug ineffective against bacteria that produce this enzyme. The number of known  $\beta$ -lactamase inhibitors is limited, clavulanic acid, tazobactam and sulbactam, being the most commonly used adducts to antibiotic therapy (Bebrone *et al.* 2010). However, these compounds do not inhibit metalloenzymes and there is evidence of reduced sensitivity amongst naturally occurring mutant forms of previously vulnerable,  $\beta$ -lactamases (Schroeder *et al.* 2002) and for this reason the search for new methods of inhibition is ongoing.

The aim of this study was to investigate whether  $H_2O_2$ , HOCl, ONOO<sup>-</sup>, and MPO generated oxidants were able to significantly reduce the activity of penicillinase. These ROS/RNS were chosen because it is feasible that they occur extracellularly in a chronic wound environment where secreted  $\beta$ -lactamases could accumulate. Though the measurement of ROS in wounds is problematic it is known that immune cells such as neutrophils release their contents (Section 1.6.2.) via degranulation (Amulic *et al.* 2012) and the production of extracellular traps, a network of strands with bound MPO (Parker & Winterbourn, 2013) that have been detected in vivo in gingival pockets (Vitkov *et al.* 2009).  $H_2O_2$  readily diffuses out of cells and could act as a substrate for the net-bound MPO to produce extracellular HOCl. Levels of these ROS, associated with neutrophil extracellular traps, have been found to be sufficient for bacterial (Brinkmann *et al.* 2004) and fungal killing (Urban *et al.* 2006).

The choice of ROS concentration was based on those measured in vivo; HOCl concentrations have been estimated to vary between 25 and 200  $\mu\text{M}$  (McCall *et al*, 2001), reaching the upper level in pathological conditions.  $\text{H}_2\text{O}_2$  levels have been measured in several human tissues and systems, including up to around 100  $\mu\text{M}$  in urine (Long *et al*, 1999) and ocular tissue (Spector *et al*, 1998), and very low  $\mu\text{M}$  levels in human plasma (Halliwell *et al*, 2000). A range of concentrations was used for each ROS, apart from  $\text{ONOO}^-$ , which was determined by the limited volume synthesised.

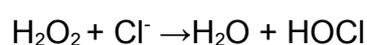
The penicillinase used in this study was from *Bacillus cereus* (*B. cereus*) and comprised two  $\beta$ -lactamases (Section 1.2.1); one had a serine at its active site, the other was a metalloenzyme that utilised two zinc ions as cofactors. In order to determine whether supplemental zinc was necessary for metalloenzyme function an assay was performed of enzyme activity with various concentrations of  $\text{ZnSO}_4$ . It was also beneficial to determine what proportion of activity was due to each  $\beta$ -lactamase, so a further assay of penicillinase activity was carried out with the addition of various concentrations of EDTA, a chelating agent. EDTA should reduce the activity of the metalloenzyme, but not the serine containing enzyme, if the EDTA bound the active site zinc ions sufficiently to disrupt function.

Trace amounts of catalytic transition metals in buffer may result in oxidative reactions, such as that produced via Fenton chemistry (Section 1.6.2.), that affect the interpretation of data from assays. It was therefore desirable to treat all buffers with a chelating agent prior to use. Chelex, an ion-exchange resin, was used to treat all vulnerable solutions. An ascorbate assay was performed to check that Chelex had successfully removed trace metals from a sample of treated buffer.

The penicillinase was exposed to ROS/RNS, and modification of activity was determined using a colorimetric assay with CENTA as a substrate. CENTA is a chromogenic cephalosporin which is readily hydrolysed by  $\beta$ -lactamases (Bebrone *et al.* 2001). It undergoes a colour change from light yellow ( $\lambda$  maximum 340 nm) to chrome yellow ( $\lambda$  maximum 405 nm) making it measurable with a spectrophotometer. Due to the possibility that ROS were able to bleach CENTA the absorbance of CENTA was measured following the addition of various concentrations of ROS.

In addition to looking at the direct effect of ROS on penicillinase activity a ROS generating model was also investigated. Initially, lactoperoxidase (LPO) was used as an oxidant generating model as it was cheaper to purchase than MPO. LPO is a haem-containing peroxidase which catalyses the oxidation of halides or pseudohalides, particularly iodide and thiocyanate, by hydrogen peroxide. In mammals it is found in milk, saliva and tears (Morrison *et al.* 1965, Thomas *et al.* 1994). The products of oxidation, iodine and thiocyanous acid, are known to be antimicrobial (Welk *et al.* 2009) as well as potential oxidising agents. However, this model was unsuccessful and it was decided to use MPO.

MPO is a haem-containing peroxidase found, predominantly, in neutrophils (Section 1.6.2.). When stimulated, neutrophils generate  $O_2^-$  and  $H_2O_2$  during the “respiratory burst” and MPO utilises  $H_2O_2$  and a halide, or pseudohalide, to produce other ROS, however, it is unique amongst the peroxidases for being able to utilise chloride as a substrate (Winterbourn, 2002) to produce HOCl:



As  $H_2O_2$  is a substrate of MPO it was necessary to include  $H_2O_2$  alone as a control to determine how much of the reduction in penicillinase activity may

have been due to H<sub>2</sub>O<sub>2</sub> directly. Catalase was used to quench H<sub>2</sub>O<sub>2</sub> and reduced glutathione was used to quench HOCl, in order to control the time that penicillinase was exposed to the oxidants. In order to estimate whether residual activity, following ROS exposure, was that of the metalloenzyme, assays were carried out with the addition of EDTA just prior to the determination of enzyme activity.

To summarise, the aim of this study was to demonstrate that ROS/RNS damage penicillinase and result in a reduction in enzyme activity. The objective was to measure penicillinase activity following exposure to various RS. This study found that the penicillinase used was vulnerable to H<sub>2</sub>O<sub>2</sub>, HOCl, ONOO<sup>-</sup>, and possibly, MPO generated oxidants.

## *2.2. Materials and methods*

### *2.2.1. Chemicals*

CENTA and myeloperoxidase (Cat# 475911) were obtained from Calbiochem (Merck Serono Ltd, Feltham, Middlesex, UK). Ethanol, glacial acetic acid, hydrochloric acid, L- ascorbic acid (sodium salt) and sodium iodide were obtained from BDH Biochemical (VWR, BDH Prolabo, Lutterworth, Leicestershire, UK). Catalase (Cat# 60635), Chelex 100 (sodium form, 200-400 mesh) DMF, DTPA, EDTA, glutathione, guaiacol, hydrogen peroxide (30%), lactoperoxidase (from bovine milk, Cat# 61328), penicillinase, sodium thiocyanate, TMB, taurine and all inorganic salts were obtained from Sigma-Aldrich (Poole, Dorset, UK). Bleach was obtained from J Sainsbury plc (London, UK). Potassium permanganate was kindly donated by Nick Crocker (University of Plymouth, Plymouth, UK). All water used was prepared in a Milli-Q system (resistivity = 18 MΩ·cm<sup>-1</sup>).

### *2.2.2. Preparation of Chelex-treated potassium phosphate buffer*

Stock solutions of 1 M potassium phosphate (monobasic) and potassium phosphate (dibasic) were prepared. Appropriate volumes of each solution were combined and then diluted with ultrapure water. The pH was checked and adjusted before making up to the final volume.

Dialysis tubing (20 mm wide) was prepared by cutting a length 10 cm long and boiling it for 5 min in 1 mM EDTA, pH 8. Once cool, the tubing was rinsed well with dH<sub>2</sub>O. For each 500 ml of buffer 2.5 g of Chelex was placed in the prepared dialysis tubing. Each end of the tubing was knotted after squeezing out any air. The tube containing the Chelex was placed in a bottle of buffer and shaken overnight, on an orbital shaker at 100 rpm. The Chelex was left in the buffer. Unless otherwise stated all buffer used in work involving penicillinase was 50 mM potassium phosphate buffer, pH 7 and was stored at 4 °C.

### *2.2.3. Ascorbate assay for trace metal contamination of buffer*

An assay, based on the method by Buettner (1988), was carried out to check the efficiency of Chelex in the removal of trace metals. Briefly, 1 ml of untreated potassium phosphate buffer was placed in a 1.6 ml volume cuvette (disposable plastic, UV – 220-900 nm, Fisher Scientific, Loughborough, Leicestershire, UK). The cuvette was placed in a UV spectrophotometer (Perkin Elmer UV/VIS, Lambda Bio 20, with UV WinLab Software, Cambridge, UK) with a circulating water bath at 25 °C. One microlitre of 100 mM sodium ascorbate solution was added to the cuvette and stirred thoroughly after which absorbance was measured at 265 nm for 15 min. The data was plotted (SigmaPlot, Systat Software Inc. Hounslow, London, UK) and using linear regression analysis, the mean reaction rate % change min<sup>-1</sup> was calculated (Table 2.1).

Table 2.1. Mean rate of ascorbate loss in treated and untreated buffer, n = 4

Buffer	% loss of ascorbate in 15 min
Untreated buffer	13.95
Chelex treated buffer	0.43

The lower percent loss of ascorbate from the treated buffer indicated that the Chelex was successfully removing transition metals from the buffer, and fell below the upper limit of 0.5 % loss of ascorbate in 15 min, which indicated non-significant metal contamination (Buettner, 1988).

#### 2.2.4. Penicillinase activity with CENTA as substrate

In order to determine the optimal concentration of penicillinase and its substrate to use for all enzyme activity assays following ROS exposure, a colorimetric assay was carried out using CENTA as a substrate. Purchased CENTA was reconstituted in buffer and stored in 1 ml aliquots of 0.4 mg ml<sup>-1</sup> at -20 °C. Penicillinase (from *B cereus*, 1,500-3000 U mg<sup>-1</sup> protein<sup>7</sup>) was dissolved in buffer and stored in 500 µl aliquots of 500 U ml<sup>-1</sup> at -20 °C. All solutions were thawed on ice immediately prior to use.

For the assay, CENTA (0.4 mg ml<sup>-1</sup>) was diluted with buffer to make 5 ml of 0.02 mg ml<sup>-1</sup>; stock penicillinase was diluted with buffer to make 5 different concentrations. Three hundred microlitres of buffer was added to a 1 ml plastic cuvette (1 cm path length) which was placed in a spectrophotometer with a circulating water bath at 30 °C; 450 µl of CENTA (0.02 mg ml<sup>-1</sup>) was then added and stirred. Continuous readings were commenced at 405 nm and 150 µl of penicillinase (13.8 U ml<sup>-1</sup>) was added to the cuvette and stirred thoroughly for approximately 10 s. Absorbance was read for 10 min. This procedure was repeated for all concentrations of penicillinase. The spectrophotometer was blanked with 1 ml buffer prior to all samples being read. This assay was

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<sup>7</sup> One unit will catalyse the hydrolysis of 1.0 µmol of substrate (benzylpenicillin) per min at pH7, at 25 °C.

repeated for all new batches of penicillinase or CENTA and the optimum concentrations were deemed as those that gave good absorbance values (> 0.05) with a reaction rate that did not level off too soon. Although it would have been preferable to obtain a linear rate of increase in absorbance for several minutes this was not always achieved.

#### *2.2.5. Effect of EDTA on penicillinase activity*

The basic penicillinase and CENTA assay was repeated with various concentrations of EDTA; a 900 µl volume in each cuvette consisting of 446 µl of 50 mM potassium phosphate buffer, pH 7, 450 µl of CENTA (0.04 mg ml<sup>-1</sup>), and 2 µl of EDTA (various concentrations). Two microlitres of penicillinase (81 U ml<sup>-1</sup>) were added to initiate the reaction and the contents were read at 405 nm for 10 min.

#### *2.2.6. Effect of zinc supplementation on penicillinase activity*

The basic penicillinase and CENTA assay was repeated with various concentrations of ZnSO<sub>4</sub>; a 900 µl volume in each cuvette consisting of 430 µl of 50 mM potassium phosphate buffer, pH 7, 450 µl of CENTA (0.04 mg ml<sup>-1</sup>), and 10 µl of ZnSO<sub>4</sub> (various concentrations). Ten microlitres of penicillinase (225 U ml<sup>-1</sup>) was added to initiate the reaction and the contents were read at 405 nm for 10 min.

#### *2.2.7. Catalase assay*

The enzyme catalase was used for the quenching of H<sub>2</sub>O<sub>2</sub> in some of the following assays:  $2 \text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2 \text{H}_2\text{O} + \text{O}_2$

An assay of catalase activity was carried out based on the decomposition of H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich, 2008). The spectrophotometer (with circulating water bath at 25 °C) was blanked with 1.5 ml of 50 mM potassium phosphate buffer, pH 7. A cuvette (UV range, plastic) containing 1.45 ml of 0.036 % H<sub>2</sub>O<sub>2</sub> was placed in

the spectrophotometer and recording was commenced at 240 nm. Fifty microlitres of suspension ( $100 \text{ U ml}^{-1}$ ) was added to the cuvette and the contents stirred well for 5-10 s; recording was continued for a further 10 min. The calculation used to determine activity can be found in Appendix 1.

#### *2.2.8. General protocol for the exposure of penicillinase to ROS*

The assay carried out for each exposure of penicillinase to ROS had the same basic structure, but as solutions and concentrations varied the assay will be described in detail in each appropriate section. Briefly, the core of the assay involved exposing penicillinase to ROS for up to 60 min with samples being removed at 10 min intervals from time zero. The oxidative reaction was quenched for each sample removed and the samples were stored on ice. At the end of sampling three replicates of each time point were placed in a 96-well plate (clear, plastic, flat-bottom) and the plate was read at 405 nm immediately following the addition of CENTA. Details of controls used will be given in each section. All data were plotted with SigmaPlot and either linear, or non-linear (exponential rise to maximum, single, 3 parameter) regression analysis performed to determine the initial reaction rate. The equation to determine initial rate can be found in Appendix 2.

Concentrations of penicillinase varied throughout the study due to batch differences in enzyme activity. The concentration used for exposure was determined. Alongside that of CENTA, from the enzyme activity assay described in Section 2.2.4. Prior to starting the assay penicillinase and CENTA were thawed, diluted with buffer to appropriate concentrations and stored on ice. All other solutions used were prepared as required and stored on ice. Details of these solutions will be given in the appropriate sections of this

chapter. All assays were carried out at least three times unless otherwise stated.

### *2.3. Exposure of penicillinase to lactoperoxidase generated oxidant*

#### *2.3.1. Lactoperoxidase activity assay*

An assay to determine lactoperoxidase activity was based on that in the Worthington Enzyme Manual (Worthington & Worthington 2007), which was a modification of that of Morrison (1970). The rate of oxidation of iodide to triiodide is measured spectrophotometrically. It was observed, however, that a fall in the rate of reaction occurred after only 30 s; after consulting the original paper it was noted that different concentrations of H<sub>2</sub>O<sub>2</sub> had been used in the two assays and as it has been suggested that an excess of H<sub>2</sub>O<sub>2</sub> may inhibit enzyme activity (Galijasevic *et al.* 2004) it was decided to investigate the effect of H<sub>2</sub>O<sub>2</sub> concentration on LPO activity.

A reaction mixture was prepared containing 50 mM potassium buffer, pH 7, 5 mM potassium iodide (KI) and 0.45 mM H<sub>2</sub>O<sub>2</sub>. One millilitre of reaction mixture was placed in a cuvette and a blank rate established at 350 nm, at 25 °C. LPO (3 µl of 66.3 U ml<sup>-1</sup>) was then added to the cuvette and stirred vigorously. Absorbance was read at 1 s intervals for 5 min. The above was repeated with the reaction mixture containing 0.3 mM and 0.15 mM H<sub>2</sub>O<sub>2</sub>, respectively. The calculation to determine U mg<sup>-1</sup> enzyme can be found in Appendix 3.

#### *2.3.2. Exposure of penicillinase to LPO generated oxidant*

Initially an assay using KI as a substrate was used, but data was extremely inconsistent so a revised protocol using thiocyanate (SCN<sup>-</sup>) was devised. Four conditions were prepared (Table 2.2) in 1.5 ml microcentrifuge tubes. At time zero, for each condition (this was staggered, with 2 min intervals), 100 µl penicillinase (23.5 U ml<sup>-1</sup>) was added and mixed well; immediately 60 µl was

transferred to a 0.7 ml microcentrifuge tube containing 1  $\mu\text{l}$  of catalase<sup>8</sup> (20 000 U  $\text{ml}^{-1}$ ). This was mixed well and stored on ice. At 10 min intervals a further 60  $\mu\text{l}$  of mix was transferred in the same manner, for all conditions. This was repeated for up to 60 min. The final concentration of penicillinase in each tube was 4.7 U  $\text{ml}^{-1}$ .

*Table 2.2.* Exposure of penicillinase to LPO-derived oxidants. Volumes and concentrations of solutions combined for each condition. Values in brackets are final concentrations. At time zero, 100  $\mu\text{l}$  of penicillinase (23.5 U  $\text{ml}^{-1}$ ) was added to each condition. The final concentration of penicillinase in each tube was 4.7 U  $\text{ml}^{-1}$ .

Condition	Potassium phosphate buffer	SCN <sup>-</sup>	H <sub>2</sub> O <sub>2</sub>	LPO <sup>9</sup>	H <sub>2</sub> O
ROS exposed penicillinase	390 $\mu\text{l}$ 50 mM pH7	5 $\mu\text{l}$ 50 mM (500 $\mu\text{M}$ )	3 $\mu\text{l}$ 25 mM (150 $\mu\text{M}$ )	2 $\mu\text{l}$ 78 U $\text{ml}^{-1}$ (0.3 U $\text{ml}^{-1}$ )	
Penicillinase activity control	390 $\mu\text{l}$ 50 mM pH7				10 $\mu\text{l}$
No enzyme control	390 $\mu\text{l}$ 50 mM pH7	5 $\mu\text{l}$ 50 mM (500 $\mu\text{M}$ )	5 $\mu\text{l}$ 15 mM (150 $\mu\text{M}$ )		
No substrate control	390 $\mu\text{l}$ 50 mM pH7			10 $\mu\text{l}$ 15 U $\text{ml}^{-1}$ (0.3 U $\text{ml}^{-1}$ )	

When all samples had been collected, 15  $\mu\text{l}$  from each tube was transferred to each of three wells in a 96-well microplate. Immediately, 250  $\mu\text{l}$  of CENTA (0.01 mg  $\text{ml}^{-1}$ ) was added to each well containing samples and also to a further three wells to provide an absorbance measurement for CENTA. The plate was read in a platereader (Optimax, Molecular Devices, Wokingham, Berkshire, UK) at 405 nm for 10 min. Data were acquired with SoftmaxPro software and plotted with SigmaPlot software.

#### 2.4. Exposure of penicillinase to MPO-generated oxidants

<sup>8</sup> 2000 U  $\text{mg}^{-1}$  protein. One unit will decompose 1.0  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub>  $\text{min}^{-1}$  at pH7 at 25 °C.

<sup>9</sup> 88 U  $\text{mg}^{-1}$  protein, 39 U  $\text{mg}^{-1}$  solid. One unit will form 1.0 mg purpurogallin from pyrogallol in 20 s at pH 6 at 20 °C.

#### 2.4.1. Chloramine production by MPO

MPO activity with chloride as the halide substrate was assayed using the method of Dypbukt *et al.* (2005). Briefly, the chlorination of taurine by HOCl results in the production of taurine chloramine, which is then detected by iodide catalysed oxidation of TMB to a blue product which is proportional to the concentration of taurine chloramine.

#### 2.4.2. MPO assay standard curves

Standard buffers were prepared with various concentrations of HOCl (Table 2.3). HOCl was prepared by diluting bleach with 50 mM potassium phosphate buffer, pH 7, and reading absorbance at 235 nm to determine concentration ( $\epsilon_{235} = 100 \text{ M}^{-1} \text{ cm}^{-1}$ ). The developing reagent was prepared (Table 2.3) immediately before use.

Table 2.3. Composition and final concentrations of standard buffer and developing reagent for the formulation of standard curves. \* TMB was dissolved in DMF (20 mM TMB) prior to addition to the developing reagent.

Standard Buffer pH 7.4	Developing Reagent
10 mM phosphate buffer	400 mM acetate buffer, pH 5.4
140 mM NaCl	2 mM TMB*
10 mM KCl	100 $\mu$ M NaI
5 mM taurine	
HOCl	

Two hundred microlitres of each standard was transferred to each of three wells of a 96 well plate; 50  $\mu$ l of developing reagent was then added to each well. The plate was left at room temperature for 5 min before reading at 650 nm. The control consisted of standard buffer with ultrapure water in place of HOCl.

### 2.4.3. MPO assay

Taurine containing buffer was prepared fresh prior to each run of the assay, the composition of which is 20 mM potassium phosphate buffer, pH 6.5, 100 mM sodium chloride and 5 mM taurine, and 6.5 ml of this buffer was transferred to a 20 ml universal container. To this was added 5.91  $\mu\text{l}$  of MPO (594 U  $\text{ml}^{-1}$ ) and the contents of the container were stirred well. Seven hundred microlitres of this mix was added to each of nine 1.5 ml microcentrifuge tubes and these were stored on ice. At time zero, 7  $\mu\text{l}$  of 2mM  $\text{H}_2\text{O}_2$  was added to one of the microcentrifuge tubes followed immediately by 3  $\mu\text{l}$  of catalase (9320 U  $\text{ml}^{-1}$ ). The contents were mixed well and these were stored on ice. This was repeated for all microcentrifuge tubes containing the taurine-buffer mix but catalase was added after a different time period for each tube. From each tube, 200  $\mu\text{l}$  was transferred to each of three wells in a 96-well plate; 50  $\mu\text{l}$  of developing reagent (as described in Section 2.4.2.) was added to each well and the plate was left at room temperature for 5 min after which it was read at 650 nm. The equation for calculating the concentration of HOCl at each time point can be found in Appendix 4.

### 2.4.4. Exposure of penicillinase to MPO-derived oxidants

Three 1.5 ml microcentrifuge tubes were prepared (Table 2.4) and left at room temperature. At time zero, 100  $\mu\text{l}$  of penicillinase (34 U  $\text{ml}^{-1}$ ) was added to one of the tubes, vortexed briefly, and immediately 60  $\mu\text{l}$  was transferred to a 0.7 ml microcentrifuge tube containing 1  $\mu\text{l}$  of catalase ( $2 \times 10^4$  U  $\text{ml}^{-1}$ ). This was stored on ice. This process was repeated for all other tubes. At 10 min, and thereafter, at 10 min intervals up to a total of 60 min; 60  $\mu\text{l}$  aliquots were transferred from each tube to fresh microcentrifuge tubes containing catalase. The final concentration of penicillinase in each tube was 6.8 U  $\text{ml}^{-1}$ .

*Table 2.4.* Exposure of penicillinase to MPO-derived oxidants. Contents of each 1.5 ml microcentrifuge tube. Penicillinase (100  $\mu\text{l}$  of 34  $\text{U ml}^{-1}$ ) was added to each tube at time zero (this was staggered to allow time to remove first sample). Values in brackets are final concentrations in the tube. The final concentration of penicillinase in each tube was 6.8  $\text{U ml}^{-1}$ .

	<b>ROS exposed penicillinase</b>	<b>Penicillinase activity control</b>	<b>No enzyme control</b>
<b>50 mM potassium phosphate buffer</b>	365 $\mu\text{l}$	365 $\mu\text{l}$	365 $\mu\text{l}$
<b>150 mM <math>\text{H}_2\text{O}_2</math></b>	5 $\mu\text{l}$ (1.5 mM)		5 $\mu\text{l}$ (1.5 mM)
<b>3 M NaCl</b>	25 $\mu\text{l}$ (150 mM)		25 $\mu\text{l}$ (150 mM)
<b>135 <math>\text{U ml}^{-1}</math> MPO</b>	5 $\mu\text{l}$ (1.35 $\text{U ml}^{-1}$ )		
<b>Ultrapure <math>\text{H}_2\text{O}</math></b>		35 $\mu\text{l}$	

When all samples had been collected, 15  $\mu\text{l}$  of each sample was placed in each of three wells of a 96 well plate. When all aliquots had been transferred, 250  $\mu\text{l}$  of CENTA (0.01  $\text{mg ml}^{-1}$ ) was added to each well. The same volume of CENTA was also added to three wells containing 15  $\mu\text{l}$  of buffer only. The plate was read at 405 nm for 10 min.

### *2.5. Exposure of penicillinase to $\text{H}_2\text{O}_2$*

As it was observed that  $\text{H}_2\text{O}_2$  with no added MPO also reduced the activity of penicillinase it was decided to investigate this effect in more detail. The method was exactly the same as that described in Section 2.4.4., except for the contents of the tubes (Table 2.5).

*Table 2.5.* Contents of tubes for exposure of penicillinase to H<sub>2</sub>O<sub>2</sub>. At time zero 100 µl of penicillinase (180 U ml<sup>-1</sup>) was added to each tube. The far left column gives the final concentration of H<sub>2</sub>O<sub>2</sub> that penicillinase was exposed to in the tube. The final concentration of penicillinase was 36 U ml<sup>-1</sup>.

Control	370 µl 50 mM potassium phosphate buffer, pH 7	30 µl H <sub>2</sub> O
15 µM H <sub>2</sub> O <sub>2</sub>	370 µl 50 mM potassium phosphate buffer, pH 7	30 µl 250 µM H <sub>2</sub> O <sub>2</sub>
150 µM H <sub>2</sub> O <sub>2</sub>	370 µl 50 mM potassium phosphate buffer, pH 7	30 µl 2.5 mM H <sub>2</sub> O <sub>2</sub>
1.5 mM H <sub>2</sub> O <sub>2</sub>	370 µl 50 mM potassium phosphate buffer, pH 7	30 µl 25 mM H <sub>2</sub> O <sub>2</sub>

### *2.5.1. The effect of EDTA on H<sub>2</sub>O<sub>2</sub> exposed penicillinase*

A modification of the protocol (Section 2.4.4) was carried out to investigate the effect of EDTA on H<sub>2</sub>O<sub>2</sub> exposed penicillinase. Two tubes were prepared (Table 2.6) and two samples were taken from each tube at time zero, as previously described. Two further samples were taken from each tube after 60 min of exposure time only.

*Table 2.6.* Contents of tubes for exposure of penicillinase to H<sub>2</sub>O<sub>2</sub>. At time zero 100 µl of penicillinase (180 U ml<sup>-1</sup>) was added to each tube. The far left column gives the final concentration of H<sub>2</sub>O<sub>2</sub> that penicillinase was exposed to in the tube. The final concentration of penicillinase was 36 U ml<sup>-1</sup>.

<b>Control</b>	370 µl 50 mM potassium phosphate buffer, pH 7	30 µl H <sub>2</sub> O
<b>1.5 mM H<sub>2</sub>O<sub>2</sub></b>	370 µl 50 mM potassium phosphate buffer, pH 7	30 µl 25 mM H <sub>2</sub> O <sub>2</sub>

One microlitre of EDTA (6 mM) was added to one sample from each tube, for the 60 min time point only. Catalase was used, as previously described, to quench the reaction. Samples were left at room temperature for 5 min. Three replicates of each sample were transferred to a 96 well plate as previously described and the plate was read at 405 nm for 10 min following the addition of CENTA. The final concentration of EDTA in the tubes was 100 µM.

### *2.5.2. The effect of H<sub>2</sub>O<sub>2</sub> on the absorbance of CENTA*

In order to rule out any bleaching effect of H<sub>2</sub>O<sub>2</sub> on CENTA, the absorbance of CENTA was measured before and after incubation with H<sub>2</sub>O<sub>2</sub>. Four concentrations of H<sub>2</sub>O<sub>2</sub> were prepared, which when 50 µl was added to 250 µl of CENTA resulted in the following concentrations: 10 µM, 100 µM, 1 mM and

10 mM. Three replicates of each concentration were transferred to a 96 well plate and three wells were included with added water as a control; CENTA (250  $\mu\text{l}$  of 0.02  $\text{mg ml}^{-1}$ ) was added to each well and the absorbance read at 340 nm. The plate was left at room temperature for 3 min and then read again.

### 2.6. Exposure of penicillinase to HOCl

The method for this exposure experiment was the same as that described in Section 2.4.4. except for the contents of the tubes (Table 2.7) and 1  $\mu\text{l}$  of reduced glutathione (60 mM) which was added to each 0.7 ml microcentrifuge tube, instead of catalase, to quench the reaction of HOCl with penicillinase.

*Table 2.7.* Contents of tubes for exposure of penicillinase to HOCl. At time zero 100  $\mu\text{l}$  of penicillinase (180  $\text{U ml}^{-1}$ ) was added to each tube. The far left column gives the final concentration of HOCl that penicillinase was exposed to in the tube. The final concentration of penicillinase was 36  $\text{U ml}^{-1}$ .

<b>Control</b>	370 $\mu\text{l}$ 50 mM potassium phosphate buffer, pH 7	30 $\mu\text{l}$ $\text{H}_2\text{O}$
<b>4 <math>\mu\text{M}</math> HOCl</b>	370 $\mu\text{l}$ 50 mM potassium phosphate buffer, pH 7	30 $\mu\text{l}$ 68 $\mu\text{M}$ HOCl
<b>8 <math>\mu\text{M}</math> HOCl</b>	370 $\mu\text{l}$ 50 mM potassium phosphate buffer, pH 7	30 $\mu\text{l}$ 136 $\mu\text{M}$ HOCl
<b>16 <math>\mu\text{M}</math> HOCl</b>	370 $\mu\text{l}$ 50 mM potassium phosphate buffer, pH 7	30 $\mu\text{l}$ 272 $\mu\text{M}$ HOCl

#### 2.6.1. The effect of HOCl on the absorbance of CENTA

A range of dilutions, from 20  $\mu\text{M}$  to 1.5 mM, of HOCl were prepared and three, 50  $\mu\text{l}$ , replicates of each dilution were placed in a 96 well plate. Fifty microlitres of 50 mM potassium phosphate buffer, pH 7, were also added to three wells. Two hundred and fifty microlitres of CENTA (0.024  $\text{mg ml}^{-1}$ ) was added to each well and the plate was read at 340 nm.

## *2.7. Exposure of penicillinase to peroxynitrite*

In order to determine whether reactive nitrogen species were also capable of reducing the activity of penicillinase, peroxynitrite ( $\text{ONOO}^-$ ) was synthesised, based on a method by Robinson and Beckman (2005).

### *2.7.1. Synthesis of $\text{ONOO}^-$*

A 250 ml conical flask containing 50 ml of 0.6 M  $\text{NaNO}_2$  was placed on a magnetic stirring plate in a fume cupboard. Fifty microlitres, containing 30 ml of 1 M HCl, 15 ml of ultrapure  $\text{H}_2\text{O}$  and 5 ml of 9.79 M  $\text{H}_2\text{O}_2$ , was added to the flask, immediately followed by the addition of 50 ml 1.2 M NaOH. This is a highly exothermic reaction and great care was taken at all times. Immediately following the addition of NaOH the contents of the flask were carefully poured into a 250 ml conical flask whose base was thinly covered with manganese dioxide. This flask was placed on ice for 15 min. The suspension was then filtered through grade No. 5 filter paper (Whatman plc, Maidstone, Kent, UK) and stored overnight in 20 ml aliquots at  $-20\text{ }^\circ\text{C}$ . The following day, the  $\text{ONOO}^-$  solutions were removed from storage and the yellow liquid supernatant was aliquoted into 0.7 ml microcentrifuge tubes and stored at  $-80\text{ }^\circ\text{C}$ .

The concentration of  $\text{ONOO}^-$  was determined by measuring absorbance at 302 nm, using 0.4 M NaOH as a blank ( $\epsilon_{302} = 1670\text{ M}^{-1}\text{ cm}^{-1}$ ).

### *2.7.2. Exposure of penicillinase to synthesised $\text{ONOO}^-$*

Due to the high pH of the  $\text{ONOO}^-$  solution, this exposure experiment was carried out with 50 mM potassium phosphate buffer, pH 4.6, to bring the pH down to that of the control. Standard buffer, pH 7 was used for control conditions. The contents of each tube are given in Table 2.8.

*Table 2.8.* Contents of tubes for exposure of penicillinase to ONOO<sup>-</sup>. The final concentration of ONOO<sup>-</sup> is given in the far left column. At time zero 100 µl of penicillinase (180 U ml<sup>-1</sup>) was added to each tube, giving a final concentration of 36 U ml<sup>-1</sup>.

<b>Control</b>	370 µl 50 mM potassium phosphate buffer, pH 7	30 µl H <sub>2</sub> O
<b>104 µM</b>	370 µl 50 mM potassium phosphate buffer pH 4.62	30 µl 1.77 mM ONOO <sup>-</sup>
<b>ONOO<sup>-</sup></b>		

One hundred microlitres of penicillinase (180 U ml<sup>-1</sup>) were added at time zero. As there was no effective way to quench the reaction between ONOO<sup>-</sup> and penicillinase, 60 µl samples were taken at time zero for the control only and after 60 min of exposure for all conditions. At the end of exposure all samples were immediately loaded into a 96 well plate as previously described (Section 2.3.2.) and 250 µl of CENTA (0.02 mg ml<sup>-1</sup>) were added to each well, including three wells containing standard buffer only. The plate was read at 405 nm for 10 min.

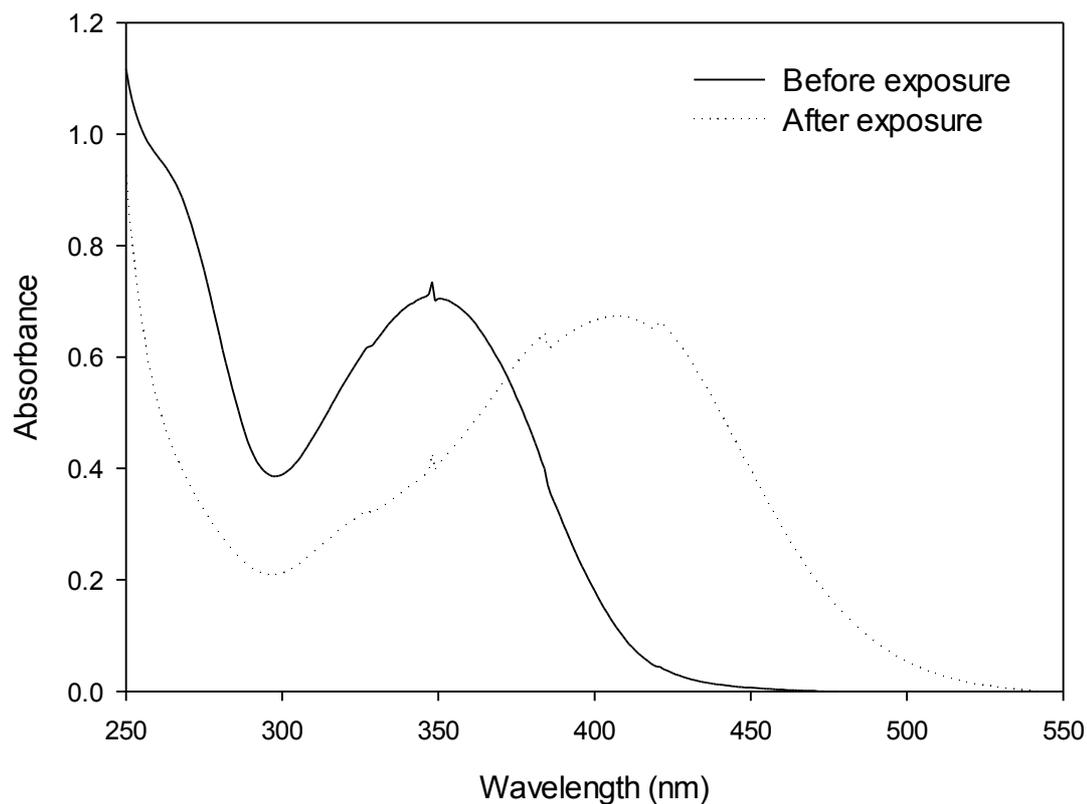
### *2.7.3. The effect of ONOO<sup>-</sup> on the absorbance of CENTA*

Fifteen microlitres of 100 µM ONOO<sup>-</sup> were added to each of three wells in a 96 well plate; 15 µl of standard buffer was placed in a further three wells. Two hundred and fifty microlitres of CENTA (0.02 mg ml<sup>-1</sup>) were added to each well and the plate was read at 340 nm.

## *2.8. Results*

### *2.8.1. Penicillinase catalysed the hydrolysis of CENTA*

A scan to demonstrate the shift in λ maximum for CENTA exposed to penicillinase was carried out based on the method of Jones *et al.* (1982). Penicillinase catalysed the hydrolysis of CENTA resulting in a product detectable at 405 nm (Figure 2.1). The measured activity of penicillinase was dose dependent (Figure 2.2).



*Figure 2.1.* Absorption spectra of CENTA ( $0.053 \text{ mg ml}^{-1}$ ) before and after exposure to penicillinase ( $4.6 \text{ U ml}^{-1}$ ). Briefly, CENTA ( $990 \mu\text{l}$  of  $0.054 \text{ mg ml}^{-1}$ ) was placed in a 1 ml plastic UV cuvette. This was scanned in a spectrophotometer (Perkin Elmer) through a wavelength range of 250-600 nm. Following the scan, penicillinase ( $10 \mu\text{l}$  of  $230 \text{ U ml}^{-1}$ ) was added to the cuvette and stirred thoroughly. The CENTA was scanned again through the same range of wavelengths.

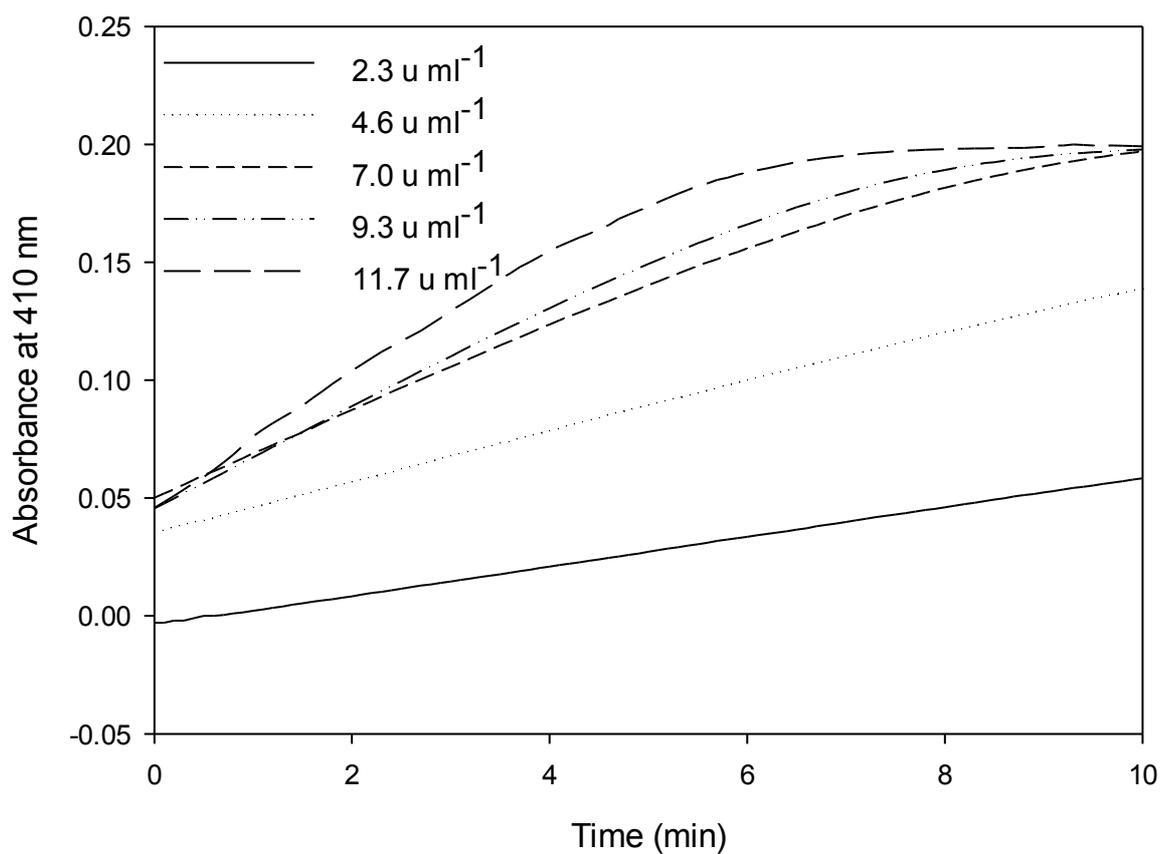


Figure 2.2. Hydrolysis of CENTA as a measure of penicillinase activity. The concentrations given are of penicillinase. The initial concentration of CENTA was 0.01 mg ml<sup>-1</sup>.

### 2.8.2. Zinc supplementation did not affect penicillinase activity

The addition of zinc sulphate to the buffer, in ten-fold dilutions, did not result in any significant changes to penicillinase activity (Fig. 2.3); it was concluded that zinc ion supplementation was unnecessary.

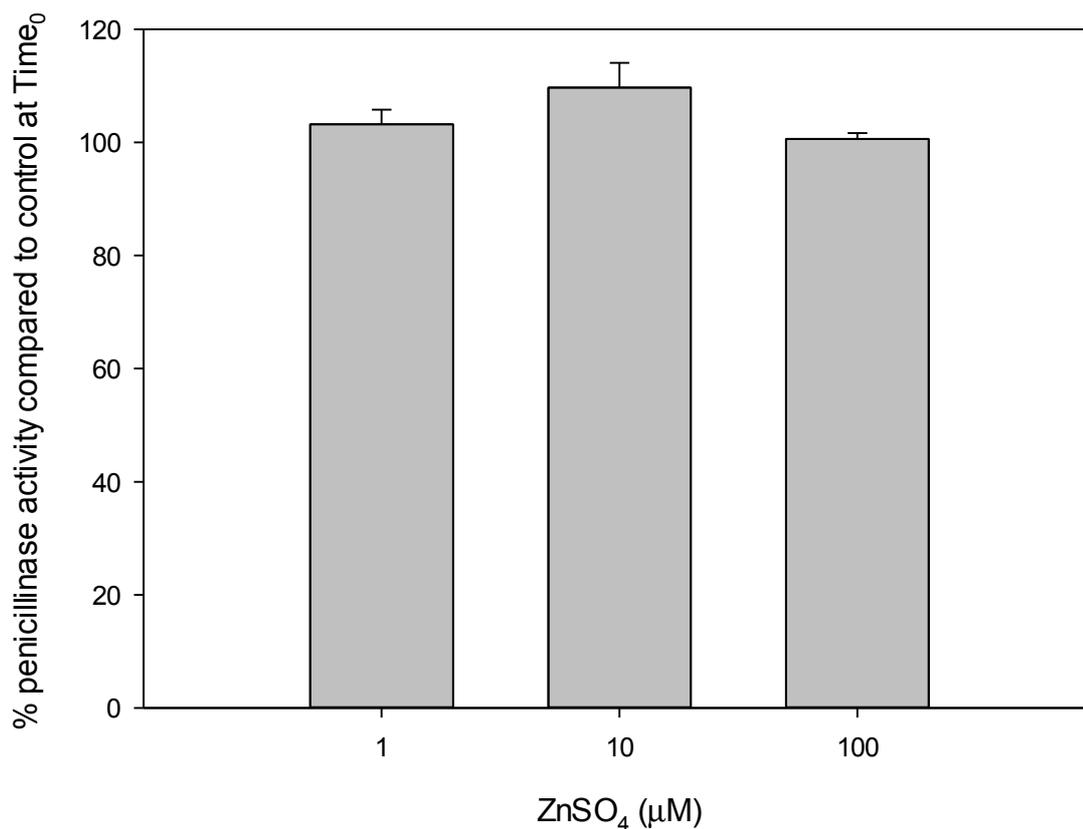
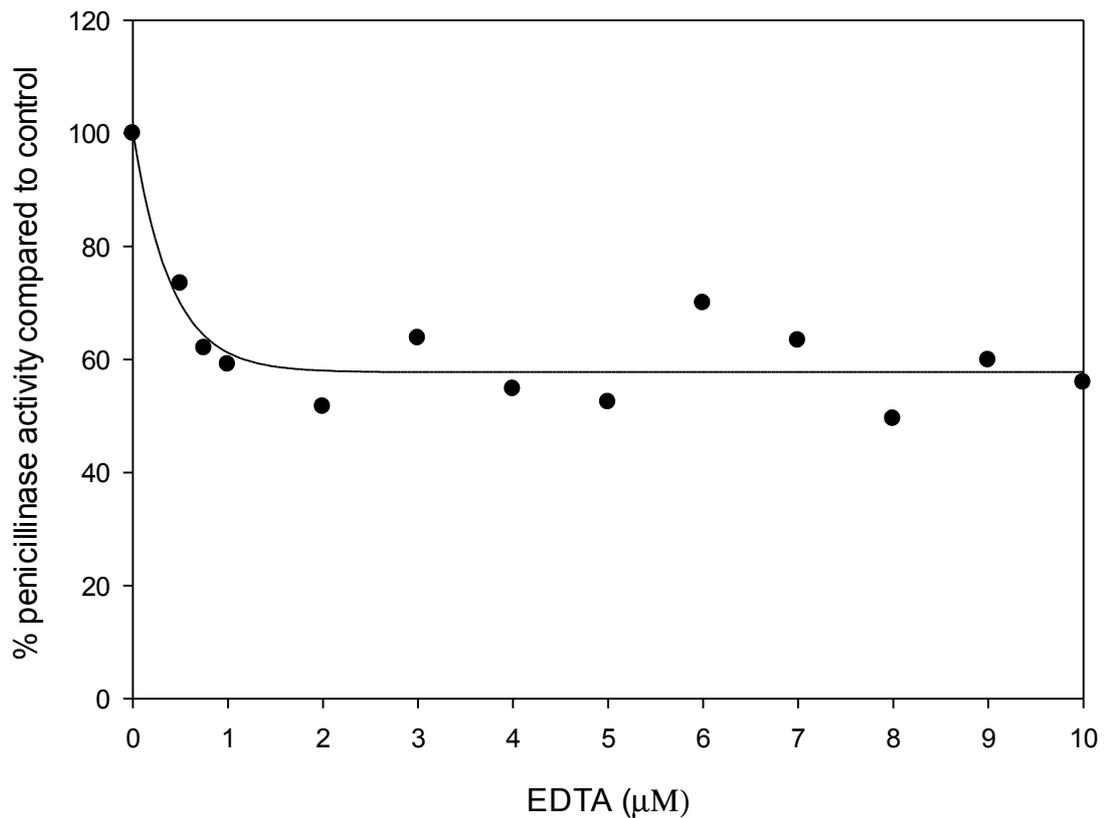


Figure 2.3. Effect of zinc ions on penicillinase activity. The penicillinase assay, with CENTA as substrate was performed with zinc sulphate added to buffer and CENTA (0.02 mg ml<sup>-1</sup>) prior to addition of penicillinase (2.5 U ml<sup>-1</sup>). Results are means ± SE, n=3. There was no significant difference between the activity under any of the conditions,  $p = 0.101$  (One way ANOVA).

### 2.8.3. EDTA reduced the activity of penicillinase

As previously mentioned (Section 2.2.6), part of penicillinase comprises a metallo-β-lactamase with zinc at its active site; it was optimal, therefore, to determine whether a chelating agent such as EDTA would affect enzyme activity. The activity of penicillinase reduced, when exposed to EDTA, before settling at a level of residual activity of approximately 60% of that of the control (Fig. 2.4).



*Figure 2.4.* Activity of penicillinase following exposure to various concentrations of EDTA, expressed as a percentage of that of the control (no EDTA). Penicillinase ( $0.18 \text{ u ml}^{-1}$ ) activity was determined by measuring the hydrolysis of CENTA ( $0.02 \text{ mg ml}^{-1}$ ) at 405 nm for 10 min. These data represent a single experiment only.

#### *2.8.4. Activity of LPO*

The concentration of  $\text{H}_2\text{O}_2$  ( $0.15 \text{ mM}$ ) in the original assay by Morrison (1970) gave the best curve for analysing the activity of LPO on the oxidation of iodide (Fig. 2.5).

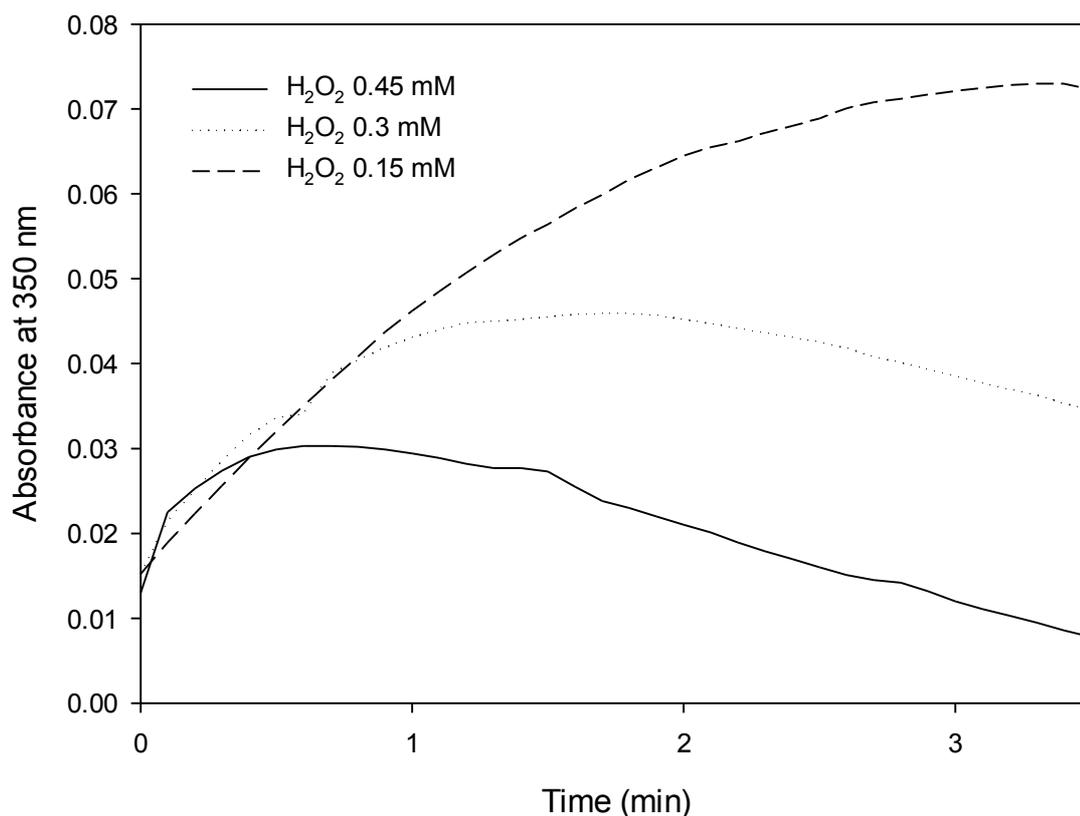
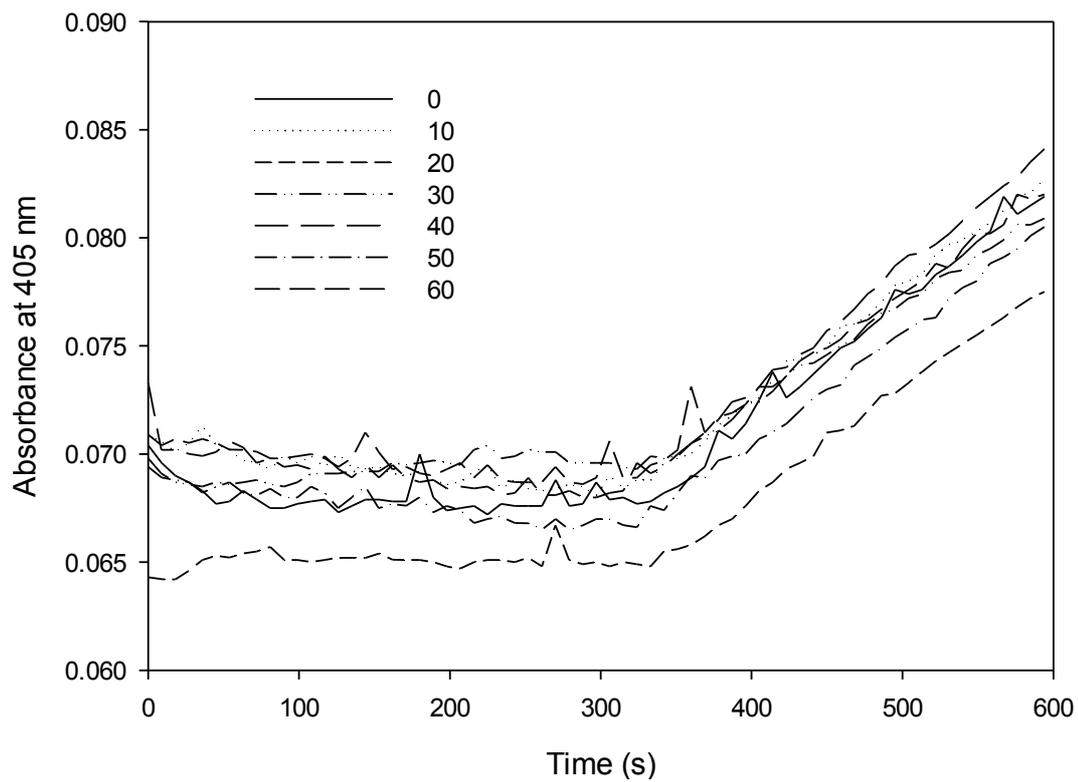


Figure 2.5. The effect of H<sub>2</sub>O<sub>2</sub> concentration on LPO activity in the oxidation of iodide. KI 5 mM, LPO 0.2 U ml<sup>-1</sup>.

### 2.8.5. LPO was a poor model enzyme for oxidant production for this assay

The data from this assay were unexpected; samples containing LPO and both its substrates showed no penicillinase activity for the first 5 min of the assay, but thereafter a low rate of absorbance increase was seen (Figure 2.6). The erratic absorbance was most likely due to small absorbance changes. It was concluded that a product of the reaction catalysed by LPO may have resulted in bleaching of the CENTA, but why this appeared to resolve after 5 min is unclear. An exposure experiment was carried out which used four different final concentrations of LPO, but, the data were too noisy and inconsistent to be of any value. It was decided to abandon LPO as an oxidant generator and to try MPO instead.



*Figure 2.6.* Penicillinase ( $4.7 \text{ U ml}^{-1}$ ) activity following exposure to LPO derived oxidants. Legend indicates exposure time in minutes. LPO,  $4 \mu\text{g ml}^{-1}$ ;  $\text{H}_2\text{O}_2$ ,  $150 \mu\text{M}$ ;  $\text{SCN}^-$ ,  $500 \mu\text{M}$ ; CENTA,  $0.01 \text{ mg ml}^{-1}$ . Each time course is the mean of 3 replicates.

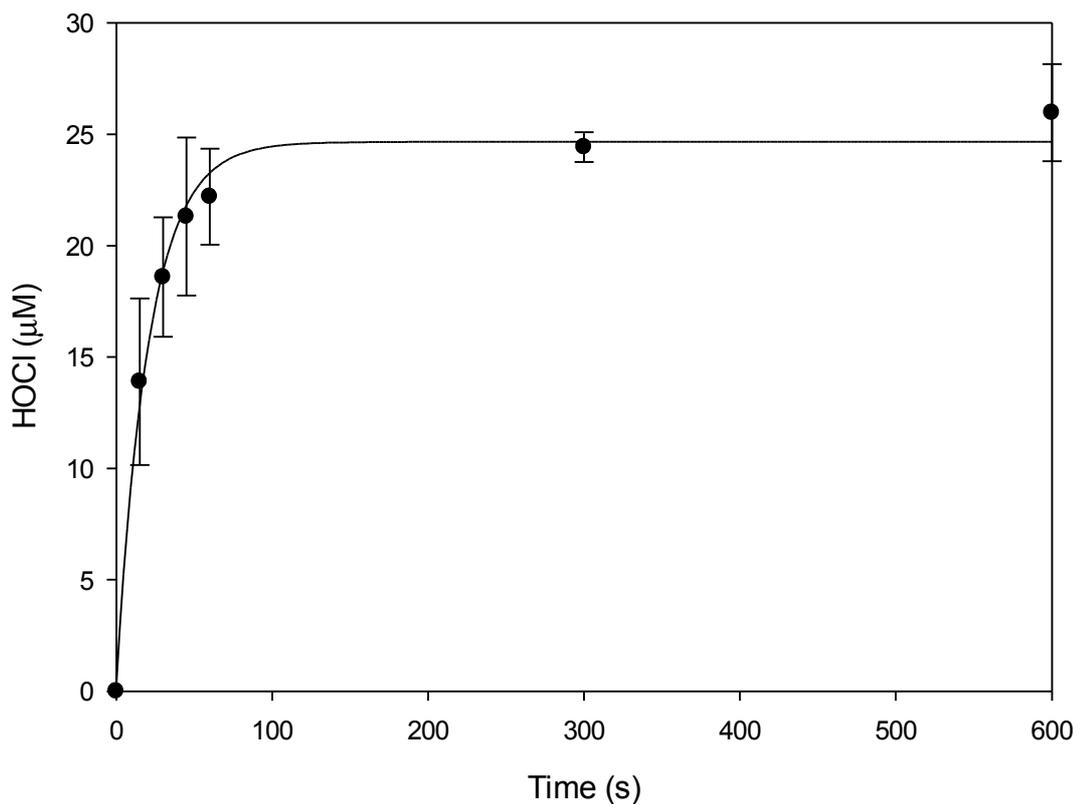
### 2.8.6. Chloramine standard curve

Oxidation of TMB is proportional to concentration of HOCl (Fig. 2.7).

*Figure 2.7.* Chloramine standard curve. Iodide catalysed oxidation of TMB (2 mM) by taurine chloramine. Taurine chloramine was produced by adding the above concentrations of HOCl to taurine which was incubated, with TMB in the presence of iodide, for 5 minutes. The oxidised TMB was read at 650 nm. The data are means, n = 3.

### 2.8.7. Catalysis of HOCl production by MPO

MPO ( $0.027 \text{ U ml}^{-1}$ ), with  $20 \text{ }\mu\text{M}$   $\text{H}_2\text{O}_2$  as a substrate, catalysed the production of HOCl at a rapid rate for the first 15 s (Fig. 2.8); the rate of production then decreased until approximately 60 s when it then declined to nothing as the supply of the substrate hydrogen peroxide ran out.



*Figure 2.8* Time course for HOCl production by MPO. Taurine ( $5 \text{ mM}$ ) was exposed to MPO ( $0.027 \text{ U ml}^{-1}$ ) with its substrates  $\text{H}_2\text{O}_2$  ( $20 \text{ }\mu\text{M}$ ) and  $\text{NaCl}$  ( $100 \text{ mM}$ ). The reaction was stopped with catalase. Absorbance at  $650 \text{ nm}$ , representing the production of oxidised TMB, and data from Figure 2.6 were used to calculate HOCl concentrations. See Appendix 2(iv) for equation. Data represent means  $\pm\text{SE}$ ,  $n = 3$ .

### 2.8.8. MPO-generated oxidants reduced the activity of penicillinase

MPO-generated oxidants reduced the activity of penicillinase by approximately 80%, compared to the control at time zero, following 60 min of exposure (Fig. 2.9).

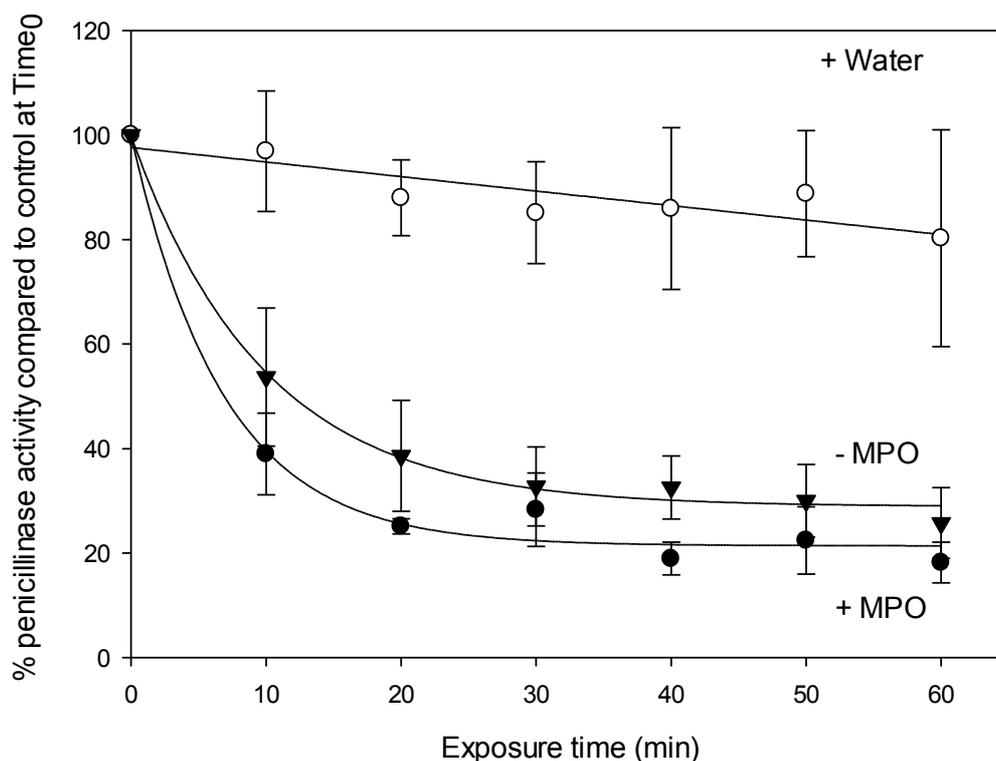


Figure 2.9. Activity of penicillinase ( $6.8 \text{ U ml}^{-1}$ ) exposed to MPO-derived oxidants, expressed as a percentage of activity of the control at time zero. Exposure time indicates the time (min) that penicillinase was exposed to each condition: water (open circle),  $\text{H}_2\text{O}_2$  and NaCl (closed triangle) and  $\text{H}_2\text{O}_2$ , NaCl and MPO (closed circle). Concentration of  $\text{H}_2\text{O}_2$  was 1.5 mM, NaCl, 150 mM, and MPO,  $1.35 \text{ U ml}^{-1}$ . Following exposure penicillinase activity was measured spectrophotometrically at 405 nm for 10 min, using CENTA ( $0.02 \text{ mg ml}^{-1}$ ) as a substrate. Data points are means  $\pm$  SE,  $n = 3$ .

A rapid loss of approximately 60% of activity occurred. A residual activity of 21.4% remained (Table 2.10). A similar loss of activity was seen following exposure of penicillinase to MPO substrates, minus MPO, though the final residual activity was 28.9% compared to control. The rate constant for MPO was higher than that for substrates minus MPO (Table 2.9).

Table 2.9. Rate constants and residual activity for loss of activity of penicillinase exposed to MPO-derived oxidants. Determined using non-linear regression (exponential decay, single, 3 parameter).

	Rate constant ( $\text{min}^{-1}$ )	Residual activity (%)
Substrates minus MPO	0.1015	28.9
Substrates plus MPO	0.1463	21.4

### 2.8.9. $\text{H}_2\text{O}_2$ reduced the activity of penicillinase

$\text{H}_2\text{O}_2$  reduced the activity of penicillinase in a dose-dependent manner (Fig. 2.10).

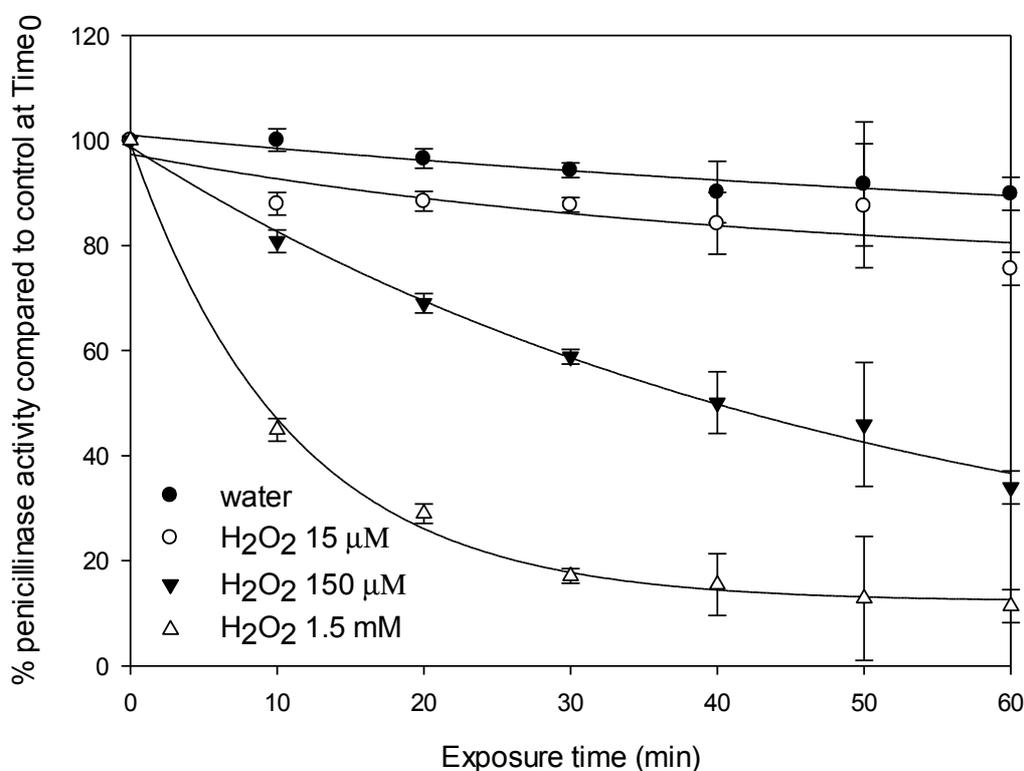


Figure 2.10. Activity of penicillinase ( $36.0 \text{ U ml}^{-1}$  in the microplate well) exposed to  $\text{H}_2\text{O}_2$ , expressed as a percentage of activity of the control at time zero. Exposure time indicates the time (min) that penicillinase was exposed to each concentration of  $\text{H}_2\text{O}_2$ . Following exposure penicillinase activity was measured spectrophotometrically at 405 nm for 10 min, using CENTA ( $0.02 \text{ mg ml}^{-1}$ ) as a substrate. Data points are means  $\pm$  SE,  $n = 3$ .

Following 60 min of exposure,  $15 \mu\text{M}$   $\text{H}_2\text{O}_2$  had reduced activity by approximately 20%,  $150 \mu\text{M}$   $\text{H}_2\text{O}_2$  by 60% and  $1.5 \text{ mM}$  by 85%. The rate constants for loss of activity are dose-dependent when a constrained fit is used

(Table 2.11). The highest concentration of H<sub>2</sub>O<sub>2</sub> showed no further reduction in activity following around 30 min of exposure.

*Table 2.10.* Rate constants and residual activity for loss of activity of penicillinase exposed to H<sub>2</sub>O<sub>2</sub>. Determined using non-linear regression (exponential decay, single, 3 parameter). Constrained fit is to the level of residual activity of 1.5 mM H<sub>2</sub>O<sub>2</sub>.

	Rate constant (min l <sup>-1</sup> )		Residual activity (%)
<b>15 μM H<sub>2</sub>O<sub>2</sub></b>	Unconstrained fit	0.0239	75.3
	Constrained fit	0.0037	
<b>150 μM H<sub>2</sub>O<sub>2</sub></b>	Unconstrained fit	0.02	9.9
	Constrained fit	0.021	
<b>1.5 mM H<sub>2</sub>O<sub>2</sub></b>		0.0921	12.2

### 2.8.10. EDTA did not remove residual penicillinase activity

The exposure of penicillinase to 100  $\mu$ M EDTA following exposure to  $H_2O_2$  for 60 min did not remove the residual penicillinase activity (Fig. 2.10).

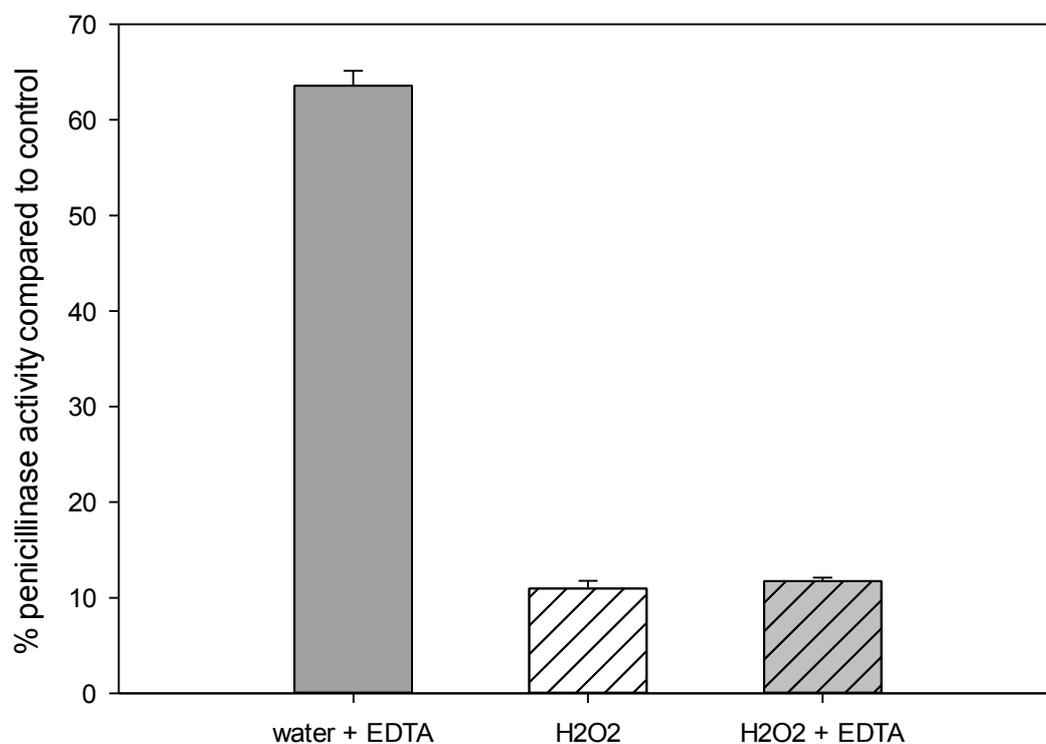


Figure 2.11. Penicillinase ( $36.0 \text{ U ml}^{-1}$ ) activity compared to control following exposure to  $H_2O_2$  and EDTA. Penicillinase was exposed to 1.5 mM  $H_2O_2$  or water for 60 min and then incubated with 100  $\mu$ M EDTA for 10 min. This data represents the means  $\pm$  SE,  $n = 3$ . There is no significant difference between the samples with  $H_2O_2$ , with or without EDTA.

### 2.8.11. $H_2O_2$ did not cause bleaching of CENTA

Exposure of CENTA to a range of concentrations of  $H_2O_2$  did not result in a reduction in absorbance values when measured at 340 nm (Fig. 2.12).

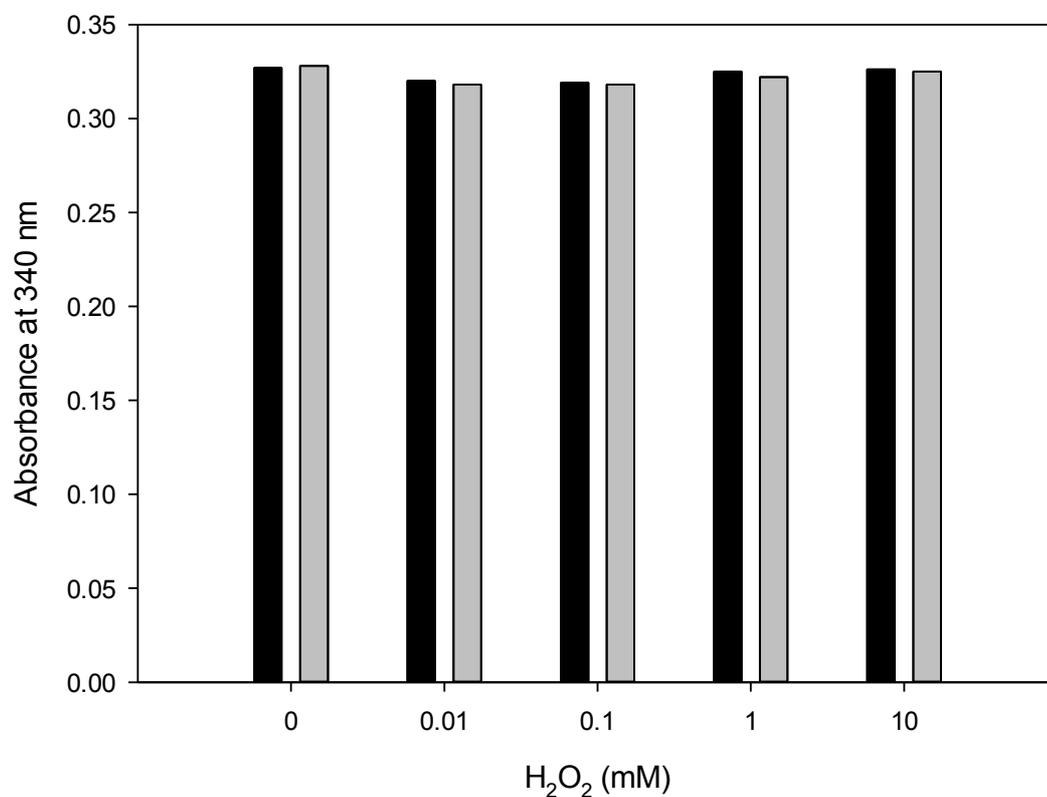
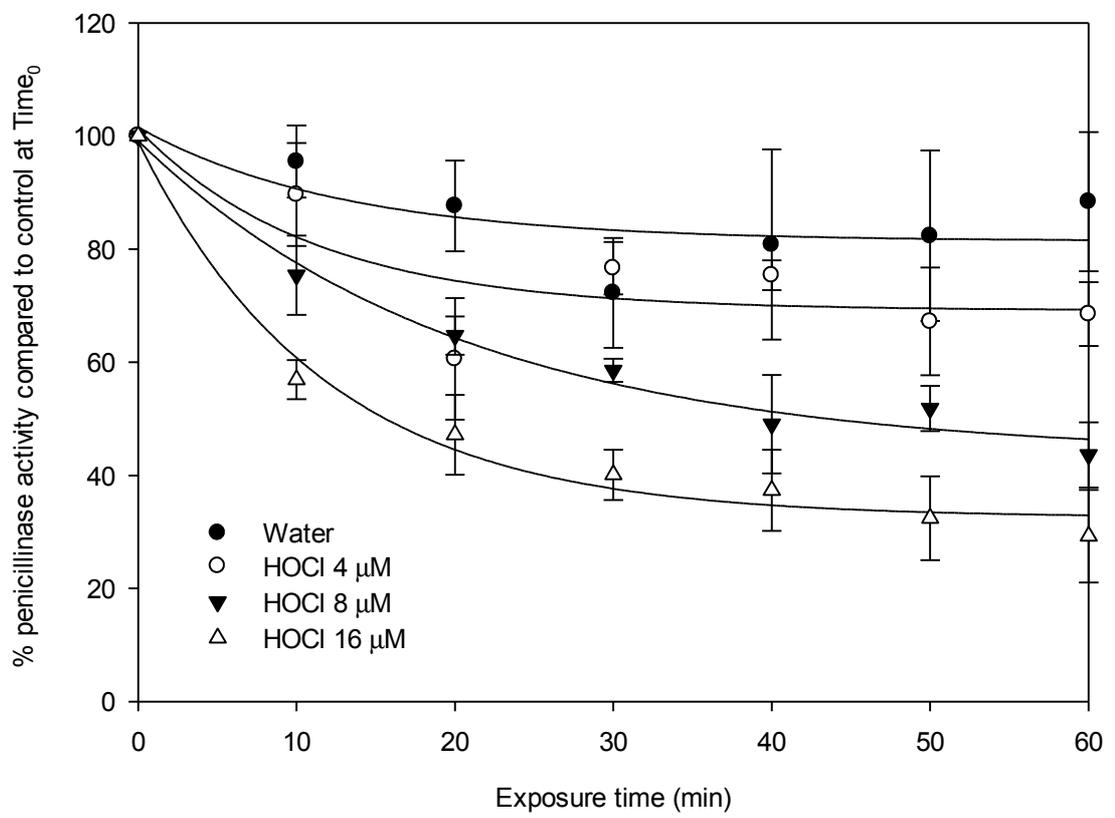


Figure 2.12. Absorbance of CENTA ( $0.02 \text{ mg ml}^{-1}$ ) when exposed to various concentrations of  $H_2O_2$ . The bars represent measurements taken immediately following addition of  $H_2O_2$  (black bars) and those taken after 3 min incubation with  $H_2O_2$  (grey bars). Values are means,  $n = 2$ .

### *2.8.12. HOCl reduced the activity of penicillinase*

HOCl reduces the activity of penicillinase in a dose dependent manner (Fig. 2.13). After 60 min of exposure to 16  $\mu\text{M}$  HOCl penicillinase activity fell by approximately 60%, compared to the control at time zero; the fall in activity was around 50% for 8  $\mu\text{M}$  HOCl and around 30% for 4  $\mu\text{M}$  HOCl. The rate constants for loss of activity are dose-dependent when a constrained fit is used (Table

2.11).



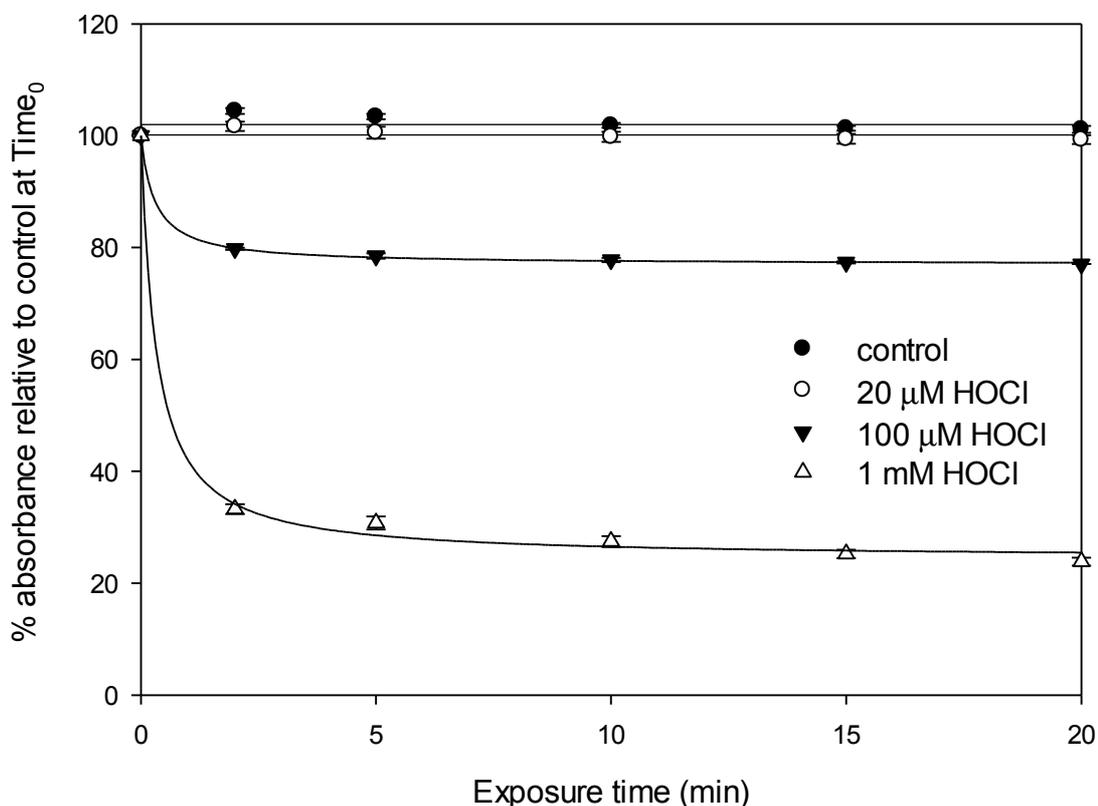
*Figure 2.13.* Activity of penicillinase ( $36.0 \text{ U ml}^{-1}$ ) exposed to HOCl. Expressed as a percentage of activity of the control at time zero. Exposure time indicates the time (min) that penicillinase was exposed to each concentration of HOCl. Following exposure penicillinase activity was measured spectrophotometrically at 405 nm for 10 min, using CENTA ( $0.02 \text{ mg ml}^{-1}$ ) as substrate. Data points are means  $\pm$  SE,  $n = 3$ .

Table 2.11. Rate constants and residual activity for loss of activity of penicillinase exposed to HOCl. Determined using non-linear regression (exponential decay, single, 3 parameter). Constrained fit is to the level of residual activity of 16  $\mu\text{M}$  HOCl.

	Rate constant ( $\text{min}^{-1}$ )		Residual activity (%)
4 $\mu\text{M}$ HOCl	Unconstrained fit	0.0905	69.2
	Constrained fit	0.0112	
8 $\mu\text{M}$ HOCl	Unconstrained fit	0.0491	43.4
	Constrained fit	0.0311	
16 $\mu\text{M}$ HOCl		0.0857	32.6

### 2.8.13. HOCl bleached CENTA in a dose dependent manner

HOCl bleaches CENTA in a dose-dependent manner (Fig. 2.14). No significant bleaching effect was detected with 20  $\mu\text{M}$  HOCl, but the absorbance of CENTA fell by approximately 20% with 100  $\mu\text{M}$  HOCl and 70% with 1 mM HOCl. Bleaching occurred rapidly after which the remaining absorbance stayed fairly constant over time. In molar terms the ratio of CENTA to 100  $\mu\text{M}$  HOCl, was 1:27.



*Figure 2.14.* HOCl bleaching of CENTA. CENTA ( $0.02 \text{ mg ml}^{-1}$ ) was exposed to various concentrations of HOCl for 20 min. Absorbance was read at 340 nm. Data are expressed as the percent absorbance, relative to control (water) at time zero. Error bars denote the range from the mean,  $n = 2$ .

#### 2.8.14. ONOO<sup>-</sup> reduced the activity of penicillinase

The concentration of synthesised ONOO<sup>-</sup> was 1.77 mM. Following 60 min exposure to ONOO<sup>-</sup>, the activity of penicillinase fell by approximately 40% compared to that of control at time zero (Fig. 2.15).

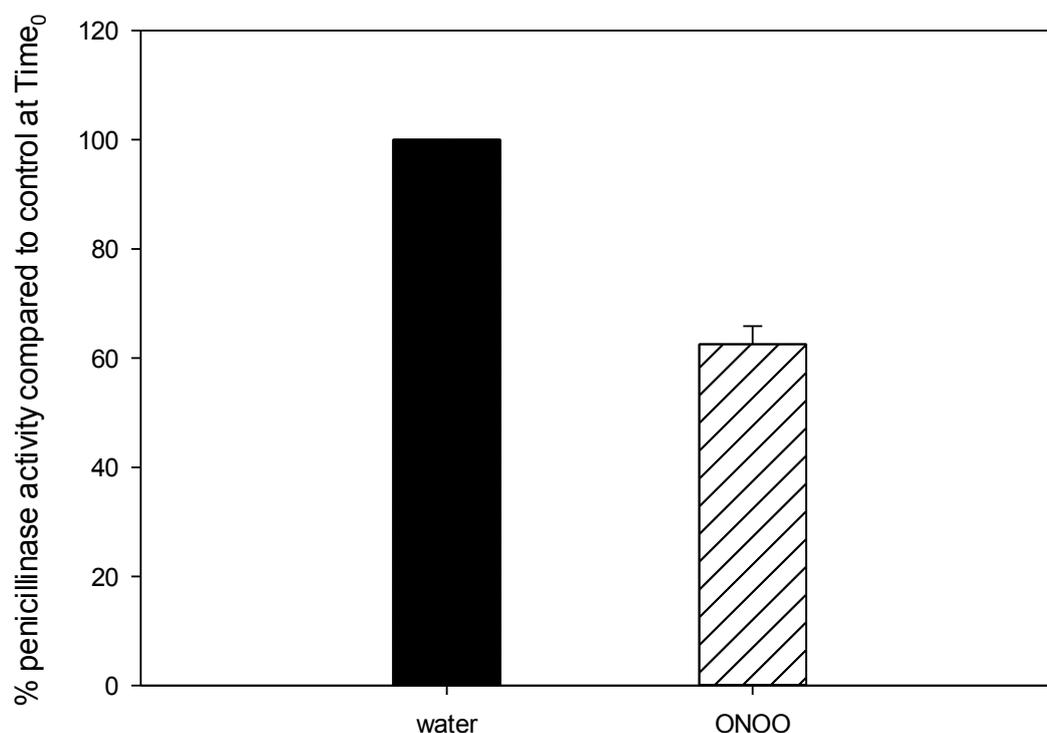


Figure 2.15. Activity of penicillinase (36.0 U ml<sup>-1</sup>) after 60 min exposure to ONOO<sup>-</sup> (104 μM), expressed as a percentage of activity of the control (water) at time zero. Following exposure penicillinase activity was measured spectrophotometrically at 405 nm for 10 min, using CENTA (0.02 mg ml<sup>-1</sup>) as a substrate. Data points are means ± SE, n=3.

#### 2.9. Discussion

This study has demonstrated that reactive species (RS) modify penicillinase to a sufficient degree to significantly reduce its activity compared to controls; to our knowledge this is the first time that this has been reported. The penicillinase used in this study comprises two β-lactamases; I, which has a molecular mass of 27,800 Da (Lloyd & Peacock, 1970) and has a serine at its active site (Hill *et*

*al.* 1980), and II, with a molecular mass of 22,500 Da (Kuwabara & Lloyd, 1971) and two binding sites for zinc cofactors, of which binding at the highest affinity site is essential for enzymic activity (Thatcher, 1975).  $\beta$ -lactamases are enzymes and numerous studies have demonstrated that enzymes, being proteins, are vulnerable to modification by RS, including the species used in this study, hydrogen peroxide (Anjem & Imlay, 2012), hypochlorous acid (Summers *et al.*, 2012) and peroxyxynitrite (Hausladen & Fridovich, 1994). The nature of enzyme modification by RS depends on the species investigated and may involve oxidation, chlorination or nitration reactions, which may be reversible (Fuentes-Almagro *et al.*, 2012), or result in inactivation of the enzyme (Hurd *et al.*, 2012). This study did not investigate the possible sites of enzyme modification by the species used so the nature of the damage caused is unknown; however, amino acid sequences of the two  $\beta$ -lactamases (Appendix 5) show the presence of several amino acids that have been shown to be particularly vulnerable to RS. Both  $\beta$ -lactamases contain cysteine and the sulphhydryl groups on these amino acid residues are readily oxidised. In a reducing environment disulphide bonds are unstable, but in an oxidising environment conditions may be conducive to the formation of these bonds which may alter the configuration of an enzyme; Shimizu-Ibuka *et al.* (2004) engineered  $\beta$ -lactamase mutants, with disulphide bridges, which showed reduced activity for some cephalosporins.

Both enzymes contain several methionines which may be vulnerable to oxidation; the possibility that this type of modification may lead to significant changes to enzyme activity is supported by Hsieh *et al.*, (2012), who demonstrated that oxidation of a methionine was a factor in the inactivation of procaspase-3. Histidine, tryptophan and tyrosine, also present in both  $\beta$ -

lactamases, are readily attacked by RS (Möller *et al*, 2012; Todorovski, *et al*, 2011; Uchida & Kawakishi, 1994).

It, perhaps, should not be surprising that these amino acids are readily modified by RS as they are frequently involved in cellular signalling and, as mentioned above, the activation/inactivation of enzymes, and they provide a possible mechanism for the reduction in activity seen in penicillinase exposed to RS. This effect may be direct, or via a radical product of amino acid oxidation/nitration/chlorination (Alvarez & Radi, 2003; Möller *et al*, 2012).

The compositional difference between the two  $\beta$ -lactamases, i.e. zinc ions in the active site of  $\beta$ -lactamase II, presented an opportunity to explore whether modification was occurring to just one or both of the enzymes comprising penicillinase. EDTA, as an effective chelating agent, was used to bind the zinc ions in  $\beta$ -lactamase II thereby significantly curtailing the enzymes' activity, as has been found previously by Davies and Abraham (1974) who observed more than 99% loss of activity in  $\beta$ -lactamase II when zinc ions were removed. Indeed, we found that EDTA (10 and 100  $\mu$ M) reduced the activity of penicillinase by 35–45% (Figs. 2.4 and 2.11), suggesting that at least 50% of the activity of this particular penicillinase is attributable to  $\beta$ -lactamase I. When penicillinase was exposed to both  $H_2O_2$  and EDTA there was no significant difference in the amount of residual activity (Fig. 2.11), suggesting that any residual activity following exposure to  $H_2O_2$  can be attributed to  $\beta$ -lactamase I. One conclusion to be drawn from this observation is that 1.5 mM  $H_2O_2$  was sufficient to fully inactivate  $\beta$ -lactamase II, and partially inactivate  $\beta$ -lactamase I. This finding is of relevance due to the inherent problem, in the clinical environment, of the paucity of drugs that are able to inhibit  $\beta$ -lactamases (Section 1.2.1). Of particular relevance is that there are, as far as we are aware,

no inhibitors of metallo- $\beta$ -lactamases in clinical use at this time, though Mollard *et al* (2001) demonstrated that thiomendelic acid was capable of inhibiting a broad spectrum of zinc metallo- $\beta$ -lactamases, including those from *B. cereus*, so, given the lengthy process of clinical trials it is possible that a drug is in development. Of interest, from the work of Mollard, is that the active site zinc ions are coordinated by three histidines, which are necessary for enzyme activation, suggesting that RS modification of histidines is a possible pathway.

The concentrations of RS used in this study require further comment; the ability of a RS to inhibit an enzyme in a clinical situation is only relevant if the concentration is likely to occur *in vivo*, however, accurate measurements of physiological levels of RS are challenging and may be relevant only under certain conditions. HOCl concentrations have been estimated to vary between 25 and 200  $\mu$ M (McCall *et al*, 2001), reaching the upper level in pathological conditions. This study found that penicillinase activity was reduced by more than 60% with just 16  $\mu$ M HOCl and so it is feasible that physiological concentrations of HOCl would be sufficient to cause significant reduction in penicillinase activity. H<sub>2</sub>O<sub>2</sub> levels have been measured in several human tissues and systems, including up to around 100  $\mu$ M in urine (Long *et al*, 1999) and ocular tissue (Spector *et al*, 1998), and very low  $\mu$ M levels in human plasma (Halliwell *et al*, 2000), however, meaningful deductions concerning the relevance of these measurements is problematic. Given the ubiquitous nature of H<sub>2</sub>O<sub>2</sub> production by many human tissues, its role in cell signalling and the presence of quenching systems *in vivo* it was difficult to rationalise a specific concentration to use for this study. An assumption was made that rapid diffusion through cell membranes, and leaking neutrophils could increase extracellular

levels of H<sub>2</sub>O<sub>2</sub> significantly in a wound environment; however, a range of concentrations from μM to mM was used.

Care was taken in the preparation of buffers, and other solutions, to eliminate as much as possible all trace metals as the study was looking specifically at the effect of H<sub>2</sub>O<sub>2</sub> on penicillinase. However, *in vivo* the environment is unlikely to be so prescribed and though a loss of approximately 10% after a 60 minute exposure of penicillinase to 15 μM of H<sub>2</sub>O<sub>2</sub> was seen, *in vivo* the real risk is that even very low concentrations of H<sub>2</sub>O<sub>2</sub> will react with transition metal ions to form the highly reactive and damaging OH• radical via Fenton chemistry. Humans are well equipped with mechanisms to bind metal ions to reduce the possibility of such reactions, however, free iron has been measured in inflammatory exudate fluid in a rat model, indicating that this may also occur in a chronic wound environment (Gutteridge *et al*, 1981). In fact H<sub>2</sub>O<sub>2</sub> itself has been shown to degrade haem proteins to free iron (Halliwell *et al*, 2000), as has HOCl (Souza *et al*, 2011).

In the case of ONOO<sup>-</sup>, the concentration used (133 μM) was determined by the limited amount of product synthesised; the equipment necessary to optimise the synthesis of ONOO<sup>-</sup> was not readily available. The method used can result in contamination with Cl<sup>-</sup>, NO<sub>2</sub><sup>-</sup>, NaOH and H<sub>2</sub>O<sub>2</sub>, which has to be considered when interpreting results, though the latter was removed with manganese dioxide. The reduction in penicillinase activity by ONOO<sup>-</sup> was much lower than the other RS examined (possibly supporting the fact that H<sub>2</sub>O<sub>2</sub> was effectively removed), 40% compared to 80-90%. However, we are not able to determine whether this was due to insufficient concentration or to a fundamental difference in the mode of enzyme modification. Also, because the synthesis of ONOO<sup>-</sup> results in a solution with a very high pH, it needed to be diluted with a buffer of pH 4.62 to

maintain the pH of the exposure conditions. The optimal pH for *B. cereus*  $\beta$ -lactamase I and II activity is around 7; catalytic activity falls rapidly at pH 8 (Waley, 1975; Bicknell *et al*, 1983). It is possible that, at this pH, ONOO<sup>-</sup> was rapidly decomposed. It is clear that there was some component of the solution, that penicillinase was exposed to, that reduced its activity. However, its true identity cannot be stated with any confidence.

The failure to be able to use LPO as an oxidant generating system was disappointing; unfortunately, time constraints meant that determining the causes of the failure was not possible. Initial thoughts were that some component of the system was causing bleaching of the CENTA, but the sudden appearance of activity at 5 min does not support this. The time courses in Figure 2.6 indicate a lag in enzyme activity for the first 5 min, which could occur if initially, CENTA, or the product of the hydrolysis of CENTA, is being bleached, but as a component becomes depleted unbleached product returns.

The MPO system appeared to work, though it could be argued that the fall in penicillinase activity was due to H<sub>2</sub>O<sub>2</sub>, one of the MPO system substrates rather than HOCl; however, the time course (Fig.2.8) indicates a very rapid production of HOCl, greater than 20  $\mu$ M by 1 min. When preparing the exposure experiment, MPO and its substrates would have been combined for at least 2 min before adding penicillinase, which would have been sufficient for significant HOCl production. It is possible, however, that there was still an excess of H<sub>2</sub>O<sub>2</sub> in the mix, so an effect of this oxidant cannot be completely discounted. In hindsight it would have been valuable to have added catalase to quench excess H<sub>2</sub>O<sub>2</sub> following a period of time allowing for HOCl concentrations to increase. The higher rate constant and lower residual activity in the presence of MPO does indicate that greater oxidant damage was occurring under these

conditions though as Figure 2.14 indicates concentrations of HOCl 100  $\mu$ M and above cause bleaching of CENTA, the penicillinase substrate; some of the effect of the MPO system could have been due to bleaching if the production of HOCl was greater than anticipated.

The rate constants for penicillinase exposure to H<sub>2</sub>O<sub>2</sub> and HOCl (Tables 2.10 and 2.11 respectively), when constrained to fit the residual activity of the highest concentration showed dose-dependency. This was not the case for unconstrained fits.

This study demonstrated that three RS are capable of modifying the activity of penicillinase from *B. cereus*. It was not able to provide evidence to identify the site or nature of the modification, e.g. oxidation, chlorination or nitration, so any further work in this area should address this omission. The use of mass spectroscopy to identify molecular changes to damaged penicillinase, following exposure to each of the RS, would be valuable. Using a method which first separates the two  $\beta$ -lactamases would also provide further insights into the differential activity, and vulnerability of the two enzymes. Repeating exposure with ONOO<sup>-</sup> would be advantageous if more appropriate equipment could be used for synthesis, resulting in a purer product; though there still remains the problem of maintaining a suitable pH. Extending the work to include  $\beta$ -lactamases from other classes and from other bacterial species would also be beneficial

## Chapter 3

### The effects of reactive oxygen species on acyl-homoserine lactones

#### 3.1. Introduction

Due to its role in the formation of biofilms (Section 1.5.4), QS can be regarded as a component of antibiotic resistance, with evidence suggesting that interfering with QS results in a reduction in biofilm with an inherent increased vulnerability to antibiotics.

##### 3.1.1. Quorum sensing molecules in *P. aeruginosa*

As discussed in Chapter 1, QS molecules are numerous and diverse; those pertinent to this study are the AHLs, particularly those produced by *P. aeruginosa*. The 5 membered lactone ring is a common feature of all AHLs, with specificity being determined by the length of an amide linked acyl side chain, and presence or absence of a side group, (usually oxo or hydroxyl) on the C3 carbon of the acyl chain (Figure 1.3.), (Fuqua *et al.* 2001). *P. aeruginosa* possesses two AHL QS regulatory circuits, termed LasI/R, with the cognate signal molecule O-C12-HSL and RhII/R, with the cognate signal molecule C4-HSL. These AHLs do not function in isolation; the QS system in *P. aeruginosa* is hierarchical with the *las* regulon playing a role in the positive activation of the *rhl* regulon (Figure 3.1.). With increasing population density (or conditions conducive to limited diffusion), O-C12-HSL enters cells, binds to LasR in a dimeric formation and activates *lasI*, producing the enzyme LasI that synthesises O-C12-HSL, resulting in a positive feedback loop. The LasR-AHL molecule also regulates the expression of the genes encoding for the virulence determinants elastase, LasA protease, alkaline protease and exotoxin A (Gambello and Iglewski, 1991, Toder *et al.* 1991, Gambello *et al.* 1993, Pearson *et al.* 1994). RhIR bound with C4-HSL regulates the expression of *rhlAB*

(rhamnolipid), *lasB* (elastase), *aprA*, *RpoS*, cyanide, pyocyanin and the lectins PA-I and PA-II (Ochsner *et al.* 1994, Brint & Ohman, 1995, Latifi *et al.* 1995, Pearson *et al.* 1995, Winson *et al.* 1995, Latifi *et al.* 1996, Winzer *et al.* 2000). LasR-AHL also controls the synthesis of a negative regulator, RsaL, which inhibits transcription of *lasI*.

Alongside the LasI/R and RhII/R QS circuits, *P. aeruginosa* also utilises a 4-quinolone signal molecule (2-heptyl-3-hydroxy-4(1H)-quinolone) termed the *Pseudomonas* Quinolone Signal or PQS. Regulation of PQS production is controlled by LasR, and in turn PQS controls the expression of *lasB*, *rhlI* and *rhlR* and several *rhl*-dependent genes (Diggle *et al.* 2003), suggesting that PQS acts a link between the two AHL-mediated QS circuits in *P. aeruginosa*.

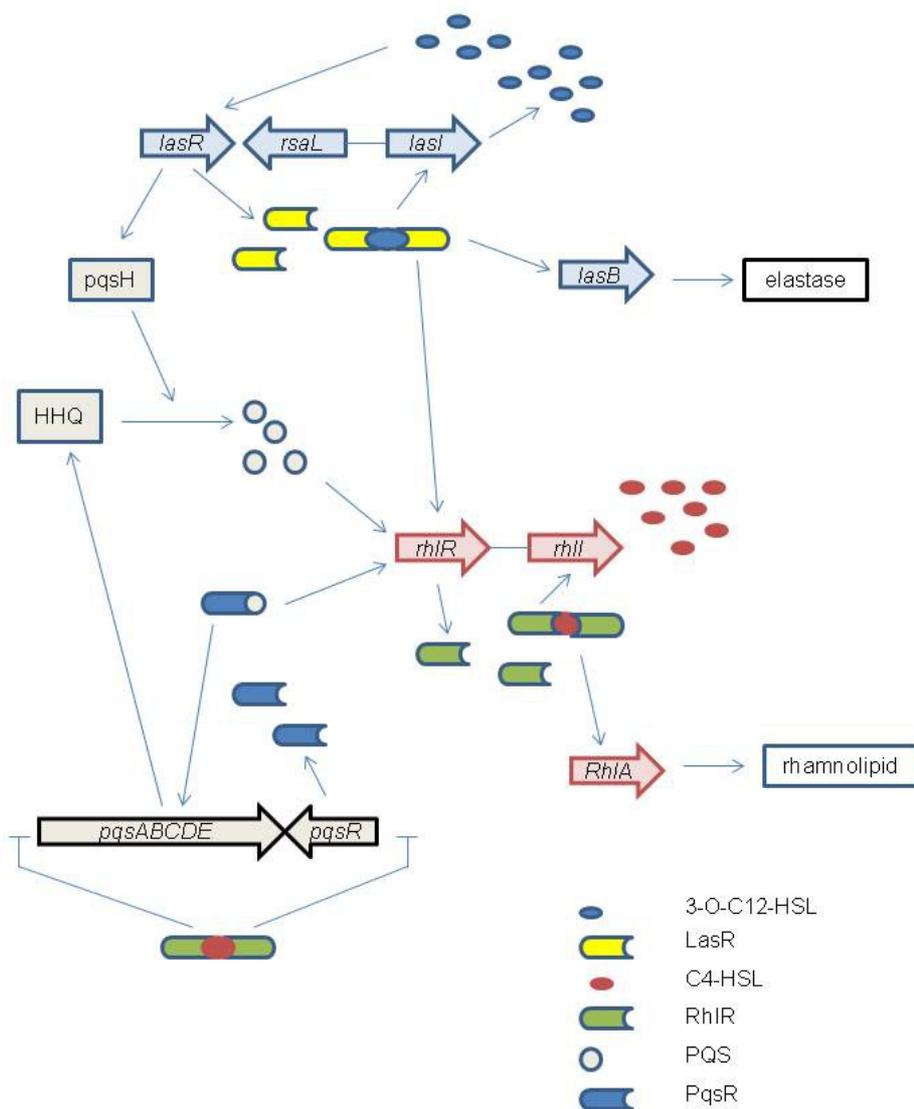


Figure 3.1. Hierarchical regulation of AHL QS in *P. aeruginosa*. O-C12-HSL enters the cell and once a quorum is reached *lasR* is activated. Its product LasR binds O-C12-HSL in a dimeric formation and this activates *lasI*, producing the enzyme LasI that catalyses the synthesis of O-C12-HSL, forming a positive feedback loop. The LasR-AHL molecule also activates *rhIR*, *rhII* and *lasB* leading to the synthesis of *rhIR*, C4-HSL and elastase respectively. RhIR-AHL activates *RhIA* leading to the synthesis of rhamnolipid. LasR positively regulates the synthesis of *pqsH* which converts HHQ (encoded by *pqsABCDE*) to PQS, which positively regulates *rhII*. The RhIR-AHL molecule negatively regulates *pqsABCDE* and *pqsR*. The PqsR-PQS molecule positively regulates *rhIR* and *pqsABCDE*.

### 3.1.2. *The role of QS in P. aeruginosa biofilms*

In Section 1.5.4. the role of QS in the formation and maintenance of biofilms was discussed. For *P. aeruginosa* in particular, evidence supports the contribution of QS to several key areas; the production of rhamnolipids (Davey *et al.* 2003; Pamp & Tolker-Nielsen 2007), lectins (Winzer *et al.* 2000; Diggle *et al.* 2006) pyoverdine (Stintzi *et al.* 1998; Banin *et al.* 2005), extracellular DNA (Allesen-Holm *et al.* 2006) and EPS (Sakuragi & Kolter 2007). Despite this evidence, the full role of QS in biofilm formation is still questioned, mainly due to the contribution that growth conditions, particularly those involving the availability of key nutrients, has on biofilm structure. This is seen in the case of swarming in *P. aeruginosa*, Shrouf *et al.* (2006) showed that swarming does contribute to early biofilm formation the extent of which was dependent on the type of carbon source supplied.

### 3.1.3. *Interfering with QS*

The search for ways to interfere with QS has intensified and this has been discussed in Section 1.4.5. ROS are also thought to damage AHL signal molecules. Michels *et al.* (2000) was the first team to demonstrate degradation of AHLs by oxidized halogens and propose a possible pathway (Fig.3.2). The pathway is very pH dependent and some products may not be detected, for example, at pH 3 only the products of reactions 1 and 2 are detectable. At a more favourable pH, halogenation of the AHL ultimately leads to splitting of the molecule and hydrolysis of the lactone ring (Fig. 3.2).

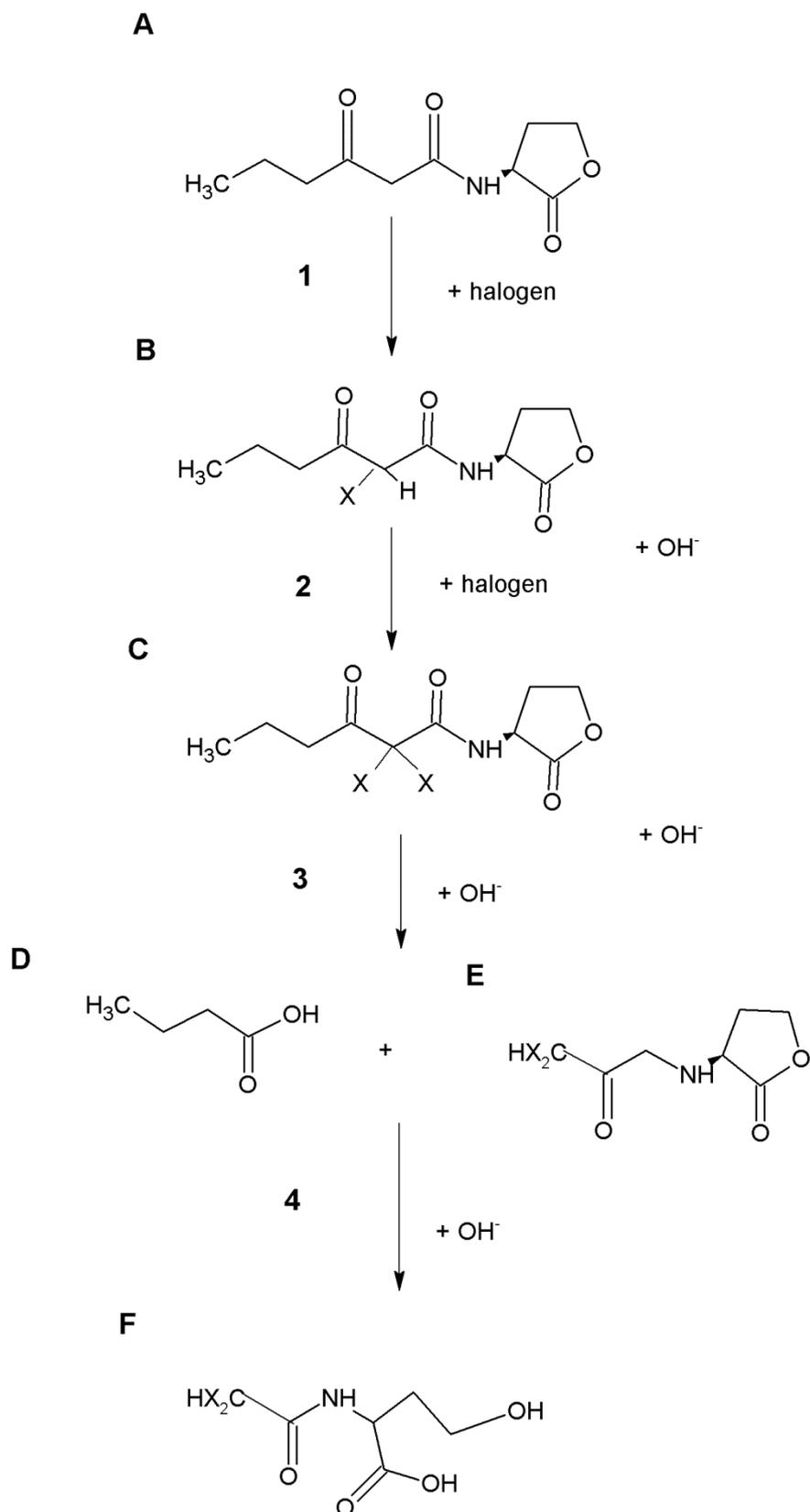


Figure 3.2. Proposed pathway of AHL (O-C6-HSL) degradation by an oxidizing halogen. The four reactions (1 to 4) lead from: **A**. Intact AHL to **F**. Hydrolysed molecule. This Figure has been adapted from that of Michels *et al.* (2000).

A microbiological approach to the same work (Borchardt *et al.* 2001) provided indirect evidence of damage to O-C6-HSL and O-C12-HSL, but not C6 or C12.HSLs. Reactivity of HSLs with oxidized halogens was determined by measuring the consumption of halogens in a colorimetric assay. Using a *Chromobacterium violaceum* CV026 bioassay they demonstrated reduced activity for O-C6-HSL but not C6-HSL, the conclusion being that the oxo side group in some way facilitates AHL modification.

Hypochlorous (HOCl) and hypobromous (HOBr) acids are non-radical derivatives of oxygen (as is H<sub>2</sub>O<sub>2</sub>); HOCl is a powerful two-electron oxidizing agent and is capable of damaging biomolecules either directly (Hannum *et al.* 1995; Park *et al.* 2008), or in the form of chlorine following decomposition. H<sub>2</sub>O<sub>2</sub> on the other hand is a weak oxidizing agent and relatively poorly reactive and yet it has been shown to damage some enzymes (Li *et al.* 2011), which this study also demonstrated in the reduced activity of penicillinase (Chapter 2).

The aim of this part of the study was to determine whether ROS were able to reduce the activity of AHLs, QS signalling molecules utilized by several Gram negative bacteria, including those found within chronic wounds, e.g. *P. aeruginosa*. Synthetic AHLs were exposed to HOCl and H<sub>2</sub>O<sub>2</sub>, two ROS that are likely to be found in a chronic wound environment, (Section 1.6.2.) and their activity compared to controls. It was also considered necessary to extend the range of AHLs tested to those other than C6 and C12, and to utilize a more reliable assay for AHLs with longer chain lengths than the *C. violaceum* assay would accommodate. AHLs with chain lengths ranging from C4 to C14 were tested and these included the dominant AHLs found in *P. aeruginosa*, C4-HSL and O-C12-HSL. Initially, to test the experimental conditions, only C8 AHLs,

with various substitutions, were exposed to ROS. Bioreporter strains of *E. coli*, relevant to AHL chain length, were used to demonstrate AHL activity.

The second aim was to determine whether ROS were able to reduce the AHL signal in growing populations of *P. aeruginosa*, and if any changes to the level of AHL signal might have an impact on the production of the RhlI-regulated virulence factors LasB (elastase) and RhlA (rhamnolipid). The final aim of the study was to attempt to ascertain the nature of the degradation of the AHL molecule by looking for evidence of a lactonase activity by examining the molecular profile of exposed and synthetic AHLs with MS-MS.

Briefly, this study demonstrated that HOCl, but not H<sub>2</sub>O<sub>2</sub>, reduced the activity of a range of AHLs in vitro, but not in vivo. MS-MS suggested that the AHL lactone ring was hydrolysed following exposure to HOCl.

### *3.2 Materials and methods*

#### *3.2.1. Chemicals*

Catalase (Cat# 60635), hydrogen peroxide (30%), LB broth powder, reduced glutathione, Bacto agar, potassium phosphate monobasic, potassium phosphate dibasic, ampicillin sodium salt and hydrochloric acid were obtained from Sigma Aldrich Company Ltd (Poole, Dorset, UK). Ethyl acetate, acetonitrile and ethanol were obtained from VWR BDH Prolabo (Lutterworth, Leicestershire, UK). Tetracycline hydrochloride was obtained from Merck Chemicals Ltd (Beeston, Nottingham, UK). Bleach was obtained from J. Sainsbury plc (London, UK).

The AHLs *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-octanoyl-L-homoserine lactone (C8-HSL), *N*-(3-oxodecanoyl)-L-homoserine lactone (O-C10-HSL) and *N*-dodecanoyl-L-homoserine lactone (C12-HSL) were obtained from Sigma Aldrich Company Ltd (Poole, Dorset, UK). *N*-butanoyl-L-

homoserine lactone (C4-HSL), *N*-decanoyl-L-homoserine lactone (C10-HSL), *N*-(3-hydroxyhexanoyl)-L-homoserine lactone (OH-C6-HSL), *N*-(3-hydroxyoctanoyl)-L-homoserine lactone (OH-C8-HSL), *N*-(3-hydroxydecanoyl)-L-homoserine lactone (OH-C10-HSL); *N*-(3-hydroxydodecanoyl)-L-homoserine lactone (OH-C12-HSL); *N*-(3-oxobutanoyl)-L-homoserine lactone (O-C4-HSL), *N*-(3-oxohexanoyl)-L-homoserine lactone (O-C6-HSL), *N*-(3-oxooctanoyl)-L-homoserine lactone (O-C8-HSL), *N*-(3-oxododecanoyl)-L-homoserine lactone (O-C12-HSL) and *N*-(3-oxotetradecanoyl)-L-homoserine lactone (O-C14-HSL) were obtained from Nottingham University.

### 3.2.2. Strains and plasmids

All strains and plasmids used in this chapter are listed in Table 3.1.

Table 3.1. Strains and plasmids used in this study.

Strain or Plasmid	Genotype and/or relative markers	Reference
<b>Strains</b>		
<i>E. coli</i>		
JM109	<i>recA1 supE44 endA1 hsdR17 gypA96 relA1 thi D (lac-proAB)</i>	Schaefer <i>et al.</i> (1996)
<i>P. aeruginosa</i>		
PAO1n	PAO1n (Nottingham WT strain)	
PAO1n/ LasI::Lux	PAO1n containing a LasI::Lux gene fusion	Kindly donated by Miguel Camara, Nottingham University
PAO1n/ LasB::Lux	PAO1n containing a LasB::Lux gene fusion	
PAO1n/ RhII::Lux	PAO1n containing a RhII::Lux gene fusion	
PAO1n/ RhIAI::Lux	PAO1n containing a RhIAI::Lux gene fusion	
<b>Plasmids</b>		
pSB401	AHL reporter plasmid; <i>luxR</i> :: <i>luxCDABE</i> (Amp <sup>R</sup> )	Winson <i>et al.</i> (1998)
pSB536	AHL reporter; <i>ahyR</i> :: <i>luxCDABE</i> (Amp <sup>R</sup> )	Swift <i>et al.</i> (1997)
pSB1075	AHL reporter plasmid; <i>P. aeruginosa lasRI</i> and <i>luxCDABE</i> from <i>Photobacterium luminescens</i> (Amp <sup>R</sup> )	Winson <i>et al.</i> (1998)

### 3.2.3. Storage and preparation of AHLs

All AHLs were stored at -20 °C, in acetonitrile at a concentration of 10 mg ml<sup>-1</sup>. Prior to each assay, each AHL was diluted with acetonitrile to 1 mg ml<sup>-1</sup>. Unless otherwise stated AHL solutions were kept on ice throughout the assay procedure. All procedures involving acetonitrile were carried out in fume cupboard conditions.

### 3.2.4. Bioreporter strain culture conditions and media preparation

All bacterial strains were grown in sterile LB broth and maintained on sterile LB agar (1.2%), with the addition of antibiotics as follows: (a) *E. coli* pSB401 and pSB1075, tetracycline 10 µg ml<sup>-1</sup> (b) *E. coli* pSB536, ampicillin 50 µg ml<sup>-1</sup> (c) *P. aeruginosa* strains, tetracycline 75 µg ml<sup>-1</sup>. LB agar was cooled to 42 °C and antibiotics added immediately prior to pouring of plates. Antibiotics were added to LB broth immediately prior to use. All cultures were incubated at 37 °C, at 150 rpm. Streak plates were prepared for each culture, on LB agar containing the appropriate antibiotic, incubated for 24 hours and stored at 15 °C. Twenty millilitres of LB broth, as above, were inoculated with a single colony from a stored agar plate and incubated overnight prior to use for each assay.

### 3.2.5. Exposure of bioreporter to synthetic AHLs

In order to determine the optimal concentration of AHL to use in the following assays a dose response curve was generated by exposing the bioreporter *E. coli* pSB1075 to serially diluted O-C8-HSL. The bioreporter was grown overnight, as described above, and diluted 1:10 in LB broth containing tetracycline. Stock AHL (10 mg ml<sup>-1</sup>) was diluted with acetonitrile to 10 µg ml<sup>-1</sup>. Double dilutions of this concentration were prepared down to 0.0195 µg ml<sup>-1</sup>. Fifteen microlitres of the highest concentration were added to each of 3 wells in a sterile, 96-well white clear bottomed polystyrene plate (Sigma-Aldrich, Poole,

Dorset, UK). This was repeated for each of the concentrations prepared. The plate was left in a fume cupboard until the acetonitrile had evaporated. One hundred and fifty microlitres of the prepared bioreporter were added to each well containing AHLs. Controls without the addition of AHLs (i.e. media only) were also included. A lid was placed on the plate which was then incubated as described for 3 h.

On completion of incubation luminescence was measured, followed immediately by measurement of absorbance at 600 nm. Luminescence was measured on a Mithras LB940 plate reader (Berthold Technologies) utilising Mikrowin software. Absorbance was measured on a VersaMax 250 Microplate Reader (Molecular Devices) utilising SoftMax PRO data analysis software. Data analysis was carried out using Microsoft Excel. The absorbance value for medium only was deducted from all other absorbance values. For each well, the final value was determined by dividing the luminescence value (RLUs) by the absorbance value. A dose response curve was calculated with SigmaPlot 12 software.

#### *3.2.6. Bioreporter response to oxidant exposed C8-HSLs*

Initially, 2.5 ng  $\mu\text{l}^{-1}$  O-C8-HSL was exposed to 15  $\mu\text{M}$  HOCl for a total of 60 min, and samples taken at 0, 10, 20, 30 and 60 min. HOCl was quenched with reduced glutathione (GSH) to terminate any effect on the AHL at each time point. Due to a rapid response to HOCl the assay was repeated to include further sampling points at 2, 4, 6 and 8 min. In order to determine whether the OH group, or a lack of a side group made any difference to the response, C8-HSL and OH-C8-HSL (2.5 ng  $\mu\text{l}^{-1}$ ) were also exposed to 15  $\mu\text{M}$  HOCl. Controls included adding HOCl, but not quenching with GSH to determine whether there was any quenching activity from ethyl acetate, and adding water instead of HOCl to determine normal AHL activity. One of the water controls was exposed

to GSH to determine whether GSH alone had any effect on the AHL bioreporters.

Neat bleach was diluted 100-fold with 50 mM potassium phosphate buffer, pH 7 and the concentration of HOCl was determined using  $\epsilon_{235} = 100 \text{ M}^{-1} \text{ cm}^{-1}$ . This solution was further diluted with buffer to 3 mM. A 60 mM solution of reduced glutathione and a 1 mg ml<sup>-1</sup> solution of O-C8-HSL were prepared; all solutions were kept on ice. Twenty four microlitres of GSH were placed in each of ten 1.5 ml microcentrifuge tubes and the tubes placed on ice. The conditions used in this assay are summarised in Table 3.2. Four 15 ml sterile tubes were filled as indicated. AHLs were added last of all and timing was commenced at this stage.

*Table 3.2.* The volumes and concentration of chemicals added to tubes. AHL were added last and this was designated time zero. For those conditions indicated, removed samples were added to aliquots of glutathione.

Condition	Volume	Chemical	Concentration added	Final concentration in tube
<b>AHL exposed to HOCl plus GSH</b>	5.955 ml	Potassium phosphate buffer pH 6.8	50 mM	
	30 $\mu$ l	HOCl	3 mM	15 $\mu$ M
	15 $\mu$ l	AHL	1 mg ml <sup>-1</sup>	
<b>AHL exposed to HOCl minus- GSH</b>	5.955 ml	Potassium phosphate buffer pH 6.8	50 mM	
	30 $\mu$ l	HOCl	3 mM	15 $\mu$ M
	15 $\mu$ l	AHL	1 mg ml <sup>-1</sup>	5 $\mu$ g ml <sup>-1</sup>
<b>Control plus GSH</b>	2.9775 ml	Potassium phosphate buffer pH 6.8	50 mM	
	15 $\mu$ l	dH <sub>2</sub> O		
	7.5 $\mu$ l	AHL	1 mg ml <sup>-1</sup>	5 $\mu$ g ml <sup>-1</sup>
<b>Control minus GSH</b>	2.9775 ml	Potassium phosphate buffer pH 6.8	50 mM	
	15 $\mu$ l	dH <sub>2</sub> O		
	7.5 $\mu$ l	AHL	1 mg ml <sup>-1</sup>	5 $\mu$ g ml <sup>-1</sup>

Immediately following the addition of AHL the tube was vortexed and 1.2 ml of this mix was then transferred to a microcentrifuge tube containing GSH (final concentration of GSH was 1.2 mM). This was then vortexed and 200  $\mu$ l transferred immediately to each of five microcentrifuge tubes already containing 200  $\mu$ l ethyl acetate, vortexed again and replaced on ice. This procedure was repeated for all tubes, but with omission of the GSH step for one exposure condition and one control. Samples were taken for 0, 10, 20, 30 and 60 min for HOCl exposure conditions, and at 0 and 60 min for controls. These steps provided 5 replicates for each condition at each time sampling point.

On completion of sample removal 150  $\mu$ l was carefully removed from the aqueous layer and transferred to a single well of a 96-well PCR plate (*Elkay* Laboratory Products, Basingstoke, UK). The PCR plate was left in the fume cupboard overnight to allow the ethyl acetate to evaporate. The following day 30  $\mu$ l of acetonitrile was added to each well of the PCR plate containing AHL. This was left at room temperature for 10 minutes. Following gentle mixing the 30  $\mu$ l aliquots were transferred to a white, clear-bottomed 96 well plate which was left in the fume cupboard to evaporate. An overnight culture of *E. coli* was diluted 1:10 with fresh LB and tetracycline and 150  $\mu$ l of this was added to each of the wells containing AHL. The same volume of broth was also added to a further 5 wells to act as a blank for the spectrophotometer. A lid was placed on the plate, which was incubated at 37 °C, 150 rpm, for 3 h. Following incubation luminescence was measured, followed immediately by absorbance at 600 nm. In order to quantify the AHL activity the luminescence value for each well was divided by the absorbance value from which the mean of the media only value had been deducted. Means of 5 replicates were calculated and the extent of

AHL activity change was measured as a percentage change relative to the control at time zero. Each assay was repeated three times and the results expressed as means and standard errors. This assay was repeated twice more to include extra sampling points at 2, 4, 6, and 8 minutes, and also for C8-HSL and OH-C8-HSL.

### 3.2.7. *Bioreporter response to oxidant exposed C4, C6, C8, C10, C12 and C14 AHLs*

In order to determine the effect of chain length on AHL activity following exposure to oxidants a range of both substituted, and unsubstituted AHLs with carbon chain lengths of 4, 6, 8, 10, 12 and 14 were exposed to HOCl (15  $\mu$ M) for 60 min. Residual AHL concentrations after 60 min were compared to the time 0 min sample point. The assay was also repeated with H<sub>2</sub>O<sub>2</sub> to determine whether other oxidants also had an effect on AHL activity. In this case, 24  $\mu$ l catalase<sup>10</sup> (20 000 U ml<sup>-1</sup>) were used to quench the reaction. These assays were carried out with only two sampling points, time zero and following 60 min exposure. The following AHLs were not included in the assays: OH-C4-HSL, C14-HSL and OH-C14-HSL. All assays were carried out twice and a two-sample T-Test was used to determine statistical difference between conditions (Minitab 16, Minitab Ltd, Coventry, UK), with a confidence of 95%.

### 3.2.8. *Effect of HOCl on gene transfer reporter fusion strains of P. aeruginosa.*

In order to determine whether the inhibitory effect of HOCl on AHL activity *in vivo*, *P. aeruginosa* variants containing *lux* fusions to the autoinducer proteins LasI (O-C12-HSL) and RhII (C4-HSL), and the RhII regulated virulence factors LasB (elastase) and RhIA (rhamnolipid), were used. These particular reporter strains demonstrate luminescence proportional to gene expression. Each

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<sup>10</sup>

2000 U mg<sup>-1</sup> protein. One unit will decompose 1.0  $\mu$ mol H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> at pH7 at 25 °C.

reporter strain was cultured with the addition of HOCl (15  $\mu\text{M}$ ) to the growth medium. Control conditions with water replacing HOCl were included to determine whether the concentration of HOCl was sufficient to inhibit bacterial population growth.

An overnight culture of each *P. aeruginosa* reporter strain was prepared as in Section 3.2.4. and diluted 1:10 with pre-warmed LB broth (Tetracycline 75  $\mu\text{g ml}^{-1}$ ). In a white, clear-bottom, 96-well plate, 190  $\mu\text{l}$  of each culture was transferred to eight wells in two consecutive columns. Ten microlitres of HOCl (300  $\mu\text{M}$ )\* were added to each well in the first column for each strain; 10  $\mu\text{l}$  of water were added to each well in the second column for each strain. Wells containing 200  $\mu\text{l}$  of LB broth only were included as a control.

The lid was replaced on the plate which was incubated at 37  $^{\circ}\text{C}$ , 150 rpm. The plate was read for luminescence and absorbance at 600 nm following one hour of incubation and thereafter every hour until growth reached stationary phase. This assay was conducted in triplicate. Growth plots were produced with SigmaPlot 12 software, data points are means with standard error. A two-sample t-test was used to determine statistical difference between conditions at each time point (Minitab 16), with a confidence of 95%.

### 3.2.9. MS-MS of ROS exposed and unexposed AHLs

Tandem Mass Spectroscopy was kindly performed by Dr M. Priston (Pharmacy Department, Plymouth Hospitals NHS Trust) on an API 300 LC/MS/MS System (Applied Biosystems, Paisley, UK) and analysed with Analyst Software (AB SCIEX UK Ltd, Warrington, Cheshire, UK). MS-MS was carried out on standard and HOCl exposed solutions of O-C8-HSL and OH-C8-HSL. Standards were diluted with acetonitrile to 1  $\mu\text{g ml}^{-1}$  and delivered at 10  $\mu\text{l min}^{-1}$ . Solutions of

exposed HSLs were diluted with methanol to 0.0625 - 0.5  $\mu\text{g ml}^{-1}$ , and delivered at 3-12  $\mu\text{l min}^{-1}$ . The declustering potential was optimized for each AHL.

### 3.3. Results

#### 3.3.1. Dose response curve for O-C8-HSL

The dose response curve (Fig. 3.3.) was used to determine the optimal concentration of AHL to use in subsequent assays as it was necessary to avoid a concentration that had achieved saturation. A concentration of 5  $\text{ng } \mu\text{l}^{-1}$  was considered to be acceptable.

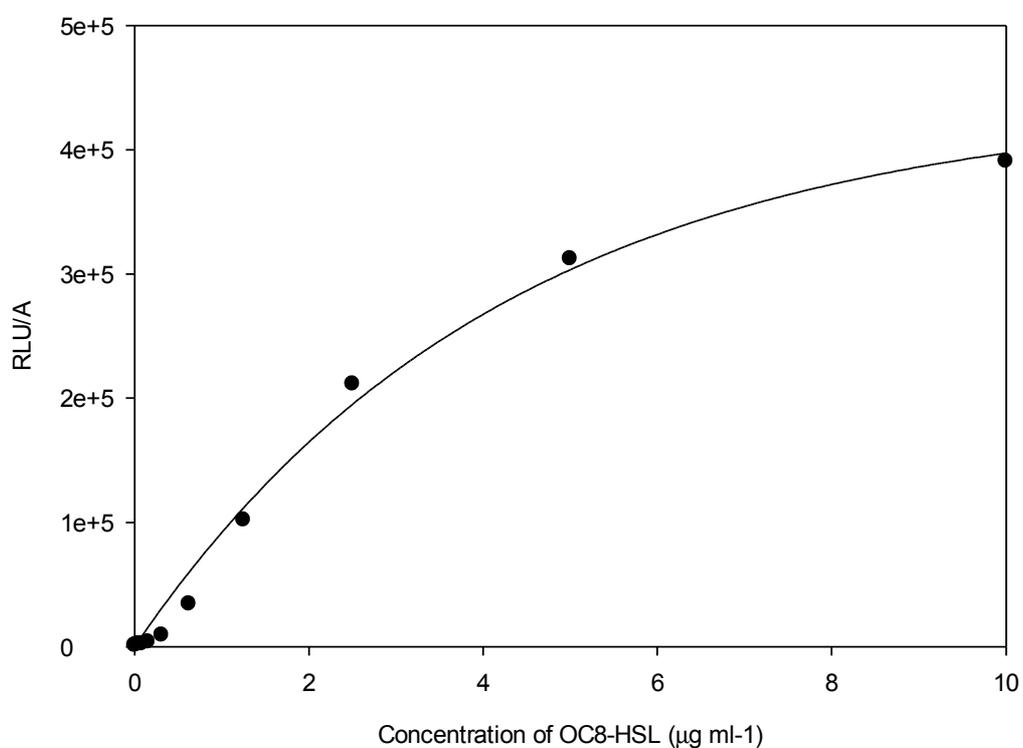
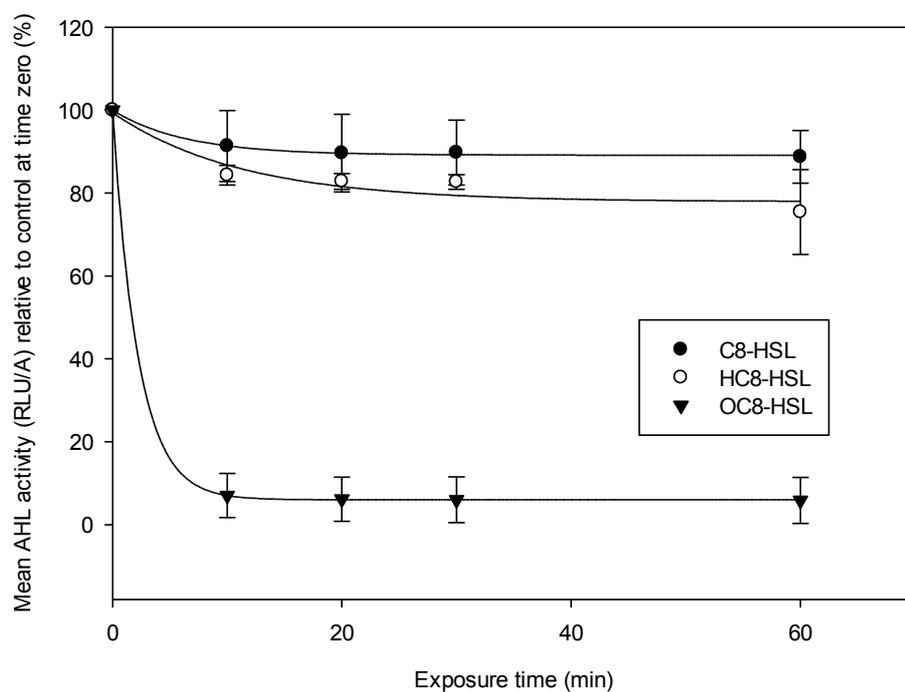


Figure 3.3. Dose response curve for O-C8-HSL. *E. coli* pSB1075, with the addition of various concentrations of AHL, was incubated for three hours.

### 3.3.2. HOCl reduced the activity of O-C8-HSL but not C8-HSL or OH-C8-HSL



The exposure of synthetic O-C8-HSL to 15  $\mu$ M HOCl, resulted in a reduction of AHL activity of approximately 90% compared to control (Fig. 3.4); reductions in activity of synthetic C8-HSL and OH-C8-HSL were approximately 10% and 20% respectively. There was no evidence that either ethyl acetate or GSH affected AHL activity, or that ethyl acetate was able to quench HOCl. The modification of O-C8-HSL activity by HOCl was rapid, achieving a 70% reduction in activity following two minutes of exposure (Fig. 5.5).

*Figure 3.4.* Exposure of C8 AHLs to HOCl (15  $\mu$ M). All AHLs were exposed for a total of 60 min with samples removed at Time 0, 10, 20, 30 and 60 min. AHLs were incubated with *E. coli* pSB1075 for 3 h, and activity was determined by measuring luminescence and optical density. Data are expressed as the mean change in activity compared to control at Time 0 ( $T_0$ ). Means  $\pm$  SE, n = 3.

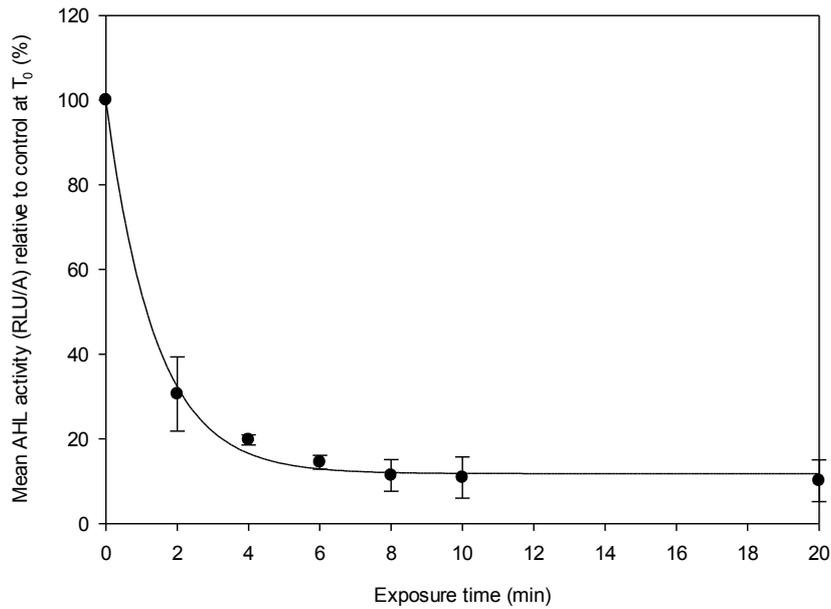
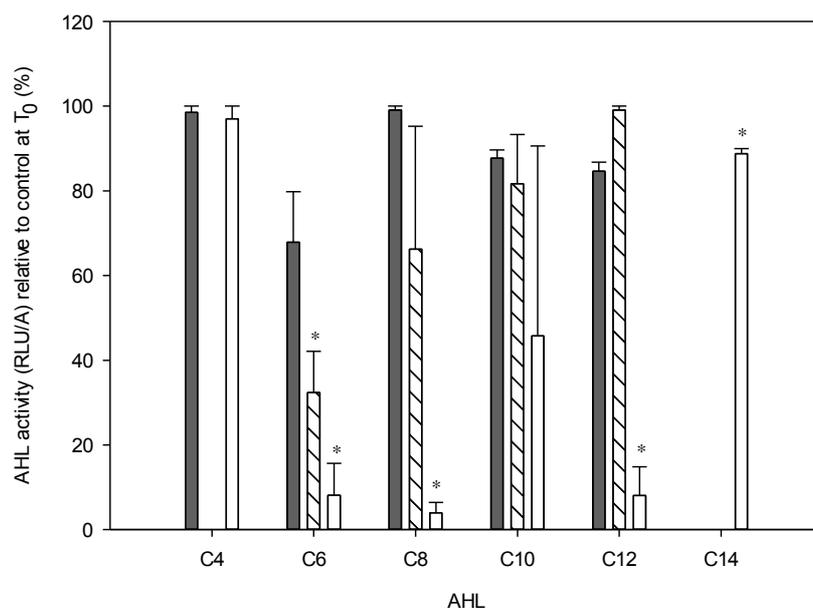


Figure 3.5. Exposure of

O-C8-HSL to 15  $\mu$ M HOCl. Samples were removed at 2 min intervals for 10 min. AHLs were incubated with *E. coli* pSB1075 for 3 h and activity determined by measuring luminescence and optical density. Data are expressed as the mean change in activity compared to control at Time zero (T<sub>0</sub>). Means  $\pm$  SE, n = 2.

### 3.3.3. HOCl reduced the activity of AHLs with different chain length

HOCl significantly reduced the activity of oxo-AHLs with acyl chain lengths of 6, 8, 12 and 14; Activity of AHLs with an OH side group was reduced in that with an acyl chain length of 6 only. The activity of all unsubstituted AHLs were not significantly reduced (Fig.3.6.).



*Figure 3.6.* Exposure of AHLs of different chain length to 15  $\mu$ M HOCl. AHLs with no side group (closed bars), AHLs with OH side group (diagonal bars) and AHLs with oxo side group (open bars). Exposure was for a total of sixty min with samples taken at time zero ( $T_0$ ) and sixty min. AHLs were incubated with *E. coli* pSB1075 for 3 h and activity was determined by measuring luminescence and optical density. Data are expressed as the mean change in activity compared to control at  $T_0$ . Means  $\pm$  SE, n = 2. \* indicates a significant difference between HOCl exposed AHL and control ( $P < 0.05$ ).

### 3.3.4. H<sub>2</sub>O<sub>2</sub> caused insignificant changes to activity of the majority of AHLs

Exposure of a range of AHLs to H<sub>2</sub>O<sub>2</sub> resulted in the significant reduction of activity in C10-HSL only; this reduction is approximately 25%. There was no significant reduction in activity in any of the remaining AHLs tested (Fig. 3.7).

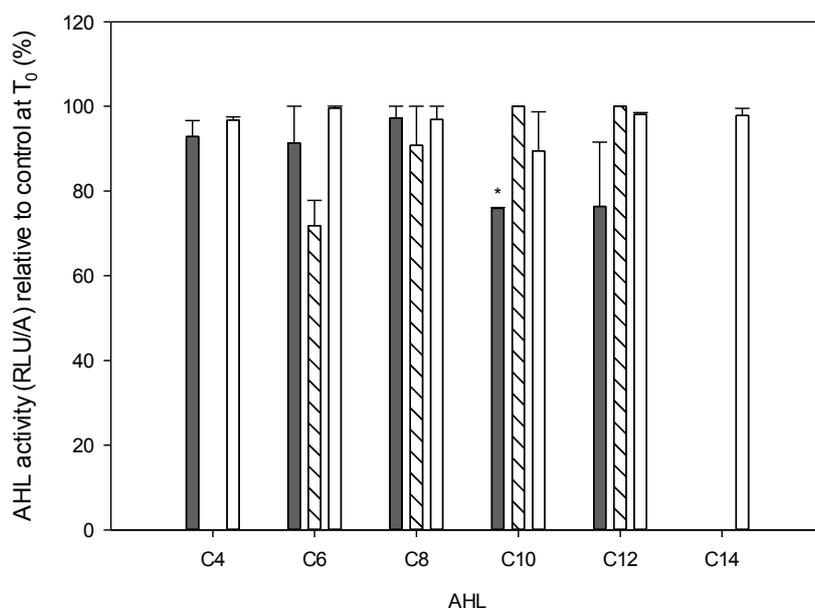


Figure 3.7. Exposure

of AHLs of different chain length to 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>. AHLs with no side group (closed bars), AHLs with OH side group (diagonal bars) and AHLs with oxo side group (open bars). Exposure was for a total of sixty min with samples taken at time zero, (T<sub>0</sub>) and sixty min. AHLs were incubated with *E. coli* pSB1075 for 3 h and activity was determined by measuring luminescence and optical density. Data are expressed as the mean change in activity compared to control at time zero. Means  $\pm$  SE n = 2. \* indicates a significant difference between H<sub>2</sub>O<sub>2</sub> exposed AHL and control ( $P < 0.05$ ).

### 3.3.5. HOCl exposure resulted in hydrolysis of lactone ring

Evidence from MS-MS suggests that exposure to HOCl resulted in hydrolysis of the lactone ring in O-C8-HSL and possibly to a lesser extent in OH-C8-HSL. The AHL OH-C8-HSL has a molecular mass of 243 Da which, with ionization during MS-MS, showed a peak at  $m/z$  (mass to charge ratio) 244 for the intact AHL (Fig. 3.8). The spectrum of the daughter ions from 244 showed a relatively strong peak at  $m/z$  101.9 (Fig. 3.9), indicating that in the control AHL the lactone ring was intact. The spectrum for the HOCl exposed OH-C8-HSL showed a strong peak at  $m/z$  244 (Fig. 3.10) indicating the presence of intact AHL, however the signal for an intact lactone ring ( $m/z$  102) was relatively weak (Fig. 3.11).

O-C8-HSL has a molecular mass of 241 which, with ionization during MS-MS, showed a peak at  $m/z$  242 for the intact AHL (Fig. 3.12). The spectrum of the daughter ions from  $m/z$  242 showed a relatively strong signal at  $m/z$  102 (Fig. 3.13), indicating that in the control AHL the lactone ring was intact. The spectrum for the HOCl exposed O-C8-HSL showed a relatively weak signal at  $m/z$  242, and the MS-MS spectrum of the daughter ions of  $m/z$  242 showed a signal at  $m/z$  231.2 indicating the presence of hydrolysed lactone rings (Fig. 3.15) on a chlorinated molecule (product F in Fig. 3.2). The presence of a hydrolysed intermediate between intact AHL and hydrolysed lactone ring (product E in Fig. 3.2) would be indicated by a signal at  $m/z$  213 so the presence of a strong signal at  $m/z$  214.2 (Fig. 3.15) was suggestive that this molecule may be present.

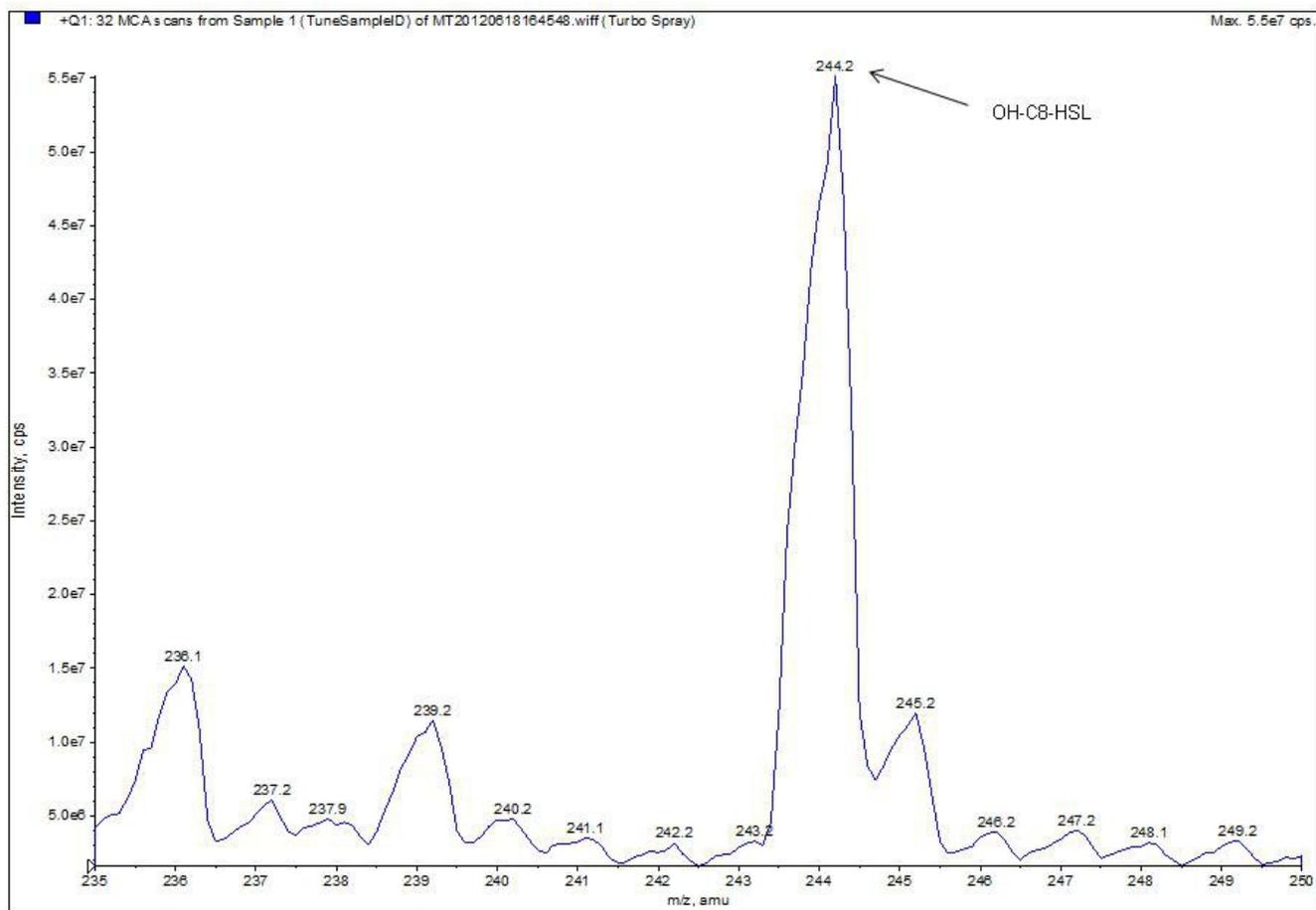


Figure 3.8. MS-MS spectrum of control OH-C8-HSL.

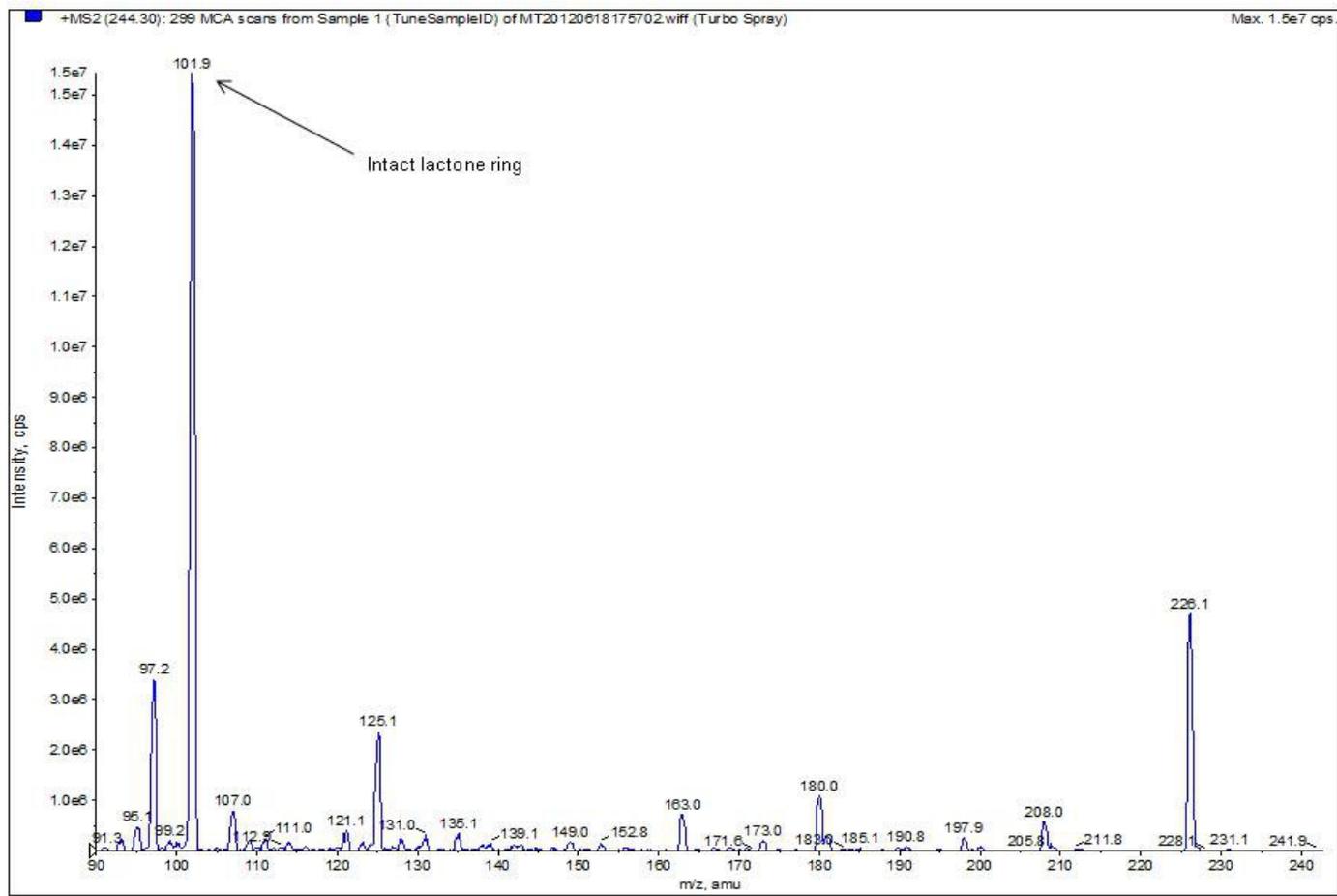


Figure 3.9. MS-MS spectrum of products of 244 from control OH-C8-HSL. The arrow indicates the presence of intact lactone rings.

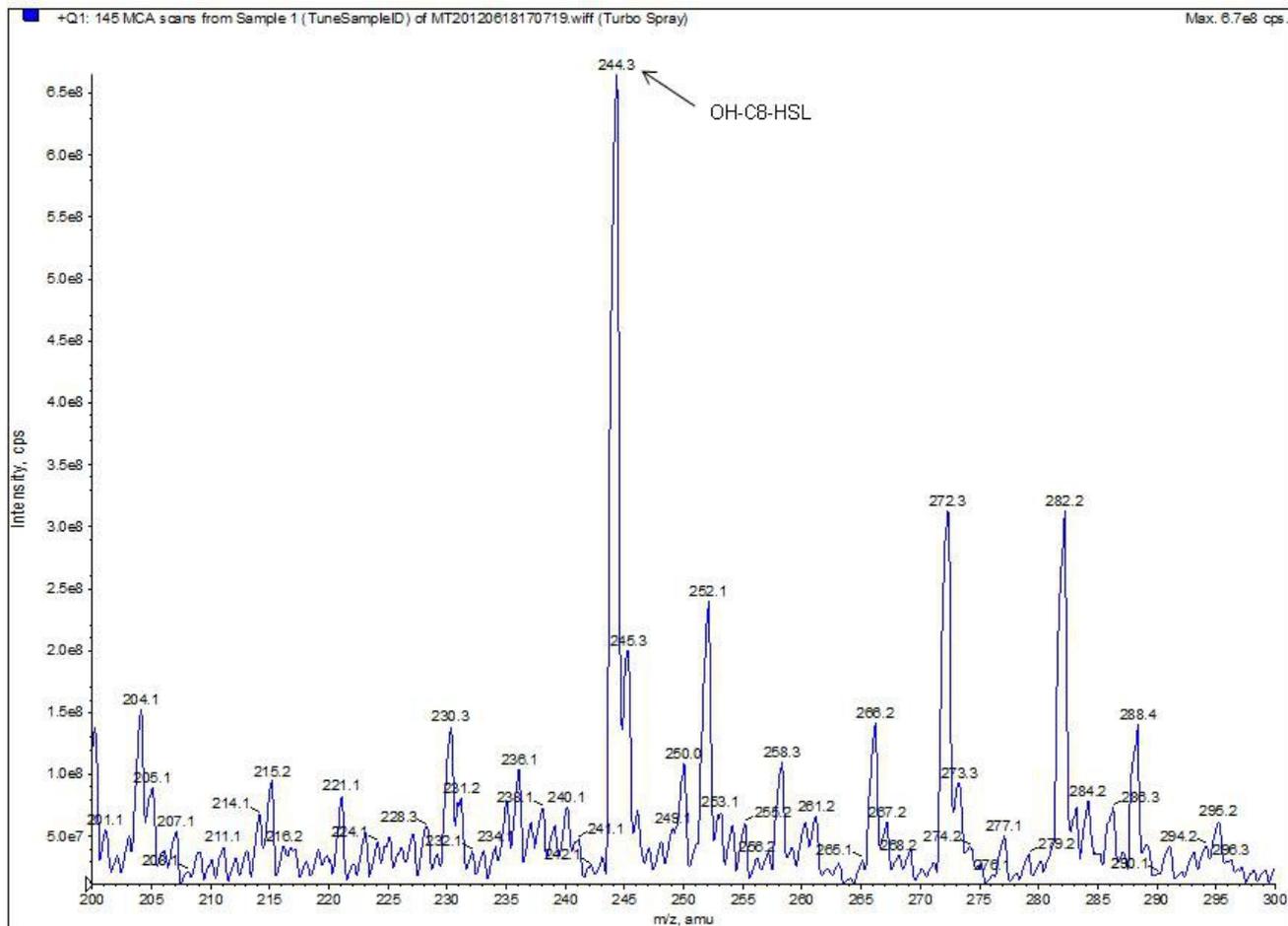


Figure 3.10. MS-MS spectrum of HOCl exposed OH-C8-HSL. The arrow indicates the presence of the intact AHL.

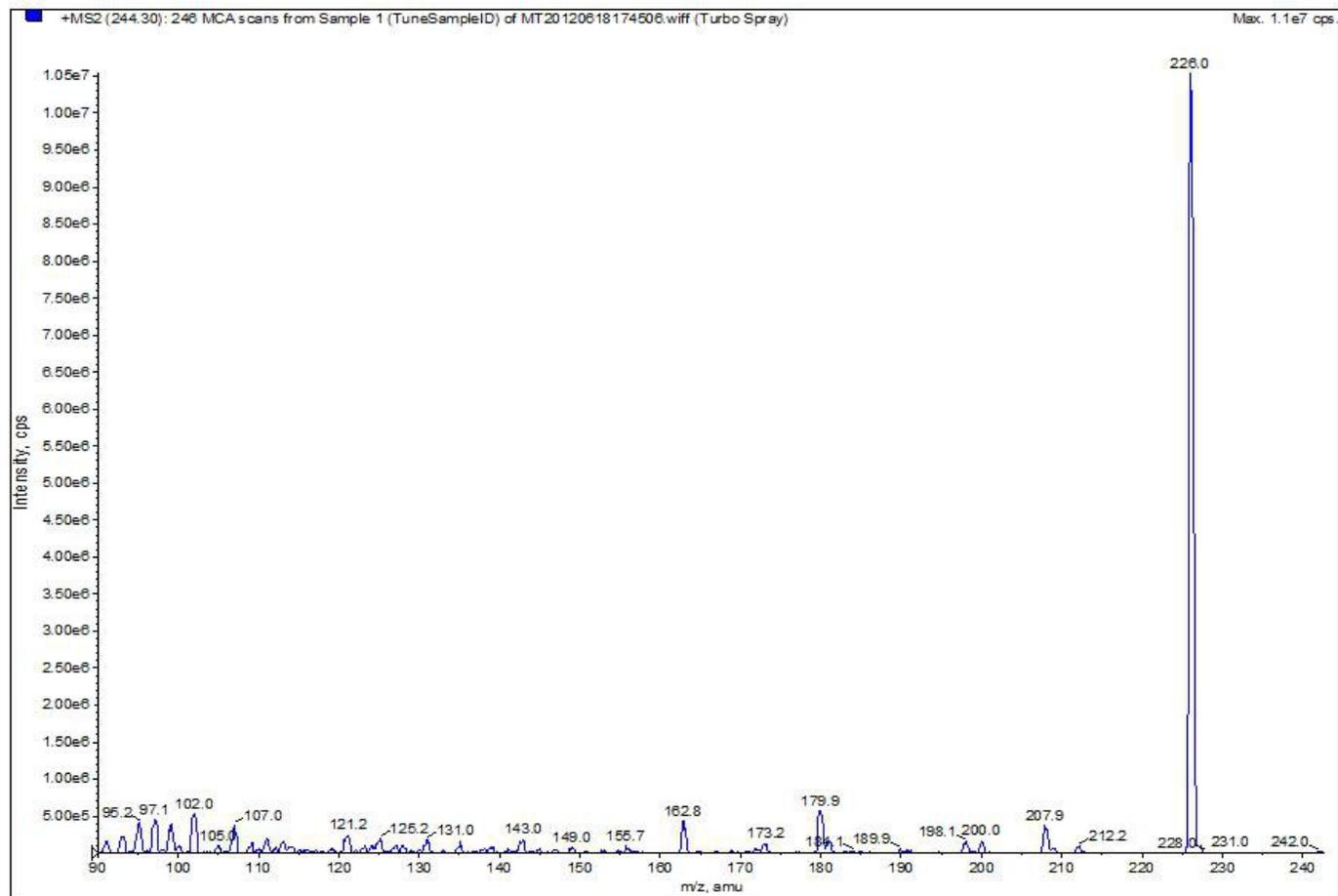


Figure 3.11. MS-MS of products of 244 from HOCl exposed OH-C8-HSL. There is a relatively low signal for 102.

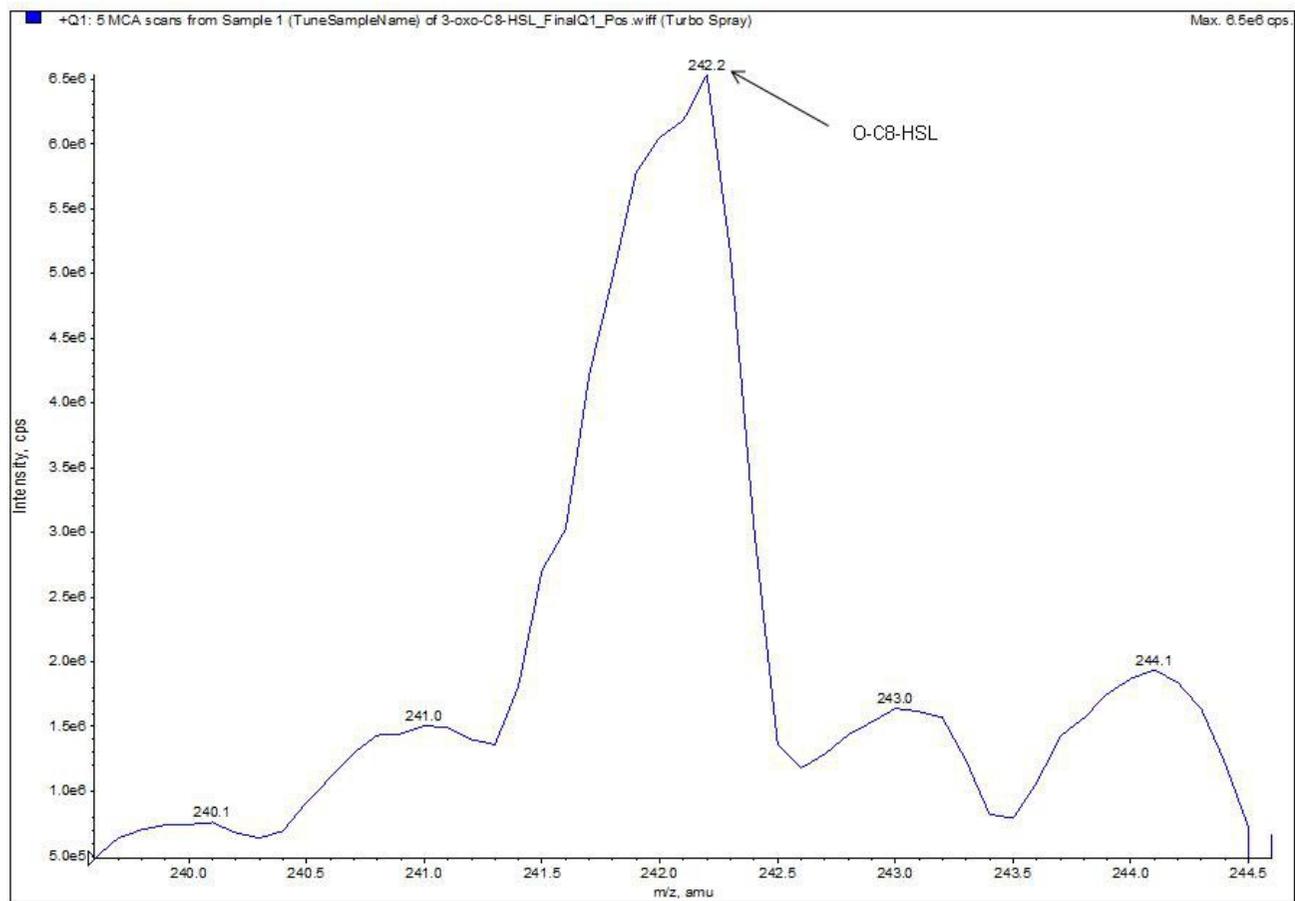


Figure 3.12. MS-MS spectrum of control O-C8-HSL. The arrow indicates the presence of intact AHL at 242.

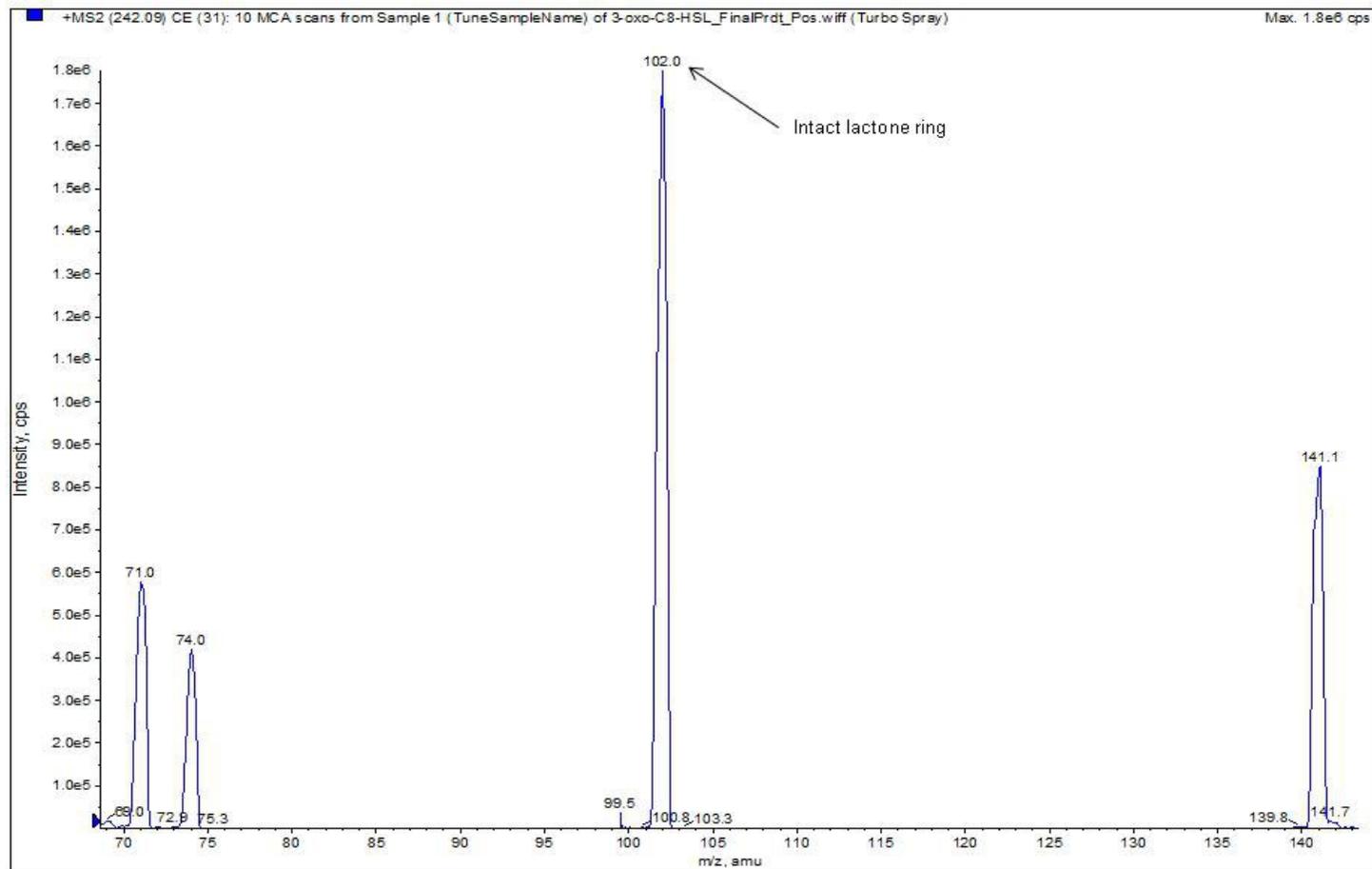


Figure 3.13. MS-MS spectrum of products of 242 for control O-C8-HSL. The arrow indicates the presence of intact lactone ring.

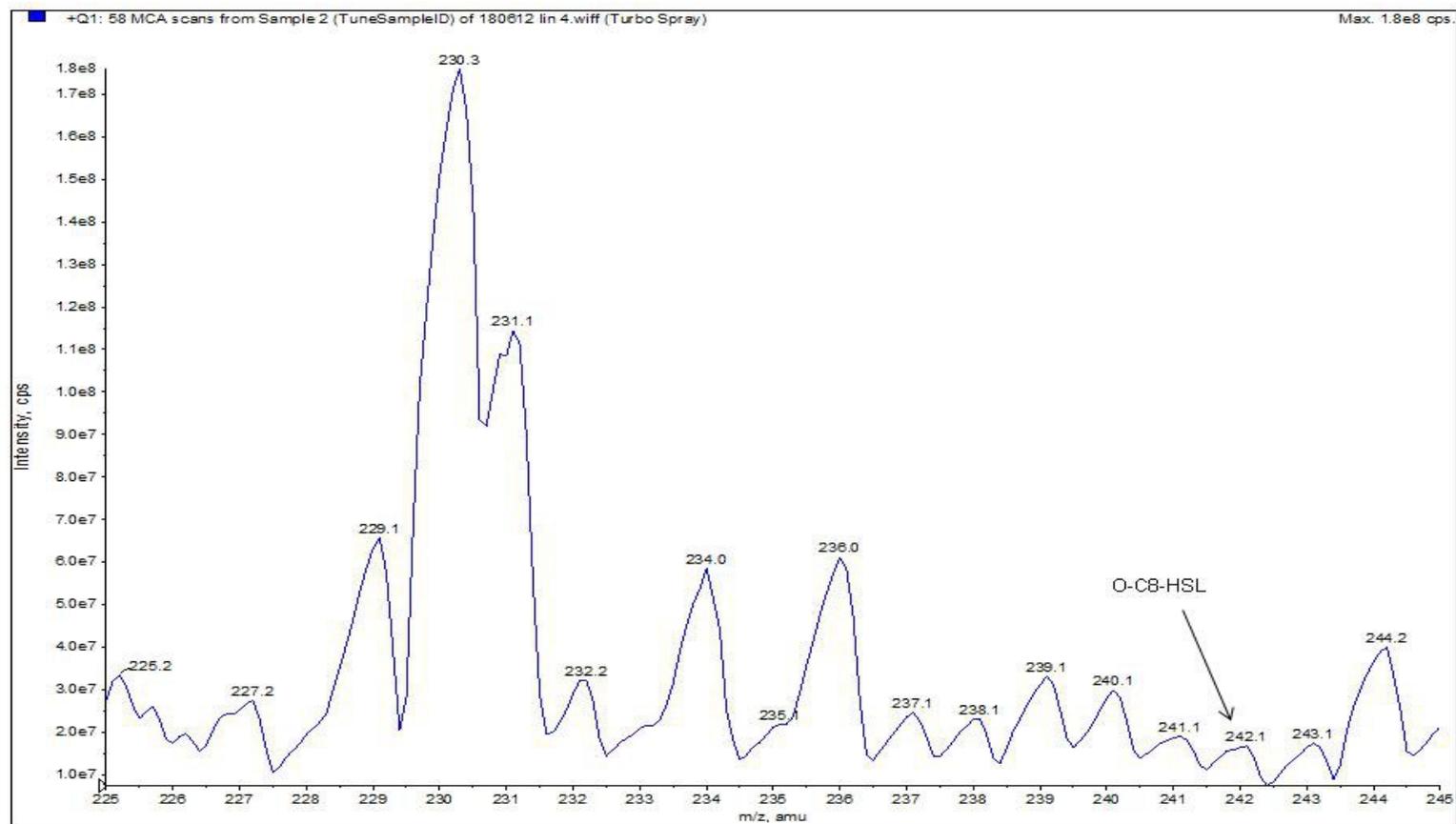


Figure 3.14. MS-MS spectrum of HOCl exposed O-C8-HSL. The arrow indicates the reduced signal for the intact AHL.

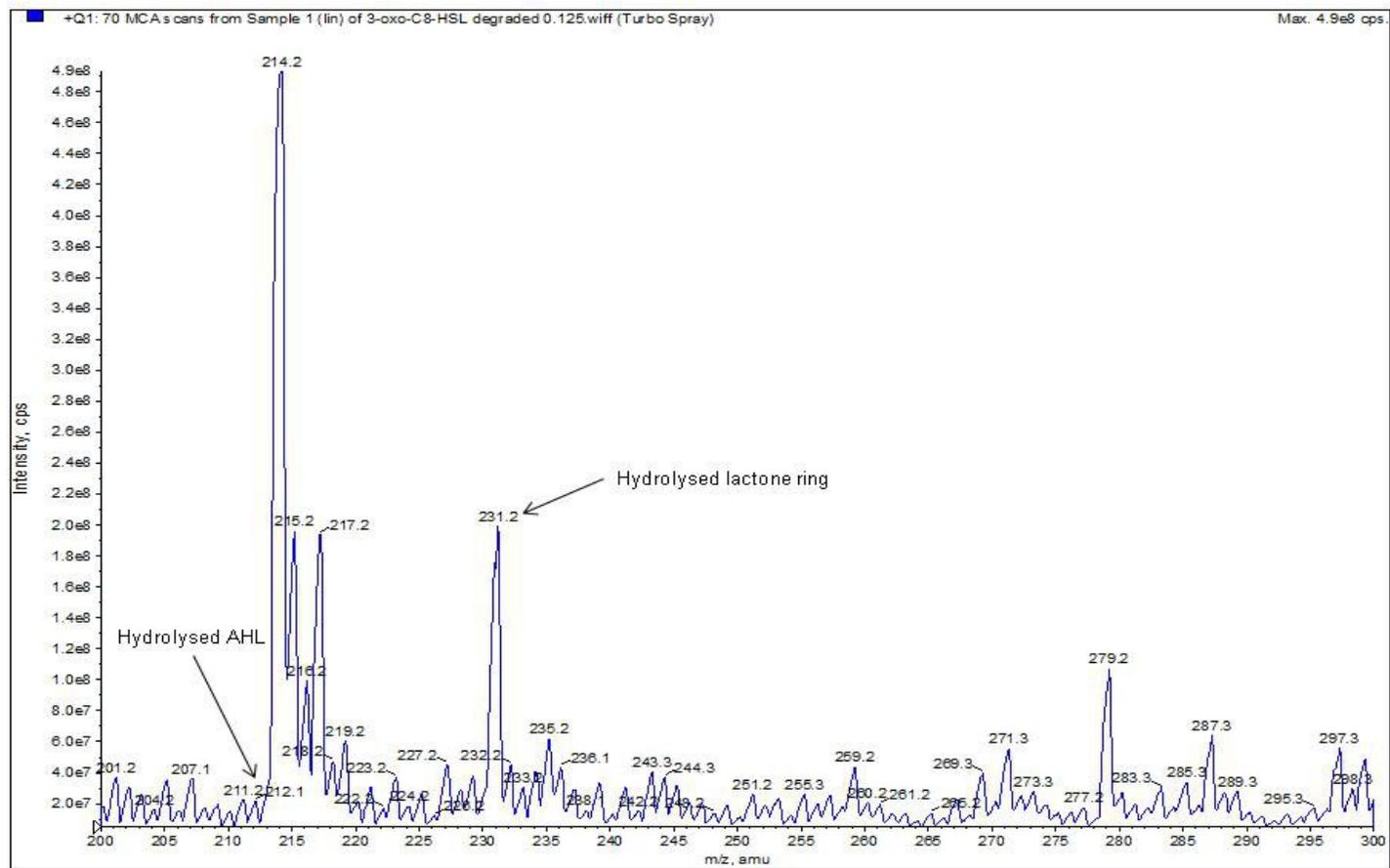
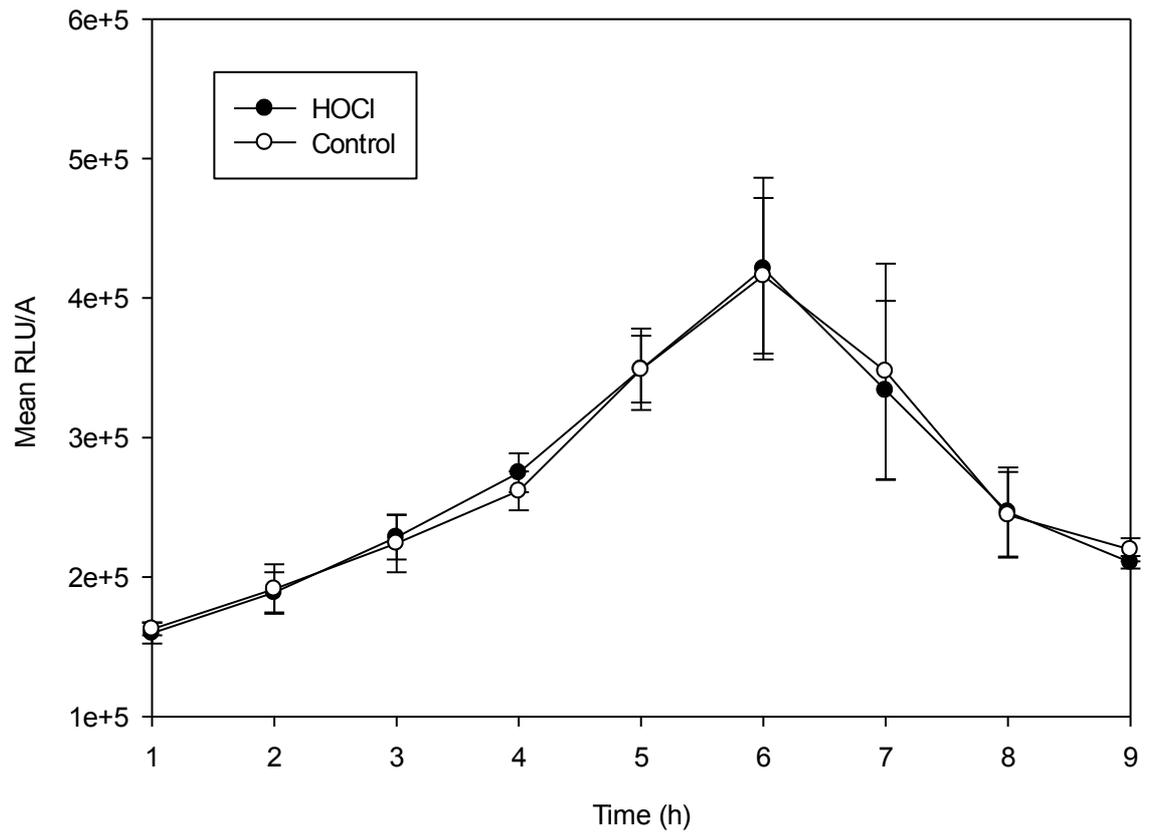


Figure 3.15. MS-MS spectrum for the products of 242 for HOCl exposed O-C8-HSL. The arrows indicate signals that represent the presence of hydrolysed AHL (at 212) and hydrolysed lactone rings (at 231).

### 3.3.6. HOCl did not reduce the signal of *P. aeruginosa* gene reporter fusion strains

HOCl, at the concentration that reduced AHL activity in Section 3.10.2., did not significantly reduce the signal from *P. aeruginosa* gene fusion reporter strains LasI::Lux, LasB::Lux, RhII::Lux or RhIA::Lux, compared to control (water). For three of the strains the largest difference in signal (not statistically significant) occurred at six hours of incubation. All strains showed a consistent increase in signal from one to six hours, following a one to two hour lag period, and all strains apart from RhIA::Lux then demonstrated reducing signal strength for the last three hours of incubation; RhIA::Lux showed a steady signal strength for a further two hours, reducing in strength by the ninth hour of incubation.

Figure 3.16. Exposure of *P. aeruginosa* reporter strain LasI to HOCl (15  $\mu$ M). HOCl was added to a 1:10 dilution of an overnight culture of the reporter strain and incubated for 9 h; luminescence and absorbance measurements were taken every hour. The data are expressed as the mean RLU/A. Mean  $\pm$  SE, n = 3. There is no significant difference between data for HOCL and control (t-test)



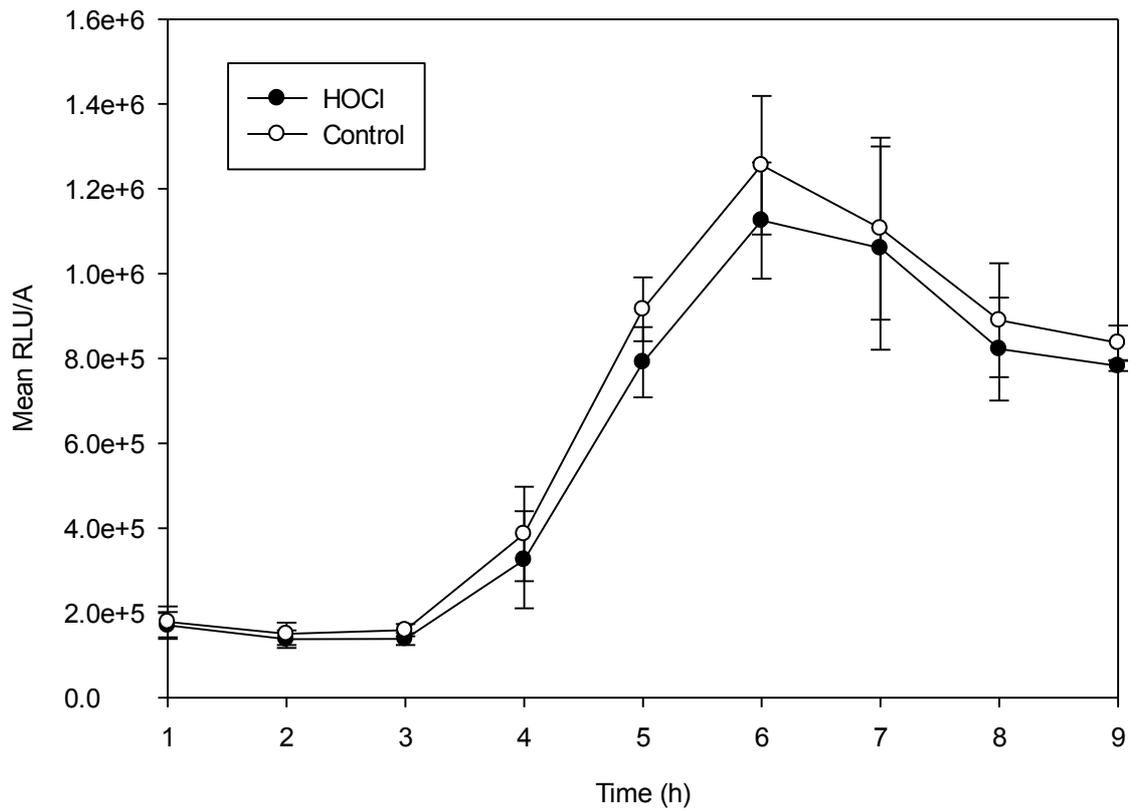


Figure 3.17. Exposure of *P. aeruginosa* reporter strain LasB to HOCl (15  $\mu$ M). HOCl was added to a 1:10 dilution of an overnight culture of the reporter strain and incubated for 9 h; luminescence and absorbance measurements were taken every hour. The data are expressed as the mean RLU/A. Mean  $\pm$  SE, n = 3. There is no significant difference between data for HOCl and control (t-test).

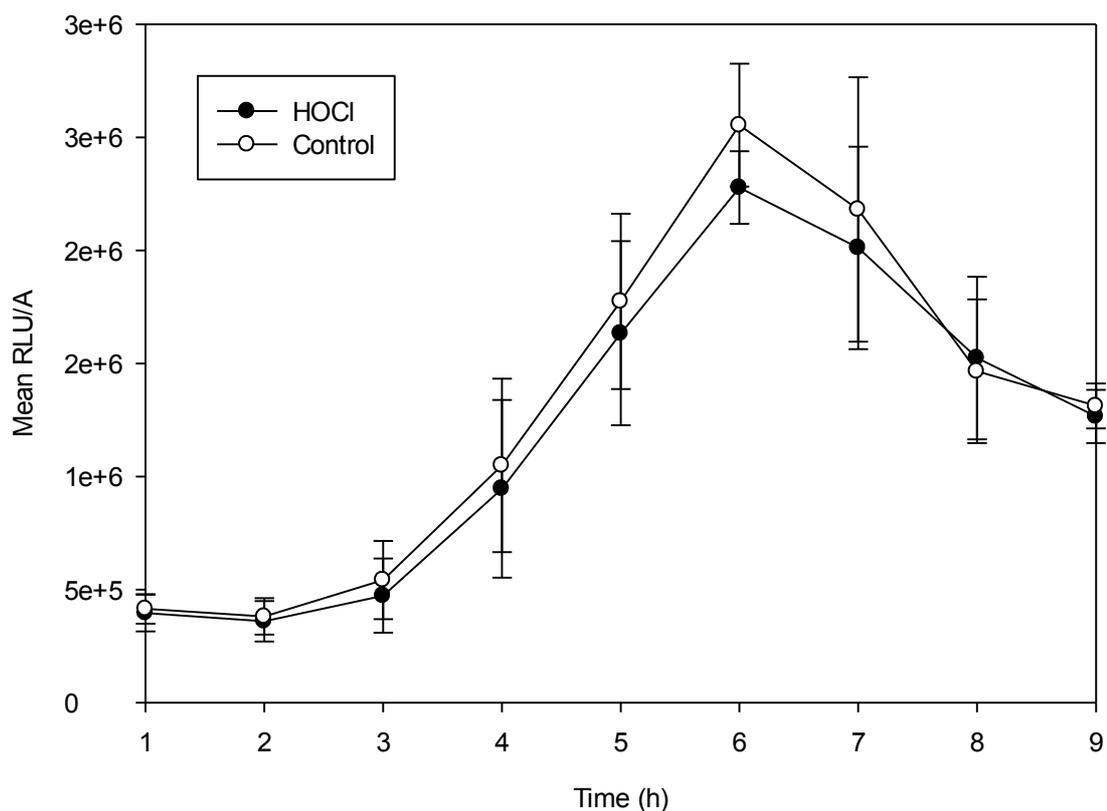


Figure 3.18. Exposure of *P. aeruginosa* reporter strain RhII to HOCl (15  $\mu$ M). HOCl was added to a 1:10 dilution of an overnight culture of the reporter strain and incubated for 9 h; luminescence and absorbance measurements were taken every hour. The data are expressed as the mean RLU/A. Mean  $\pm$  SE, n = 3. There is no significant difference between data for HOCl and control (t-test).

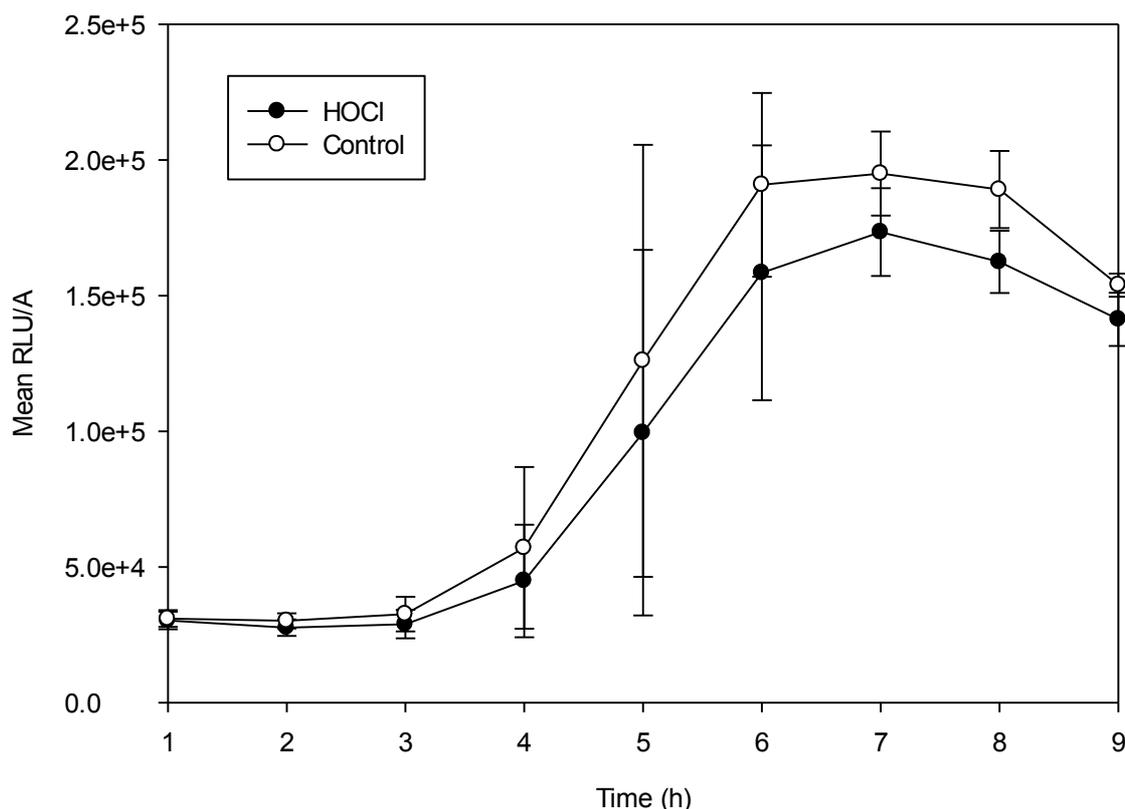


Figure 3.19. Exposure of *P. aeruginosa* reporter strain RhIA to HOCl (15  $\mu$ M). HOCl was added to a 1:10 dilution of an overnight culture of the reporter strain and incubated for 9 h; luminescence and absorbance measurements were taken every hour. The data are expressed as the mean RLU/A. Mean  $\pm$  SE, n = 3. There is no significant difference between data for HOCl and control (t-test).

### 3.4. Discussion

This study has demonstrated that HOCl significantly reduces the activity of a range of AHLs *in vitro*. Though other groups have observed halogen damage to AHLs (Michels *et al.* 2000, Borchardt *et al.* 2001), this is the first time that damage to AHLs with various chain lengths and substitutions has been systematically assessed and reported. It was also demonstrated that, generally, the activity of the same AHLs is not affected by H<sub>2</sub>O<sub>2</sub>. The *in vivo* study demonstrated that HOCl, at the concentration that damaged AHLs *in vitro*, did not reduce the signal of *P. aeruginosa* reporter strains, which to our knowledge has yet to be reported. A summary of the results from various sources, including

this study, can be seen in Table 3.3. Where AHLs have been repeated this study confers with other groups in all instances except that involving O-C10-HSL; this study found no reduction in activity when exposed to HOCl, a result at odds with that of Michels *et al.* (2000). However, even though the mean reduction in activity did appear as though it should be significant (Fig. 3.6), the large error bars ruled out arriving at this conclusion.

The overriding observation from all three studies was that substitution on the 3C of the carbon chain rendered the AHL molecule vulnerable to modification by oxidizing halogens, with the oxo side group being the predominant target (Fig. 3.6, Table 3.3). This does not appear to be specific to halogens; both substituted and unsubstituted AHLs were oxidized by OH<sup>•</sup> radicals, but the activity of substituted AHLs were noticeably less than that of unsubstituted molecules following oxidation (Frey *et al.*, 2010). An insightful observation from Frey's study and also that of Cui *et al.* (2009) was that the product of C6-HSL oxidation by OH<sup>•</sup> radicals was O-C6-HSL.

Table 3.3. A summary of results from three studies investigating AHL modification by oxidizing halogens.

AHL	Reduction in activity	Test agent	Reference
C4-HSL	No	HOCl	This study
O-C4-HSL	No	HOCl	This study
C6-HSL	No	HOCl	This study
	No	HOCl, HOBr	Michels <i>et al.</i> 2000
	No	HOCl, HOBr	Borchardt <i>et al.</i> 2001
O-C6-HSL	Yes	HOCl	This study
	Yes	HOCl, HOBr	Michels <i>et al.</i> 2000
	Yes	HOCl, HOBr	Borchardt <i>et al.</i> 2001
OH-C6-HSL	Yes	HOCl	This study
C8-HSL	No	HOCl	This study
O-C8-HSL	Yes	HOCl	This study
OH-C8-HSL	No	HOCl	This study
C10-HSL	No	HOCl	This study
O-C10-HSL	No	HOCl	This study
	Yes	HOCl, HOBr	Michels <i>et al.</i> 2000
OH-C10-HSL	No	HOCl	This study
C12-HSL	No	HOCl	This study
	No	HOCl, HOBr	Borchardt <i>et al.</i> 2001
O-C12-HSL	Yes	HOCl	This study
	Yes	HOCl, HOBr	Borchardt <i>et al.</i> 2001
OH-C12-HSL	No	HOCl	This study
O-C14-HSL	Yes	HOCl	This study

The results from the MS-MS on C8 AHLs concurred with those of Michels' group (Michels *et al.* 2000) in that evidence of a hydrolysed lactone ring was present (Fig. 3.15.), and also a possible intermediate molecule, for HOCl exposed O-C8-HSL. Using the estimated pseudo first-order rate constants in the Michels paper, at the pH conditions used for this study (6.8), it can be postulated that reactions 1 and 2 in the pathway (Fig. 3.2) were too fast for molecules A and B to be detected, which is supported by the lack of peaks at

the expected  $m/z$  values (242 and 276.5, respectively). Much slower rate constants for reactions 3 and 4 (Fig. 3.2) mean that observed daughter ions at  $m/z$  231 and possibly 213 are the equivalent of products E and F in the pathway if chlorination occurred as indicated.

The lack of any significant changes to AHL activity following exposure to  $H_2O_2$  indicated that these molecules were not as vulnerable to oxidation as they were to chlorination (Fig. 3.7), in terms of affecting activity. It is possible that an insufficient concentration was used, though a similar concentration reduced the activity of the bacterial enzyme penicillinase (Chapter 2). Another possibility is that oxidation of unsubstituted AHLs resulted in substituted AHLs, as in that seen by Cui (Cui *et al.*, 2009) and Frey (Frey *et al.*, 2010), the modified molecules themselves being capable of normal activity. So, C8-HSL would become O-C8-HSL and O-C8-HSL would become OH-C8-HSL. It is possible that the fully substituted AHL is then less vulnerable to further oxidation, indeed Frey's group demonstrated that the rate constants for the reactions between AHLs and  $OH^\cdot$  were higher for unsubstituted AHLs than substituted ones (Frey *et al.*, 2010).

A finding of this study was that the *in vitro* effect of HOCl on AHLs was not seen *in vivo* for any of the *P. aeruginosa* reporter strains used (Figs. 3.9 to 3.12). The concentration of HOCl was the same as that used for the *in vitro* experiments, but the unknown factor with the *in vivo* experiments was the concentration of AHLs being produced, it could have meant that the concentration of HOCl was insufficient to cause a measurable effect. Increasing the concentration of HOCl would have introduced the problem of direct bacterial cell toxicity.

The main question as to why no effect was seen therefore relates to the fate of HOCl in the bacterial culture; the most probable answer being that the HOCl

was being quenched by components in the culture medium, or by molecules produced by the *P. aeruginosa* strains. HOCl can readily damage biomolecules such as proteins (Malle *et al.*, 2006), so it is possible that the sub-lethal concentration used was quenched by proteins within the growth medium. GSH, an efficient quencher of HOCl is not as widespread in bacteria as other antioxidant molecules such as SOD and catalase, being found in most Gram-negative bacteria but relatively few Gram-positive bacteria (Fahey *et al.*, 1978). It is, along with other thiols, found in *P. aeruginosa* (Newton *et al.*, 1996) which would equip the bacteria with a sound defence against HOCl. However, even though this may have explained the protective role of GSH against intracellular AHL damage, unless the molecule is actively secreted by the organism, extracellular AHLs would still have been vulnerable and a reduction in intact AHLs should have occurred. Export of GSH by some strains of *Salmonella typhimurium* and *E. coli* has been reported (Owens and Hartman, 1986), and it was observed that extracellular levels of GSH rose in parallel with cell density, when, by the early stationary phase, there was more extracellular GSH than intracellular. Evidence that *P. aeruginosa* exports this molecule has not been found, but it is feasible that they do employ this extra level of oxidative defence, especially as the results of this study demonstrate that, at the concentration used, HOCl does not reduce the signal from reporter strains.

Future work could examine the effects of other ROS/RNS on AHLs, and to investigate further the relationship between damaged AHLs and the formation of biofilms. For example, mutant strains of *P. aeruginosa* that do not produce endogenous AHLs could be incubated in media containing HOCl exposed AHLs, in conditions that allow quantification of biofilm mass. Performing MS-MS on AHLs exposed to H<sub>2</sub>O<sub>2</sub> could confirm that oxidation was taking place.

Modification of AHLs, perhaps not being considered as degraded or damaged, might still affect the behaviour of the test organism if it is no longer in the form normally synthesised or recognized by the bacteria. So, even though this study did not see evidence of “damage” to AHLs by H<sub>2</sub>O<sub>2</sub>, it would be valuable to determine whether H<sub>2</sub>O<sub>2</sub> exposed AHLs resulted in different physiological and morphological responses of the bacteria to the modified AHL *in vivo*.

## Chapter 4

### The effects of oxygen on the formation of bacterial biofilms and the effects of oxygen and oxidants on 24 h bacterial biofilms

#### 4.1. Introduction

Though the general concept of antibiotic resistance in bacteria relates to genetically transferable mechanisms, the sessile form of a bacterial community, known as a biofilm, can also be considered to be a means of resistance in itself. How bacteria within biofilms are able to resist the action of antibiotics is being widely investigated and several mechanisms have been proposed, including; altered gene expression, which may result in the production of matrix components that bind antibiotics (Sadovskaya *et al.* 2010, Beaudoin *et al.* 2012), and slower growing bacteria within biofilm structures (Werner *et al.* 2004), which may be less vulnerable to antibiotics whose action requires that cells are actively growing (Section 1.2.). Biofilms, therefore, need to be considered when investigating potential anti-resistance strategies. In the context of HBOT it is worth considering whether improvement in tissue oxygenation directly affects the action of antibiotics, rendering them more effective against biofilms, or whether oxidant generation acts as an adjunct to antibiotic therapy. Wound microflora are diverse (Section 1.3.2.) and several species might have been applicable to use in this study. However, *P. aeruginosa* and *S. aureus* were chosen due to their predominance in chronic wounds and the clinical relevance of their antibiotic resistance. Two strains were obtained for each species, one, exhibiting antibiotic sensitivity and the other, resistance; this enabled a comparison of the properties between the two strains in different oxygen environments. Both species readily form biofilms and are known to be ubiquitous in the environment. They have been identified in chronic wounds

(Davis *et al.* 2008) and are thought to be a factor in non-healing (Bjarnsholt *et al.* 2008).

A protocol using broth cultures in 96-well microplates was devised based on that published by the National Committee for Clinical Laboratory Standards (Ferraro, 2003). This was to allow for high replication numbers for the various conditions and the limitation of the size of the sealed oxygen boxes.

The first aim of this study was to investigate whether oxygen concentration impacted the effectiveness of antibiotics against planktonic bacterial cultures. This was achieved by exposing cultures of sensitive and resistant strains of *P.aeruginosa* and *S. aureus* to a range of antibiotic concentrations, in oxygen concentrations of 5%, 21%, 40% and 100%. This trial was also necessary to ascertain the MICs for planktonic cells to be able to estimate the concentration necessary to kill the majority of biofilm cells.

The second aim of this study was to determine the effect of oxygen concentration on the formation of biofilm mass in both sensitive and resistant strains of bacteria. Both strains of *P.aeruginosa* and *S. aureus* were cultured for 24 h and their biofilm mass quantified by staining with crystal violet. The crystal violet was solubilised in acetic acid and measured with a spectrophotometer.

The final aim was to determine whether oxygen concentration was able to modify the effects of antibiotics and oxidants on live biofilm cells. Twenty-four hour pre-formed biofilms of an antibiotic sensitive strain of *S. aureus* were exposed to (i) the antibiotic oxacillin, (ii) the oxidant generating system xanthine oxidase, and (iii) both of these, in different oxygen incubation conditions (.5%, 21%, 40% and 100%) for a further 24 h. NADPH oxidase is the phagocytic oxidant generator likely to be found in a wound environment but a simpler system was required for this study. Xanthine oxidase, like NADPH oxidase uses

oxygen as a substrate and the rationale for its use is that with increasing oxygen availability (as during HBOT) there will be an increased production of oxidant and improved killing of bacterial cells in the biofilm. Xanthine oxidase catalyses the oxidation of xanthine as follows (Lacy *et al.* 1998):



The superoxide radical ( $\text{O}_2^-$ ) in buffer will dismutate into  $\text{H}_2\text{O}_2$  as below:



Xanthine was the substrate used for this study. Xanthine oxidase is found in vascular smooth muscle cells and endothelial cells in cardiac and skeletal muscle, and is also found in human macrophages and mast cells (Hellsten-Westling, 1993). Xanthine oxidase activity has been linked with inflammatory processes occurring following skeletal muscle damage (Hellsten *et al.* 1997), so it is reasonable to consider that it may also play a role in the generation of oxidants in a chronic wound environment. However, in this study the enzyme is being used as a surrogate for neutrophil NADPH-oxidase.

Live biofilm cells were quantified by incubating with the fluorescent dye resazurin. Blue resazurin is reduced to pink resorufin by metabolically active cells, a change that was measured by colorimetry in this study.

## 4.2. Materials and methods

### 4.2.1. Chemicals

Acetic acid and ethanol were obtained from VWR BDH Prolabo (Lutterworth, Leicestershire, UK). Crystal violet, Mueller Hinton II broth (cation adjusted), sodium chloride, oxacillin (sodium salt), inorganic salts, xanthine and xanthine oxidase (Cat. # X4500, from bovine milk) were obtained from Sigma Aldrich (Poole, Dorset, UK). Resazurin (Acros Organics), streptomycin sulphate,

tryptone soya broth (TSB) and tryptone soya agar (TSA) were obtained from Fisher Scientific UK Ltd (Loughborough, Leicester, UK).

#### 4.2.2. Bacterial strains used in this study

The bacterial strains used in this study are listed in Table 4.1. *P. aeruginosa* 8626 and *S. aureus* 6571 were kindly donated by Plymouth University. *P. aeruginosa* 10848 was obtained from NCIMB (Aberdeen, Scotland, UK). *S. aureus* 33591 was obtained from Fisher Scientific UK Ltd.

Table 4.1. The bacterial strains used and their antibiotic sensitivity.

\* National Collection of Industrial and Marine Bacteria. \*\* American Type Culture Collection.

Strain	Culture collection	Antibiotic sensitivity
<i>Pseudomonas aeruginosa</i> 8626	NCIMB*	Streptomycin sensitive
<i>Pseudomonas aeruginosa</i> 10848	NCIMB	Streptomycin resistant
<i>Staphylococcus aureus</i> 6571	NCIMB	Oxacillin sensitive
<i>Staphylococcus aureus</i> 33591	ATCC**	Oxacillin resistant

#### 4.2.3. Maintenance of bacterial cultures and preparation of media

All strains of bacteria were stored in 15% glycerol, at -80 °C and in liquid nitrogen. For short-term storage, streak plates of bacteria (on TSA) were sealed with Parafilm®M and stored at 4 °C. Fresh plates were prepared every 4-6 weeks. All liquid cultures, to provide inoculation material, were prepared by incubating the bacteria in TSB at 37 °C for *P. aeruginosa* and 35 °C for *S. aureus*. All media, dH<sub>2</sub>O and other solutions were autoclaved at 121 °C for 15 min and stored at 4 °C. All solutions containing heat sensitive components, such as antibiotics, were filter sterilised prior to use (0.22 µm filter pore size).

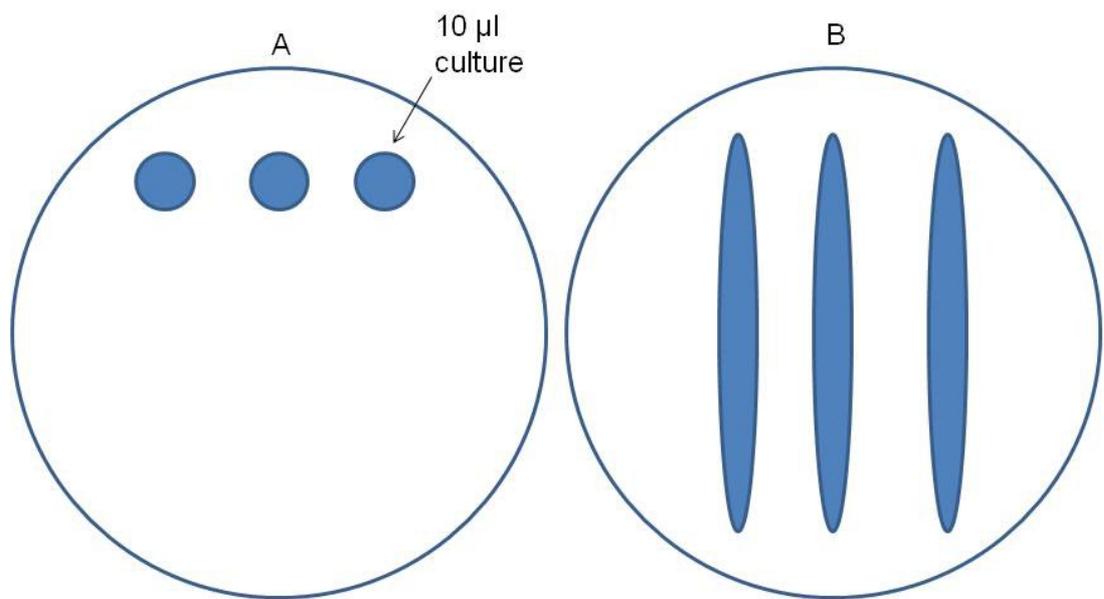
#### 4.2.4. Determination of bacterial population density

Bacterial population density was determined by measuring the absorbance, at 600 nm, of a 1 ml sample of liquid culture and using a growth calibration curve

to estimate the CFU ml<sup>-1</sup>. A 1 ml aliquot of the growth medium was used to blank the spectrophotometer.

#### *4.2.5. Preparation of bacterial population growth calibration curves*

Fifty millilitres of TSB was inoculated with a single colony from a streak plate and incubated overnight, at the appropriate temperature at 150 rpm. The following morning, 50 ml of fresh TSB was inoculated with 500 µl of the overnight culture and incubated in a shaking water bath (150 rpm) at the appropriate temperature. Every hour, for 5-6 h, 1 ml aliquots of culture were removed and the absorbance measured (Section 4.2.4.). At the same time, 100 µl of culture was added to 900 µl of medium ( $1 \times 10^{-1}$  dilution); further dilutions were performed down to  $1 \times 10^{-12}$ . For each time point, four dilutions were chosen and 10 µl, from a single dilution, was dropped onto the top of a TSA plate three times (Figure 4.1.A). The plate was gently tilted to allow each aliquot of culture to flow, in a straight line, down the plate (Figure 4.1.B). This was repeated for all dilutions chosen. All plates were incubated for 24-48 h.



*Figure 4.1.* Plate-saving method of applying liquid culture to agar plates to obtain CFU counts. There were three replicates per plate, each of 10 µl aliquots. The plate was tilted to allow a flow of culture across the plate. CFUs were counted in each streak.

Following incubation the CFUs were counted in each streak on each plate and the mean for each plate was calculated, discounting plates that had too many or too few colonies (the ideal number would have been between 10 and 50 colonies per streak). The following equation was used to determine CFU ml<sup>-1</sup>:

$$\text{CFU ml}^{-1} = \text{plating factor (100)} \times \text{CFU count} \times \text{dilution factor}$$

Plots were constructed of CFU ml<sup>-1</sup> × absorbance, and a line of best fit obtained. This was repeated for all bacterial species used.

#### *4.2.6. Preparation of cultures for inoculation of microplates*

All bacterial strains (Table 4.1) were cultured overnight in TSB (Section 4.2.3) and 50 ml of fresh TSB was inoculated with 50 µl of culture and incubated at the appropriate temperature until population growth had reached 1 × 10<sup>8</sup> CFU ml<sup>-1</sup>,

as determined by growth calibration curves for each strain. An aliquot of this culture was further diluted with medium to  $1 \times 10^6$  CFU ml<sup>-1</sup>. This was the correct density of bacterial cells, with which to inoculate microplates (sterile, 96-well, polystyrene, clear, flat bottom, 400 µl, Thermo Scientific Nunc), to give a final density of  $5 \times 10^5$  CFU ml<sup>-1</sup> following a further 1:2 dilution in the well (with antibiotic solution or medium). The medium used was dependent on the trial and will be discussed in each relevant section.

#### *4.2.7. Sealed box system for control of oxygen exposure*

Sealed plastic boxes were used to provide specified oxygen concentrations for the incubation of bacterial species (Figure 4.2). Four boxes were prepared, one for each oxygen concentration (5%, 21%, 40% and 100%). Boxes were cleaned with Virkon disinfectant after each use.



*Figure 4.2.* An example of a sealed box used in this study. The box pictured (234 × 234 × 95 mm) was fitted with an oxygen sensor to determine filling times for the different oxygen concentrations and to measure oxygen consumption of bacterial inoculants in a microplate over a period of 24 h to ensure that oxygen concentration would not be significantly depleted during the incubation period, or that gas would not leak out. The boxes used in the main part of the study did not contain an oxygen sensor.

#### *4.2.8. Determination of gas flow time*

Four gas cylinders, each filled with air and/or oxygen, to the required concentration, were prepared by DDRC. A cylinder was attached to its partner box with flexible tubing and gas was run continuously into the box (this was carried out in a fume cupboard) until the oxygen analyser recorded the correct concentration in the box. This process was timed for each concentration. At the end of the filling period the taps on the box were closed and the tubing to the cylinders disconnected.

#### 4.2.9. *Oxygen consumption during culture conditions*

Two microplates were prepared in the same manner as that for a trial and placed in the box containing the oxygen sensor. The box was filled with gas as previously described (Section 4.2.6), to achieve 21% oxygen, and was incubated at 37 °C for 24 h. During this period the oxygen sensor was attached to a recording device which monitored any change in oxygen concentration.

#### 4.2.10. *Micro-broth dilution protocol for planktonic MICs in different oxygen concentrations*

Bacterial cultures were prepared for plate inoculation as described in Section 4.2.5. Double dilutions of antibiotic (streptomycin for *P. aeruginosa* and oxacillin for *S. aureus*) were prepared by initially dissolving antibiotic solid in sterile water and further dilution in Mueller Hinton II broth (MHB) to achieve a concentration double that of the highest concentration being tested (this allows for a further 1:2 dilution when the antibiotic solution was added to the bacterial culture). These solutions were filter sterilised and serial double dilutions performed with MHB to obtain 11 concentrations. In all studies involving oxacillin, MHB was supplemented with 2% (w/v) NaCl (Dillon, 1984). Seventy five microlitres of each antibiotic concentration were added to each of 3 wells in a sterile microplate (flat-bottomed). Seventy five microlitres of bacterial culture were added to each well containing antibiotic. Three wells were prepared with culture and MHB only to act as a growth control and a further 8 wells were prepared with MHB only to act as contamination controls. A total of four plates were prepared for each strain, one for each oxygen concentration.

All plates were read in a plate reader at 600 nm and then covered with Breathe-Easy sealing film; two plates were placed in each oxygen box, one containing the antibiotic sensitive strain and one the resistant strain. The boxes were filled

with gas (Section 4.2.8) and incubated at the appropriate temperature, at 150 rpm, for 18 h for *P. aeruginosa* and 24 h for *S. aureus*. All plates were read at 600 nm, at the end of the incubation period.

#### *4.2.10.1. Data analysis*

The absorbance value for MHB only was deducted from all other values and means calculated. The equation to calculate the mean % reduction in population growth is given in Appendix 11. Means from three replicate experiments were calculated, and the MIC expressed as the lowest concentration of antibiotic to achieve a 90% reduction in population growth.

#### *4.2.11. Quantitation of 24 h biofilm mass formation grown in different oxygen concentrations*

Bacterial cultures were prepared for plate inoculation as described in Section 4.2.5. All dilutions for this trial were with TSB. A series of wells containing medium only was used as contamination control. Eight replicates were prepared for each strain of bacteria and four plates were prepared for each species. The plates were sealed with film and one plate was placed in each oxygen box. The boxes were filled with gas as previously described and incubated for 24 h. All wells were washed twice with sterile distilled water and 200  $\mu$ l of 95% (v/v) ethanol was added to each well which were then left for 15 min. Ethanol was removed and wells were left to dry at room temperature before adding 200  $\mu$ l of 0.1% (w/v) crystal violet. After 5 min at room temperature, the crystal violet was removed and the wells were washed twice with sterile distilled water before adding 200  $\mu$ l of 33% (v/v) acetic acid. The plates were read at 570 nm.

#### *4.2.11.1. Data analysis*

The absorbance value for acetic acid was deducted from all other values and means calculated. Means  $\pm$  SE were calculated from 3 runs for each bacterial

species and visualised using SigmaPlot software. One-way ANOVA was used for statistical analysis with a probability level of  $P < 0.05$  accepted as the determination of statistical significance. For analysis of individual replicate experiments the Mann-Whitney Rank Sum Test was used to determine significant differences between O<sub>2</sub> concentrations and control (21%).

#### *4.2.12. Effect of oxygen concentration on antibiotic decrease in live cells in pre-formed 24 h S. aureus biofilms*

##### *4.2.12.1. Production of 24 h biofilms*

Four microplates were inoculated with culture as previously described (Section 4.2.5) with TSB as the growth medium and using *S. aureus* strain 6571. Plates were sealed with film and incubated at 35 °C for 24 h, at 150 rpm. Following incubation all medium was gently removed from each well and biofilms were washed with sterile dH<sub>2</sub>O.

##### *4.2.12.2. Addition of antibiotic*

MHB (150 µl) containing 12.5 µg ml<sup>-1</sup> oxacillin was placed in eight wells containing biofilm, and MHB without oxacillin was added to eight more wells containing biofilm. MHB was added to eight empty wells to provide a contamination control. Plates were sealed with film, placed in each oxygen box and flushed with gas as previously described. Boxes were incubated at the appropriate temperature for 24 h following which, all medium was removed and biofilms were washed with sterile dH<sub>2</sub>O.

##### *4.2.12.3. Determination of live cell decrease with resazurin*

Fresh MHB (150 µl) containing 40 µM resazurin was added to each well containing biofilm, plus 8 empty wells (this is to provide a value for R<sub>0</sub> in the equation to calculate live cell reduction, see Section 4.2.11.4). The plates, after resealing to prevent contamination and wrapping in aluminium foil to eliminate

light, were incubated in air at 37 °C for 30 min. All plates were then read at 570 nm (maximum absorbance of reduced resazurin) and 600 nm (maximum absorbance for oxidised resazurin).

#### *4.2.12.4. Data analysis*

Raw data were analysed with Microsoft Excel. The equation to calculate the mean % reduction in live cells can be seen in Appendix 12. Means  $\pm$  SE were calculated from three runs and graphed using SigmaPlot software. One-way ANOVA was used for statistical analysis with a probability level of  $P < 0.05$  accepted as the determination of statistical significance.

#### *4.2.13. Exposure of pre-formed 24 h S. aureus biofilms to oxacillin and xanthine oxidase derived oxidants*

Four plates of biofilms were prepared as previously described (Section 4.2.11.1), using *S. aureus* strain 6571. A 750  $\mu$ M xanthine solution was freshly prepared, immediately prior to all runs, by adding 100  $\mu$ l aliquots of 1 M NaOH to 11.4 mg of xanthine until the xanthine had fully dissolved. Sterile MHB was then added slowly, to check for precipitation, to a volume of 90 ml. The pH was checked to ensure a value of between 7.0 and 7.5 and the volume made up to 100 ml with MHB. Xanthine oxidase solution was prepared by diluting the purchased enzyme suspension with sterile dH<sub>2</sub>O to the required concentration immediately prior to use. Oxacillin solutions were prepared by dissolving solid in dH<sub>2</sub>O to the required concentrations, immediately before use. Eight replicates were prepared, of each of the conditions summarised in Table 4.2.

Table 4.2. Conditions prepared for each run of *S. aureus* 6571 biofilm exposure to xanthine oxidase derived oxidants. The final volume was that for each well of the microplate and 8 replicates were prepared for each condition.

Condition	Contents	Final volume
Contamination control	MHB	150 $\mu$ l
Growth control	MHB + <i>S. aureus</i>	150 $\mu$ l
Oxidant exposure	MHB + <i>S. aureus</i> 0.067 U ml <sup>-1</sup> xanthine oxidase 100 $\mu$ M xanthine	150 $\mu$ l
Antibiotic exposure	MHB + <i>S. aureus</i> Oxacillin 12.5 $\mu$ g ml <sup>-1</sup>	150 $\mu$ l
Oxidant and antibiotic exposure	MHB + <i>S. aureus</i> 0.067 U ml <sup>-1</sup> xanthine oxidase 100 $\mu$ M xanthine Oxacillin 12.5 $\mu$ g ml <sup>-1</sup>	150 $\mu$ l

All plates were sealed with film and a single plate was placed in each oxygen box. All boxes were filled with gas as previously described and incubated at 35 °C, 150 rpm, for 24 h. Following incubation all biofilms were washed with sterile dH<sub>2</sub>O and live cell decreases determined as described in Section 4.2.11.3. Data was analysed as in Section 4.2.12.4.

### 4.3. Results

#### 4.3.1. Oxygen consumption during culture conditions

The O<sub>2</sub> concentration in the box immediately prior to incubation was recorded as 21.2%. This had fallen to 21% after 24 h of incubation.

#### 4.3.2. Oxygen concentration did not affect planktonic MICs for *P. aeruginosa* or *S. aureus*

For all four strains of bacteria tested, the oxygen concentration did not affect the MICs for their respective antibiotics (Table 4.3). As expected, the resistant strains had higher MICs than sensitive strains.

*Table 4.3.* MICs for resistant and sensitive strains of *P. aeruginosa* and *S. aureus* at different oxygen concentrations. The MIC is defined as the lowest concentration of antibiotic that achieves a 90% reduction in population growth (measured as absorbance at 600 nm); all values are  $\mu\text{g ml}^{-1}$ .

	5% O <sub>2</sub>	21% O <sub>2</sub>	40% O <sub>2</sub>	100% O <sub>2</sub>
<i>P. aeruginosa</i> 10848 (R)	40	40	40	40
<i>P. aeruginosa</i> 8626 (S)	20	20	20	20
<i>S. aureus</i> 35591 (R)	> 128	> 128	> 128	> 128
<i>S. aureus</i> 6571 (S)	0.125	0.125	0.125	0.125

#### 4.3.3. Oxygen concentration did not affect biofilm mass of *S. aureus*

Oxygen concentration, during biofilm development, did not result in a significant difference in the final mass of 24 h biofilms of *S. aureus* strains 35591 or 6571, as determined by staining with crystal violet (Fig. 4.3).

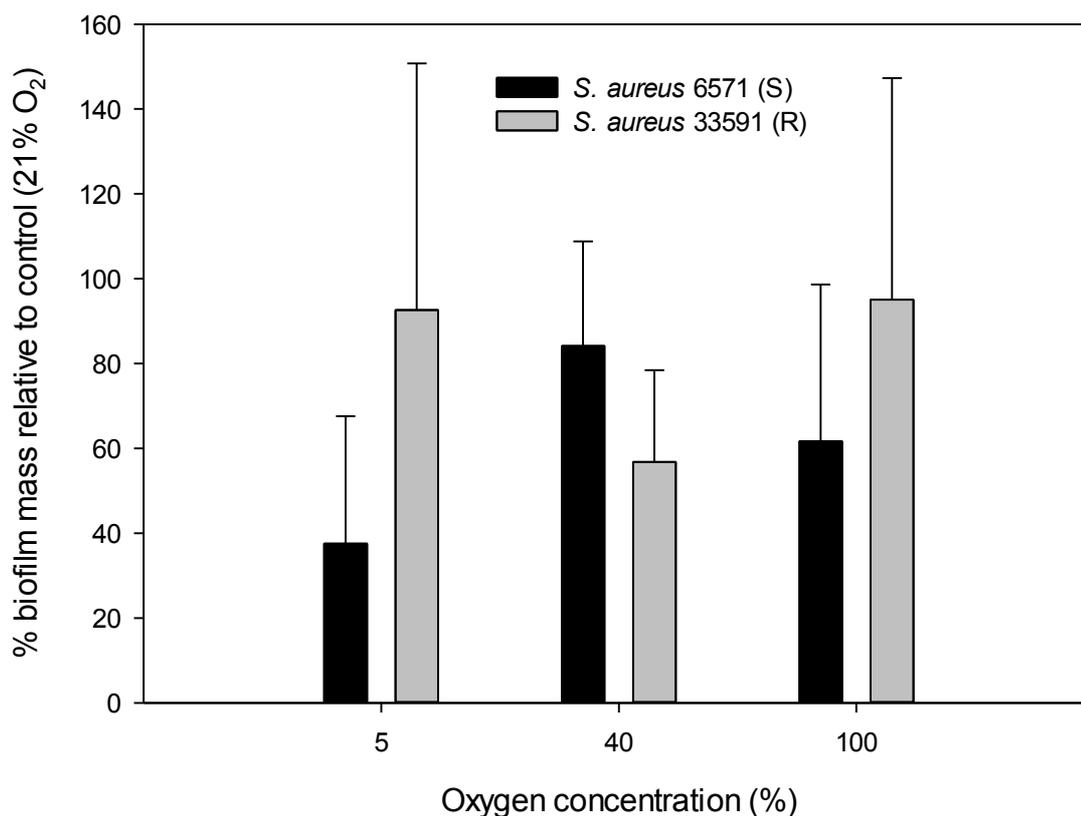
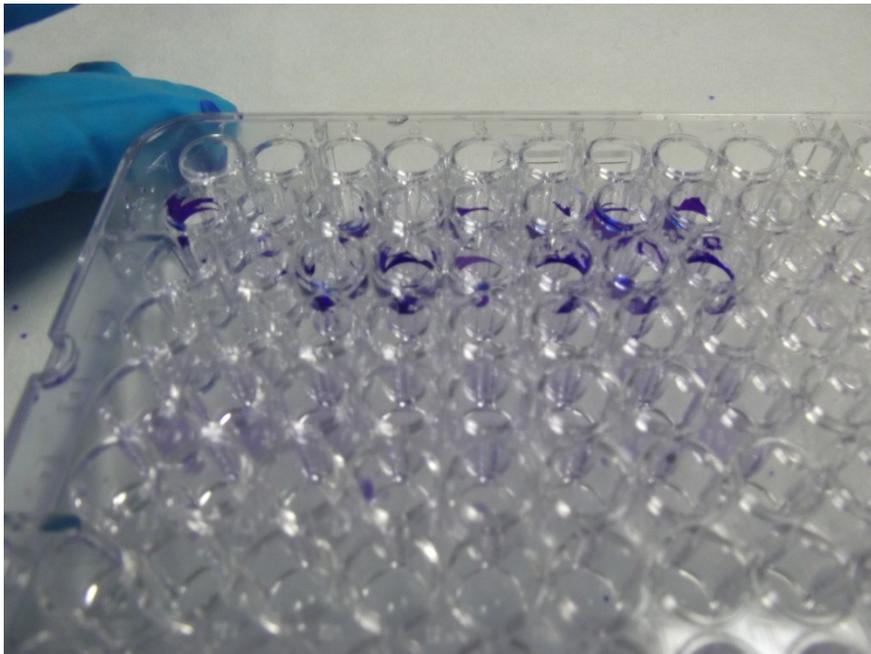


Figure 4.3. Mean relative mass of 24 h biofilms of *S. aureus* strains 6571 and 33591, to that of control biofilms grown in air. Biofilms were stained with crystal violet, the absorbance of which, solubilised in acetic acid, was measured at 570 nm. Results are expressed as means  $\pm$  SE,  $n = 3$ . Differences are not significant, as determined by one-way ANOVA.

#### 4.3.4. Biofilm mass for *P. aeruginosa* could not be quantified

Despite trying a variety of methods, acceptable biofilms of *P. aeruginosa*, strains 10848 or 8626, could not be produced. These strains produced copious flocculation on the surface of the medium rather than attaching to the surface of the microplate well, resulting in a ring of unattached bacterial material that clung to the upper part of the well (Figure 4.4). The inconsistency of the volume of material meant that accurate measurements could not be attained.



*Figure 4.4.* Photograph of *P. aeruginosa* flocculations adhering to the tops of wells following crystal violet staining. Some wells are clean, indicating that the flocculations were washed away during the washing steps.

#### *4.3.5. Oxygen concentration did not affect the activity of oxacillin on S. aureus biofilms*

In 24 h *S. aureus* 6571 biofilms exposed to oxacillin the O<sub>2</sub> concentration did not affect the activity of oxacillin against biofilm cells (Fig. 4.5). Oxacillin, at a concentration  $\times 100$  that for the planktonic MIC for this strain, resulted in a decrease in live biofilm cells of approximately 50%; there was no significant difference in cell death between oxygen concentrations.

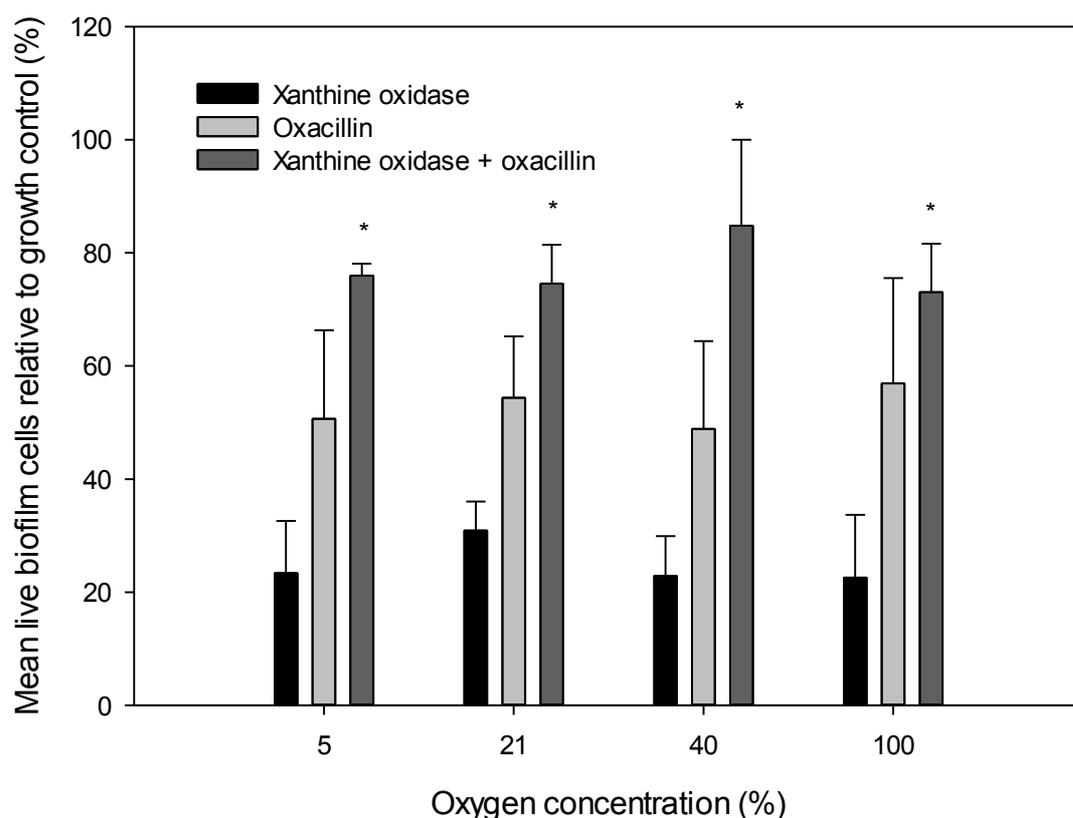


Figure 4.5. Mean live biofilm cells in 24 h *S. aureus* 6571 biofilms, relative to growth control, following exposure to xanthine oxidase derived oxidants, oxacillin and a combination of these components. Biofilms were grown in air for 24 h prior to exposure at different oxygen concentrations. Concentrations of solutions: xanthine, 100  $\mu\text{M}$ , xanthine oxidase 0.067 U  $\text{ml}^{-1}$ , oxacillin, 12.5  $\mu\text{g ml}^{-1}$ . Reduction in live biofilm cells was determined by incubation with resazurin (40  $\mu\text{M}$ ). Results are expressed as means  $\pm$  SE,  $n = 3$ . \* indicates a significant difference compared to xanthine oxidase generated oxidants,  $P < 0.05$ .

#### 4.3.6. Oxygen concentration did not affect the ability of xanthine oxidase derived oxidants to kill *S. aureus* biofilm cells

The oxygen concentration, in which 24 h *S. aureus* 6571 biofilms were exposed to xanthine oxidase derived oxidants, did not affect the reduction in live cells (Fig. 4.5). Xanthine oxidase generated oxidants resulted in a 20-30% reduction

in live biofilm cells compared to a growth control, but there was no significant difference in cells loss between oxygen concentrations.

The combination of oxacillin and xanthine oxidase generated oxidants resulted in a mean cell loss of approximately 75%, with no significant differences between oxygen concentrations (Fig.4.5). For all oxygen concentrations there was a significant difference in cell death due to xanthine oxidase alone compared to xanthine oxidase in combination with oxacillin (Fig. 4.5), but no significant difference between oxacillin alone and oxacillin combined with xanthine oxidase, as determined by one-way ANOVA with Holm-Sidak All Purpose Multiple Comparison Procedure.

#### *4.4. Discussion*

This study compared the effects of O<sub>2</sub> concentration on the antibiotic MICs between sensitive and resistant strains of *S. aureus* and *P. aeruginosa*. Few studies have examined the effect of hyperoxia on antibiotic efficiency and as far as we are aware this is the first time that a study of both sensitive and resistant strains has been reported. In air, the MICs for the resistant strains were lower than those for the sensitive strains, and this differential was maintained in all conditions. Increasing O<sub>2</sub> concentration did not affect MICs for any of the strains, indicating that O<sub>2</sub> alone is insufficient to enhance antibiotic action under these conditions. However, it is necessary to consider the properties of the antibiotics used; Muhvich *et al.* (1989) postulated that their observed reduction in MICs for trimethoprim, at 98% O<sub>2</sub>, was due to keeping critical enzymes, in a synthetic pathway, in an oxidised state. This study used streptomycin against *P. aeruginosa* strains; this is an aminoglycoside, a group of antibiotics that require oxygen-dependent transport into cells (Taber *et al.* 1987). Reduced bacterial uptake of aminoglycosides has been observed under anaerobic conditions

(Miller *et al.* 1980), so it might be reasonable to consider that increased O<sub>2</sub> concentrations could facilitate increased bacterial uptake of the drug. However, neither this study, nor that of Muhvich (1989) showed such an effect. No increase in MIC was seen at 5% O<sub>2</sub> for streptomycin, but it is possible that both strains of *P. aeruginosa* had been able to utilise alternative electron acceptors to maintain cell membrane potential. Unchanged MICs for resistant bacteria also indicated that O<sub>2</sub> conferred no benefit or hindrance on the mechanisms of resistance in these species.

This study has also examined the relationship between oxygen availability and the ability of an oxidant generating enzyme to kill cells in biofilms; as far as we are aware, this is the first time that this has been reported. In order to consider any changes in biofilm mass due to O<sub>2</sub> concentration alone and to ensure that the culture conditions resulted in sufficient biofilm formation for subsequent studies, biofilms were grown in different O<sub>2</sub> environments and their mass measured with crystal violet.

It was at this point in the study that the ability to produce good quality, reproducible biofilms for all four strains of bacteria was challenged; wide variability of biofilm mass was a persistent problem, particularly for *P. aeruginosa* strains. All stages of the protocol, which was based on those published by numerous authors (Peeters *et al.* 2008, Pettit *et al.* 2005, Sandberg *et al.* 2009, Tré-Hardy *et al.* 2008), were scrutinised and alternative materials were trialled, but with no significant improvement. During the staining phase of the protocol (following the removal of crystal violet but before the addition of acetic acid) attention was paid to the visual qualities of the biofilms; wells containing *S. aureus* had easily visualised, uniform bacterial material adhered to the whole surface of the well where culture had been in contact.

However, wells containing *P. aeruginosa* were clear of any material apart from a ring that would have been at the liquid-air interface (Fig. 4.4).

Further investigation led to the conclusion that this material was not attached to the well but was accumulating in the medium at the air interface, and was sticking to the well when liquid culture was removed. During washing steps this material was easily washed away, resulting in some wells having no visible material present at all (Fig. 4.4). The following trials were conducted: (i) incubating culture for 4 h, replenishing medium and incubating for a further 24 h, (ii) incubating in a tissue-treated 96-well plate, (iii) incubating culture for 24 h, replenishing medium and incubating for a further 24 h, (iv) incubating for 6 h, (v) incubating culture on glass microscope slides, (vi) incubation of *P. aeruginosa* PAO1n (Nottingham PAO1 strain). None of these methods produced adhered, uniform biofilms of *P. aeruginosa* on the surfaces (data not shown). Peeters *et al.* (2008) also found substantial variability in *P. aeruginosa* biofilm mass using crystal violet. It was decided to discontinue with *P. aeruginosa* strains but persist with *S. aureus* as there was adherent biofilm on the walls of the wells for this species. There was, however, still variability between separate runs for this organism (Figs. 4.3 and 4.4) despite maintaining standardised inoculation cell density and culture conditions. The large SE values mean that there are no significant differences in biofilm mass between different O<sub>2</sub> concentrations, a result at odds with that of Xu *et al.* (1998) who found that mean thickness in biofilms grown in 100% O<sub>2</sub> were approximately 33 µm thicker than those grown in air. It was expected that increased O<sub>2</sub> availability during biofilm formation might result in increased biofilm mass proportional to O<sub>2</sub> concentration, due to an increase in metabolically active cells (Xu *et al.* 1998). The biofilms measured in this study and those in that of Xu were comprised of different species, which

may have had a different response to modified O<sub>2</sub> environments. It is possible that a limitation to biofilm growth was nutrient depletion in the culture medium; had the system included a means to continually replenish medium usable biofilms might have been produced.

Because of the problems mentioned above, this study focused on investigating the effects of O<sub>2</sub> environment on the number of live cells, in pre-formed 24 h biofilms, when exposed to an antibiotic, an oxidant, and both of these together; the first time this has been reported. For all of these conditions there were reductions in live cells, compared to a growth control, but the reduction was stable at all O<sub>2</sub> concentrations (Fig. 4.5), indicating that increased O<sub>2</sub> availability did not confer an advantage to the killing/inhibition mechanism of the antibiotic or oxidant. The result with oxacillin, though not directly comparable with that for planktonic culture MICs, suggests that O<sub>2</sub> does not affect the effectiveness of this antibiotic at decreasing live cells despite the fact that as a  $\beta$ -lactam antibiotic, the lethality of oxacillin is reliant on the presence of actively growing cells. It was hypothesised that increasing the O<sub>2</sub> gradient would result in an increase in the number of metabolically active, and therefore more vulnerable, cells. This result is inconsistent with other studies that have demonstrated that O<sub>2</sub> and nutrient limitations in biofilms result in slower growing and/or stationary phase cells that show increased tolerance to antibiotics (Anderl *et al.* 2003, Walters *et al.* 2003); it is possible that nutrients, such as amino acids or carbon sources were the limiting factor. Consistent with other studies (Moskowitz *et al.* 2004, Rose & Poppens, 2009) the concentration of antibiotic needed to reduce biofilm live cells was much greater ( $\times 100$  in the present study) than that for planktonic cultures, supporting the role of biofilm as a mechanism of antibiotic resistance. However, cell density in the biofilm was likely to have been much

higher than that used for the determination of planktonic MICs, which has been shown to overestimate biofilm MICs (Cerca *et al.* 2005).

Oxidants, generated by the activity of xanthine oxidase, resulted in a loss of approximately 20% of live cells in the biofilms, which again, was stable at all O<sub>2</sub> concentrations (Fig. 4.5), a result which was initially puzzling as it was expected that an increase in the concentration of one of the substrates (O<sub>2</sub>) would increase the amount of oxidants produced with a concomitant increase in cell death. However, the  $K_m$  for O<sub>2</sub>, as a substrate for xanthine oxidase, is  $2.7 \times 10^{-5}$  M (Fridovich & Handler, 1962), which, given that in 21% O<sub>2</sub>, at 35 °C, the level of O<sub>2</sub> in the medium would have been around 800  $\mu\text{M}$ <sup>11</sup> (US. Geological Survey) it is likely that the enzyme had already reached saturation, therefore, increasing the concentration of O<sub>2</sub> would not have resulted in a higher rate of oxidant production. Xanthine oxidase has been used successfully to generate oxidants (Aitken *et al.* 1993, Kelly *et al.* 2010), at O<sub>2</sub> levels of 21% or below. Though useful in these conditions perhaps xanthine oxidase was not, in retrospect, a suitable tool for the investigation of the effects of oxidant production at O<sub>2</sub> concentrations above 21%. Glucose oxidase with a  $K_m$  of  $2 \times 10^{-4}$  M for O<sub>2</sub> would probably not be much of an improvement.

An interesting observation is that the effect of combined xanthine oxidase and oxacillin is additive, resulting in an approximately 80% decrease in live cells in *S. aureus* biofilms, irrespective of O<sub>2</sub> concentration. This could be due to each component having a different mechanism for killing or inhibiting bacteria, or that one of the components is somehow facilitating the action of the other. It would be useful for future work to determine whether the effect is seen in planktonic cultures, and to the same degree; this would help to differentiate between an

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This figure is based on dissolved oxygen in water, at sea level, at 35 °C.

intracellular effect and an effect on the biofilm matrix. A possible scenario for  $\beta$ -lactamase producing species is that an accumulation of  $\beta$ -lactamases in the matrix are damaged by oxidants (Chapter 2), increasing the effectiveness of antibiotics. The strain used in this study though a  $\beta$ -lactamase producer, indicated a different mode of action. Future work could include testing multiple species, sensitive and resistant strains, to expose in greater detail the additive behaviour of oxidants and antibiotics.

The greatest disappointment of this study was the inability to produce good quality biofilms; this could have been the result of an individual failing, or in accordance with other groups who have experienced similar problems but that are under-reported. The main challenge to future work however, is in translating this work to relate to real wound biofilms that are mixed communities of bacteria (and fungi) and exist in a much more dynamic environment than in the well of a microplate.

## Chapter 5

### The dynamics of chronic wound microflora during HBOT

#### *5.1. Introduction*

In the South West of England diabetic patients with non-healing foot ulcers may be referred for HBO therapy (HBOT) at the DDRC in Plymouth. Some patients' ulcers heal well during treatment, however, some do not and it is not fully understood why this is so. Analysis of the potential contributors to non-healing and the evidence for direct correlations are problematic due to the numerous variables involved; smoking, hypertension, compliance, and infection are just a few of the many possible confounding factors that may influence healing. The provision of appropriate controls is problematic; wounds not receiving HBOT may not be directly comparable to wounds that are undergoing HBOT as wounds referred for HBOT may be in a worse condition.

Our aim was to audit the microflora of non-healing chronic wounds during HBOT, to determine the dynamics of bacterial and fungal communities, and to investigate any correlation between healing or non-healing by the end of treatment. As discussed in Section 1.3.1., infection is thought to contribute to non-healing and as far as we are aware, this is the first time that an audit of wound microorganisms during a course of HBOT has been reported. In brief, diabetic patients with a non-healing foot ulcer were recruited from the Diabetic Foot Clinic (DFC) at Derriford Hospital in Plymouth. Initial wound swabs were taken after consent to join the study had been obtained. Wounds that failed to heal with routine antibiotic treatment were referred to DDRC for HBOT. Wound swabs and wound tissue samples were taken at regular intervals throughout the course of treatment.

Wound sample DNA was isolated and analysed, which involved polymerase chain reaction (PCR) amplification followed by denaturing gradient gel electrophoresis (DGGE). A range of bands were removed for sequencing and subsequent identification.

Yeast colonisation of chronic wounds has also been observed and may be a factor in the non-healing state (Section 1.3.3.). To determine the presence of yeasts, fungi specific primers were used to amplify DNA, clone and identify species present in the collected samples. Due to time constraints samples investigated for the presence of fungi were limited to all wounds pre-HBOT and two further samples during HBOT, for each of two wounds only.

Due to the small number of patients recruited (6) and therefore number of wounds (7), it was not feasible to perform statistical analysis on the results. Some of the swabs taken during HBOT were sent to the microbiology department at Derriford hospital for routine culture and identification; these results form part of a comparison of bacterial species identified, between routine culture procedures, and molecular analysis.

## *5.2. Materials and methods*

### *5.2.1. Chemicals*

Acrylamide/bis-acrylamide (37.5:1, 40% solution), ampicillin (sodium salt), boric acid, bromophenol blue, Na-EDTA (disodium salt dihydrate), formamide (deionised), GenElute™ PCR Clean-Up Kit, glycerol, IPTG (isopropyl  $\beta$ -D-1-thiogalactopyranoside), LB dried medium, sodium hydroxide, sucrose, TEMED (*N, N, N', N'*-tetramethylethylenediamine), Trizma Base, urea, X-Gal (5-bromo-4-chloro-indolyl- $\beta$ -D-galactopyranoside), xylene cyanol FF were obtained from Sigma Aldrich Company Ltd (Poole, Dorset, UK). Ammonium persulfate tablets (APS) and water (sterile, DNA grade) were obtained from Fisher Scientific

(Loughborough, Leicestershire, UK). Ethanol, glacial acetic acid and hydrochloric acid were obtained from BDH Biochemical (VWR, BDH Prolabo, Lutterworth, Leicestershire, UK). Agarose and Hyperladder II were obtained from Biorline Reagents Ltd (London). FTA Purification Reagent and Indicating FTA cards (FTA™ Nucleic Acid Collection, Purification and Storage) were obtained from Whatman Ltd (Maidstone, Kent, UK). SYBR® Gold Nucleic Acid Gel Stain (10,000× in DMSO) and SYBR®Safe DNA Gel Stain (10,000× in DMSO) were obtained from Invitrogen (Life Technologies, Paisley, UK). HotStarTaq Master Mix was obtained from Qiagen Ltd (Crawley, West Sussex, UK). GoTaqFlexi DNA polymerase, pGEM T-Easy Vectors and JM109 Competent Cells were obtained from Promega UK (Southampton, UK). Nutrient and tryptone soya broth (TSB) and agar dried media were obtained from Oxoid Ltd (Basingstoke, Hampshire, UK). Fresh yeast (*Saccharomyces cerevisiae*) was obtained from J. Sainsbury plc (London, UK). Bacterial DNA extracted from *Bacillus subtilis* (*B. subtilis*) and *Clostridium perfringens* (*C. perfringens*) were kindly donated by Matthew Emery (Microbiology Technician, University of Plymouth).

### 5.2.2. Primers

All primers (Table 5.1) were purchased from Eurofins (Eurofins MWG Operon, Ebersberg, Germany).

Table 5.1. Primers used in this study. \*These primers were also used with GC clamps (see text).  
GC clamp: CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG

Species	Primer pair	Sequence (5' - 3')	Target	Reference
Bacteria	9bfm	GAGTTTGATYHTGGCTCAG	1500 bp	Mühling <i>et al.</i> 2008
	1512uR	ACGGHTACCTTGTTACGACTT	16S	
Bacteria	341F*	CCTACGGGAGGCAGCAG	<i>rDNA</i> 550 bp	Ishii & Fukui, 2001
	907R*	CCGTCAATTCCTTTRAGTTT	16S	
Fungal	NS1	GTAGTCATATGCTTGTCTC	<i>rDNA</i> 1400 bp	Wu <i>et al.</i> , 2002
	NS6	GCATCACAGACCTGTTATTG	18S	
Fungal	FF1	CCTC GTTAAAAGCTCGTAGTTGAAC	<i>rDNA</i> 630 bp	Zhou <i>et al.</i> , 2000
	FR1	CTCTCAATCTGTCAATCCTTATT	18S	
			<i>rDNA</i>	

### 5.2.3. Wound study

The study received approval from the NHS Cornwall & Plymouth Research Ethics Committee (REC Ref: 08/H0203/93). The study protocol, as submitted to the ethics committee, can be found in Appendix 6.

#### 5.2.3.1. Patient recruitment

Patient recruitment took place at the DFC at Derriford Hospital (Derriford, Plymouth, UK). The principal inclusion criteria were that the patients were over 18 y and had at least one non-healing diabetic foot ulcer. In total, 8 patients were identified as being suitable candidates for HBOT, and met the inclusion criteria; however, two patients withdrew from HBO treatment before completion and were not included in the analysis. Six patients completed the HBOT regimen; one of these patients had two diabetic foot wounds resulting in a total of seven wounds analysed for the study. It is accepted practice that patients are referred for HBOT if wound healing does not respond to a specific programme of antibiotics, determined by local policy, a summary of which can be found in Appendix 7.

### 5.2.3.2. HBOT protocol

HBOT in the South West is carried out at the Diving Diseases Research Centre (DDRC) in Plymouth. The centre has a large 9-place chamber (Fig. 5.1), two smaller 3-place chambers and one monoplace chamber.



Figure 5.1. The 9-place HBO chamber at DDRC, Plymouth.

Though HBOT regimens vary, depending on the clinical circumstances, they always involve the delivery of 100% oxygen, via a hood or face mask, for a period of time followed by 'air breaks' when patients remove their masks and breathe air. The regimen for the treatment of the patients in this study consisted of breathing 100% oxygen at 2.4 ATA for 80 min per day (plus air breaks), Monday to Friday, for a total of 40 treatments.

All patients were assessed at DDRC before HBOT commenced; this is normal practice and is to determine that patients are suitable candidates for treatment (i.e. they do not have any contraindications for HBOT).

#### *5.2.3.3. Transcutaneous oxygen monitoring*

Transcutaneous oxygen monitoring (TCOM) is completed as part of the initial assessment at DDRC to determine whether patients are likely to benefit from HBOT (Section 1.6.1). TCOM is a non-invasive procedure which quantifies tissue oxygenation at multiple sites on the body with the use of Clark electrodes. Probes are placed near the wound site, at the equivalent site on the opposite limb, and over the second intercostal cavity on the chest. Measurements are of transcutaneous partial pressure of oxygen ( $tcpO_2$ ) and the units are mmHg.

#### *5.2.3.4. Wound sampling*

Wounds were sampled prior to the first HBOT session, and then at regular intervals throughout HBOT. Figure 5.2. shows a model programme for taking wound samples though this was not achieved for the majority of patients. This was due to unexpected hospital admissions or illness that interrupted HBOT. It was not possible to obtain samples from wounds not undergoing HBOT and the majority of pre-HBOT samples were those taken during the HBOT assessment. Wound exudate was obtained by swabbing the deepest, central area of the wound with a sterile cotton swab, according to the Levine technique (Levine *et al.* 1976). The swab tip was broken off and inserted into a cryotube (sterile, polypropylene, 1.8 ml, Fisher Scientific UK, Ltd, Loughborough, Leicester, UK) containing 1 ml of lysing buffer (Table 5.2). Wound tissue was collected only if the wound required debriding; a small sample of non-viable tissue was removed with a scalpel and transferred to a cryotube containing 1 ml lysing buffer (Table 5.2). All samples were stored at  $-80\text{ }^{\circ}\text{C}$ . In the DFC all samples were taken by Graham Bruce (podiatrist) and in DDRC all samples were taken by nurses, supervised by Alexandra Bishop (DDRC Research Nurse). At three time points

(Fig. 5.2), two samples were obtained, one was stored as described for molecular analysis and the other was sent to the microbiology laboratory at Derriford Hospital for routine culture and identification.

*Table 5.2.* Composition of lysing buffer for wound samples. \* The pH was adjusted to 8.3 with HCl prior to the addition of EDTA and sucrose. The buffer was autoclaved at 121 °C for 15 min. Modified from Maniatis *et al.* (1982).

<b>Reagent</b>	<b>Quantity (500 ml)</b>
Tris*	3.03 g
EDTA	8.09 g
sucrose	128.36 g
H <sub>2</sub> O	to 500 ml

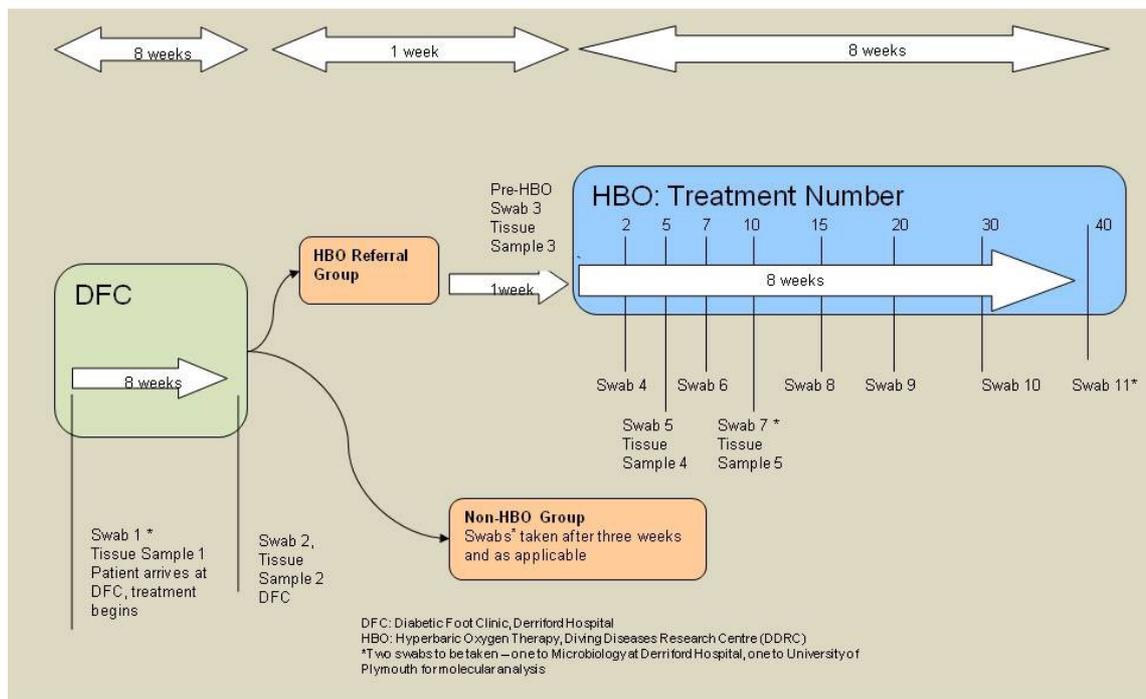


Figure 5.2. Model sampling schedule for patients recruited to the wound study.

During the first few weeks of the study Derriford Microbiology Laboratory provided us with cultures of strains isolated from some of the wound samples; these included: *Enterococcus faecalis* (*E. faecalis*), *S. aureus*, *Corynebacterium diphtheriae* (*C. diphtheriae*), an *Enterococcus* species and a non-lactose fermenting (NLF) *E. coli*. These cultures were maintained on agar slopes, stored at 4 °C. Liquid cultures of these organisms were prepared by inoculating nutrient broth with a single colony and incubating at 35 °C overnight. Some of these cultures were used in the study to produce a molecular ladder to aid identification of bands and to determine DGGE sensitivity.

#### 5.2.4. DNA isolation

##### 5.2.4.1. Application of samples and bacterial cultures to FTA cards

Whatman, FTA™ Nucleic Acid Collection cards were used for the isolation and storage of wound sample DNA. Samples were thawed and the contents of each cryotube were vortexed vigorously for 5 s; 125 µl of liquid was applied to the centre of a single circle on a FTA card and left to dry at room temperature for approximately 1 h. Following overnight incubation of wound cultures from Derriford Microbiology Laboratory, 125 µl of each culture were transferred to separate FTA cards. Similar cultures of *P. aeruginosa* 10848 (NCIMB Ltd, Aberdeen, UK) and *S. aureus* 6571 (kindly donated by University of Plymouth), grown in TSB broth, were also applied to FTA cards for use as positive controls. The cards were labelled and stored in an air-tight container at room temperature. Gloves were worn at all times when handling the cards.

#### *5.2.4.2. Preparation of FTA card punches for analysis*

For each sample, the card was placed on to a small self-healing cutting mat that had been cleaned with detergent followed by 70% ethanol and allowed to dry. Using a Harris uni-core, 2 mm punch, a single punch was removed from within the circle (indicating cards were used so that the area saturated with sample could readily be identified) on the card and transferred to a sterile PCR tube (0.2 ml, domed cap, Elkay Laboratory Products UK Ltd, Basingstoke, Hampshire, UK). Following the addition of 200 µl of FTA Purification Reagent to each tube they were incubated at room temperature for 5 min, with manual shaking. The FTA Purification Reagent was then removed and discarded. This step was repeated to give a total of two washes. The washing steps were repeated a further two times with TE<sup>-1</sup> buffer (a TE buffer with a lower concentration of EDTA to avoid the chelation of Mg<sup>2+</sup> ions during PCR, Table 5.3) but without manual shaking. Immediately following the washing steps the card punches were used for PCRs.



Table 5.3. Composition of TE<sup>-1</sup> buffer. \*Tris solution adjusted to pH 8.8 with HCl prior to addition of EDTA. Final pH adjusted to 8.0.

Reagent	Quantity (500 ml)	Final concentration
TrisHCl* 1M stock solution	5 ml	10 mM
EDTA 0.5 M stock solution pH	100 µl	0.1 mM
8.0		
H <sub>2</sub> O	Volume to make total of 500 ml	

#### 5.2.5. Preparation of bacterial PCR products

The controls used for each PCR are listed in Table 5.4. All samples from a single wound were processed in one session and also included in each session were punches from wound isolates *S. aureus* and *E. faecalis*, and cultures of *P. aeruginosa* and *C. perfringens*. These were used to produce a molecular ladder to aid in identification of bands and to determine DGGE sensitivity and optimal DGGE conditions.

Table 5.4. Controls used for each PCR.

Control	Rationale
Negative control – no punch added	To confirm absence of contamination
Negative control – washed card punch that does not carry a sample	To determine that the punch does not cause a positive result
Positive control – bacterial DNA standard ( <i>B. subtilis</i> )	To confirm that PCR conditions will amplify bacterial DNA
Positive control – washed card punch carrying bacterial culture	To confirm that the punch does not inhibit PCR

#### 5.2.5.1. Nested PCR using 16S rDNA primers

Nested PCR was used to amplify bacterial DNA from wound samples and cultures, as a single stage of PCR cycling repeatedly failed to produce visible bands on agarose gels. For the first round of PCR the universal bacterial primer pair 9bfm and 1512 uR were used (Table 5.1). These primers target bacterial 16S rDNA to produce a PCR product approximately 1500 bp which included hypervariable regions V1 to V9. PCR tubes were prepared with punches from

samples and controls as described in Section 5.2.4.2, and reagents were added, as listed in Table 5.5.

*Table 5.5.* Composition of PCR. Total volume for each reaction was 50  $\mu$ l. \*Final concentration in 50  $\mu$ l reaction is 2.5 units DNA polymerase, 200  $\mu$ M of each dNTP and 1.5 mM MgCl<sub>2</sub>.

<b>Component</b>	<b>Volume</b>
<b>HotStarTaqMaster Mix*</b>	25 $\mu$ l
<b>9bfm primer 10 pmol <math>\mu</math>l<sup>-1</sup></b>	10 $\mu$ l
<b>1512 uR 10 pmol <math>\mu</math>l<sup>-1</sup></b>	10 $\mu$ l
<b>RNase free water</b>	5 $\mu$ l for tubes with punches, 4 $\mu$ l for tubes with bacterial DNA
	standard
<b>Template DNA</b>	One punch <b>or</b> 1 $\mu$ l of bacterial DNA standard (10 ng $\mu$ l <sup>-1</sup> )

Throughout the procedure aseptic conditions were maintained and a PCR dedicated laminar flow cabinet was used; all pipette tips contained filters (Elkay Laboratory Products UK Ltd, Basingstoke, Hampshire, UK), and all equipment and solutions, except those containing genetic material (DNA template, primers etc), were UV irradiated for 20 min prior to use. Immediately following addition of reagents the tubes were placed in the thermal cycler (Techne, Bibby Scientific Ltd, Staffordshire, UK), using the cycling protocol in Table 5.6. At the end of cycling, PCR products were stored at 4 °C. Before continuing with stage two of nested PCR, aliquots of the products from stage one were subjected to agarose gel electrophoresis (Section 5.2.5.3) to determine that amplicons were present and of the correct size.

Table 5.6. PCR cycling protocol for primer pair 9bfm/1512uR. 30 cycles were completed.

Step	Temperature	Time
Initial activation	95 °C	15 min
Denaturation	94 °C	1 min
Annealing	52 °C	1 min
Extension	72 °C	1 min
Final extension	72 °C	10 min

The second stage of PCR utilised the primer pair 341F and 907R (Table 5.1), which targeted bacterial 16S rDNA to produce an amplicon of 550 bp, containing hypervariable regions V3 to V5. A gradient PCR was performed to determine: (i) the optimal annealing temperature and (ii) the optimal position of a GC clamp, i.e. on the forward primer, or the reverse primer. Twelve punches were prepared, as previously described, for each of the following; a single wound sample, a positive control (*P. aeruginosa*) and a negative control. PCR reagents were added (Table 5.7) resulting in six copies for each primer pair, representing tubes for six different annealing temperatures, 52, 53, 54, 55, 56 and 57 °C. The basic PCR protocol (Table 5.6) was used, except for the annealing temperature, where each block in the cycler annealed for 1 min at the temperatures stated above.

Table 5.7. Composition of gradient PCR. Total volume for each reaction was 50  $\mu$ l. Wound (W), positive control (+C), negative control (-C), number of punches ( $\times 6$ ).

Component	Volume	W $\times 6$	W $\times 6$	+C $\times 6$	+C $\times 6$	-C $\times 6$	-C $\times 6$
HotStarTaqMaster Mix	25 $\mu$ l	^^	^^	^^	^^	^^	^^
341F primer 10 $\mu$ M	10 $\mu$ l	^^	x	^^	x	^^	x
341F-GC primer 10 $\mu$ M	10 $\mu$ l	x	^^	x	^^	x	^^
907R primer 10 $\mu$ M	10 $\mu$ l	x	^^	x	^^	x	^^
907R-GC primer 10 $\mu$ M	10 $\mu$ l	^^	x	^^	x	^^	x
RNase free water	5 $\mu$ l	^^	^^	^^	^^	^^	^^
Template DNA	1 punch	^^	^^	^^	^^	^^	^^

Using the data from the gradient PCR, the second stage PCR conditions were determined (Table 5.8). PCR products from stage one were diluted 10 fold with sterile DNA grade water. The cycling protocol was the same as that in Table 5.6 except for the annealing temperature which was set at 54  $^{\circ}$ C.

Table 5.8. Composition of stage 2 PCR. Total volume for each reaction was 50  $\mu$ l.

Component	Volume
HotStarTaqMaster Mix	25 $\mu$ l
341F primer 10 $\mu$ M	10 $\mu$ l
907R-GC primer 10 $\mu$ M	10 $\mu$ l
PCR products from stage 1	5 $\mu$ l

#### 5.2.5.2. Confirmation of PCR products with agarose gel electrophoresis

PCR products from all stages were visualised using agarose gel electrophoresis. A 1.3% agarose gel was prepared by adding 0.8 g of agarose to a 250 ml conical flask containing 60 ml of 1  $\times$ TAE buffer (Table 5.9.); the flask was swirled gently to mix the agarose and buffer together. The flask was plugged with a roll of paper towel and placed in a microwave oven. The mixture was heated to boiling point and removed from the oven for gentle mixing. This step was repeated until the agarose had completely dissolved in the buffer.

Table 5.9. Composition of 50  $\times$  stock solution of TAE buffer, pH 8.3.

Reagent	Quantity (1 litre 50 $\times$ stock solution)
Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA 0.5 M, pH 8.0	100 ml
H <sub>2</sub> O	Volume to make up to 1 l

Whilst the gel was cooling an electrophoresis tray was prepared; both ends of the tray were sealed with tape and the tray placed on a level surface. Ten microlitres of SybrSafe (10,000 ×) were added to the agarose solution just prior to pouring and swirled gently to mix well. The agarose was carefully poured into the tray and a comb was located securely at one end. The agarose solution was left to set at room temperature. When the agarose gel was fully set the tape was removed and the tray placed in an electrophoresis tank, sufficient 1 × TAE buffer was added to cover the gel. The comb was carefully removed from the gel. Five microlitres of Hyperladder II were loaded into the first slot of the gel (50 to 2000 bp). Samples were prepared for electrophoresis by mixing 8 µl of PCR product with 2 µl of 6 × gel-loading buffer (Table 5.10) and 8 µl of sample were loaded into a single slot. When all samples were loaded electrophoresis was performed at 100 V until the loading dye had migrated to the opposite end of the tray. On completion of electrophoresis the gel was carefully transferred to an imager (Gel Doc XR, Bio Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) for analysis.

Table 5.10. Composition of 6 × gel loading buffer.

Component	Quantity (10ml 6 x solution)
Bromophenol blue	0.025 g
Xylene cyanol FF	0.025 g
Glycerol (30 % in H <sub>2</sub> O)	3 ml
H <sub>2</sub> O	7 ml

Agarose gels were observed to confirm that positive control lanes demonstrated clear bands, negative control lanes showed no visible bands, wound samples produced clear bands and all bands corresponded to the ladder at the correct length.

#### 5.2.5.3. Production of molecular 'ladder' for DGGE

DNA was extracted, using FTA cards, from overnight cultures of *P. aeruginosa*, *S. aureus*, and *E. faecalis*. These, along with extracted DNA from *C. perfringens* underwent PCR with the primer pair 341F/907R-GC. Various dilutions of the ladder were separated on DGGE gels to determine the optimal concentration and gradient of denaturant to give clear migration to four distinct bands on the gel. A 10 × dilution was used for all subsequent gels containing wound sample PCR products. Equal volumes (100 µl) of diluted PCR products from each culture were combined and stored at -20 °C. This was sufficient to complete all DGGE gels to ensure reproducibility of the ladder.

#### 3.2.6. DGGE analysis of bacterial PCR products

Following nested PCR, the PCR products of all wound samples were analysed with DGGE. DGGE is an electrophoretic method that separates PCR products according to their sequence and was first described by Fischer and Lerman (1980). Double stranded PCR products migrate through a linear gradient of increasing denaturant concentration which partially 'melts' the DNA fragment, slowing further migration. The concentration of denaturant required to melt the fragment is dependent on sequence so that gene fragments halt at positions in

the gel corresponding to their melting temperature. A GC-clamp, attached to one of the primers, results in a GC rich sequence as part of the amplicon; this modifies the melting behaviour of the amplicon and prevents complete denaturation and migration out of the gel as single strands (Myers *et al.* 1985). This study amplified fragments containing the hypervariable regions V3 to V5 of 16S rDNA and used DGGE to separate those fragments according to sequence composition.

#### *5.2.6.1. Preparation of DGGE solutions*

The solutions prepared for DGGE analysis were TAE buffer (Table 5.9), 0% denaturant (Table 5.11), 80% denaturant (Table 5.11), APS (10% w/v) and 10 × gel-loading buffer (Table 5.12). Acrylamide solutions were wrapped in foil, to protect from light, and stored at 4 °C. Any remaining solution was discarded after 4 weeks. APS was made fresh, immediately before preparing a DGGE gel, by dissolving one tablet (100 mg) in 1 ml dH<sub>2</sub>O. Gel-loading buffers were stored at 4 °C in 500 µl aliquots.

Table 5.11. Composition of 6% polyacrylamide solutions.

Reagent	Quantity (100ml)
<b>0% denaturant</b>	
40% acrylamide 37.5:1 acrylamide:bisacrylamide	15 ml
50 × TAE buffer	2 ml
dH <sub>2</sub> O	83 ml
<b>80% denaturant</b>	
40% acrylamide 37.5:1 acrylamide:bisacrylamide	15 ml
50 × TAE buffer	2ml
Urea	33.6 g
Deionised formamide	32 ml
dH <sub>2</sub> O	To make up to 100 ml

Table 5.12. Composition of 10 × gel loading buffer.

Component	Quantity (10ml 6 × solution)
Bromophenol blue	0.025 g
Xylene cyanol FF	0.025 g
Glycerol (50% in H <sub>2</sub> O)	3 ml
H <sub>2</sub> O	7 ml

#### 5.2.6.2. Preparation of DGGE equipment

The equipment used for DGGE analysis was the Bio-Rad DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK). Parallel denaturing gradient gels were produced where the denaturing gradient was parallel to the electric field and the denaturing conditions were a combination of a linear denaturant gradient and a uniform temperature of 60 °C. The system was prepared and assembled according to manufacturer's instructions (Bio-Rad Laboratories, 1996).

### 5.2.6.3. Preparation of acrylamide gels

Six percent polyacrylamide gels were used throughout the study being the concentration most suited for larger (300-1000 bp) PCR products (Green *et al*, 2009). Gradient optimisation resulted in the final gradient used for all wound sample analysis being 35%-65%. High and low gel solutions were prepared by combining denaturant solutions in two sterile 50 ml centrifuge tubes (Table 3.13); 120  $\mu$ l of APS and 18  $\mu$ l of TEMED were added to each tube and gently mixed.

Table 5.13. Composition of high and low denaturant polyacrylamide solutions.

Reagent	35% (low)	65% (high)
80% denaturant	6.5 ml	12.1 ml
0% denaturant	8.5 ml	2.9 ml

The contents of each tube were transferred to two syringes and secured in the appropriate position on the gradient former (part of the DCode system) which combines the denaturant solutions, so that a gradient forms from low at the top of the plate to high at the bottom. The top of the glass plates were covered in cling film to seal the plates and the gel was left to polymerise, at room temperature, for at least 1 h.

After polymerisation the top of the gel was rinsed with 1  $\times$  TAE buffer. A stacking gel was prepared by adding 40  $\mu$ l of APS, 6  $\mu$ l of TEMED and 10  $\mu$ l of 10  $\times$  gel loading buffer to 5 ml of 0% denaturant polyacrylamide and this was poured on top of the gel; a comb was carefully placed in the stacking gel, being careful to avoid bubbles at the base of the teeth. The plate was sealed with cling film and left for a further 1 h to polymerise. The complete frame, consisting of glass plates, polymerised gel and holding clamps was locked in place on the DCode core apparatus. The core holds two frames so that two gels can be run concurrently and for this study both frames were used, with each gel containing

the collective samples for an individual wound. The core was placed into the electrophoresis tank and the comb carefully removed. Heated buffer from the tank was used to flush each well to remove unpolymerised acrylamide.

#### *5.2.6.4. Sample loading and electrophoresis conditions*

All wound sample PCR products were diluted  $\times 10$  and 16  $\mu\text{l}$  of each dilution were added to 4  $\mu\text{l}$  of  $\times 10$  gel-loading buffer and mixed well; 16  $\mu\text{l}$  of molecular ladder was added to 4  $\mu\text{l}$  of  $\times 10$  gel-loading buffer and mixed well. Twenty microlitres of the ladder/buffer mix were loaded into a well 4 to 5 wells from the left side of the gel, using a gel pipette tip (flat, extra length, Elkay Laboratory Products). Starting from the next well on the right, 20  $\mu\text{l}$  aliquots of all wound sample PCR products were loaded, each well containing a single sample. The samples were loaded in temporal order from the beginning to the end of HBO treatment. The lid of the DCode system was secured over the core and electrophoresis was performed at 100 V for 5-10 min to migrate the PCR products into the stacking gels. The gels were then electrophoresed at 60 V and 60 °C for 16 h.

#### *5.2.6.5. Gel staining*

Gels were checked to ensure that the loading dye had run through the gel suggesting that electrophoresis had been completed. The frames were removed from the tank and the glass and gel sandwich was placed in a tray containing sufficient TAE buffer to just cover the gel; the top plate of glass was lifted free of the gel. SybrGold gel stain was added (10  $\mu\text{l}$  per 100 ml) and the tray was agitated gently in the dark, at room temperature, for 30 min. Gels stained with SybrGold did not require de-staining.

#### *5.2.6.6. Gel imaging*

The gel was carefully transferred from the glass plate to the imaging platform of the imager. An image of the gel was obtained using UV transillumination (as rapidly as possible to reduce UV exposure). The image was optimised with Quantity One<sup>®</sup> software (Bio-Rad) to identify bands required for sequencing.

#### *5.2.7. Identification of bacterial species within DGGE resolved bands*

Representative bacterial species were identified by removing bands from DGGE gels, re-amplifying the contents and using the BLAST database (NCBI) to identify the sequences.

##### *5.2.7.1. Band removal from acrylamide gels*

Using a printed image of the gel with marked bands of interest, a scalpel was used to excise the band. An acrylic UV protector screen was used whilst visualising the bands during removal and the procedure was carried out as quickly as possible. Each individual band was placed in 50  $\mu$ l ultra-pure water in a 0.7 ml microcentrifuge tube and all bands were stored at 4 °C, overnight.

##### *5.2.7.2. Preparation of band contents for sequencing*

Each tube was centrifuged briefly and 5  $\mu$ l of the contents were removed and re-amplified using the primer pair 341F/907R (no GC clamp), under the conditions previously described. The PCR products were screened on an agarose gel (Section 5.2.5.2) prior to being cleaned with GenElute<sup>™</sup> PCR Clean-Up Kit. The DNA content of the cleaned PCR products was measured with a nano-drop spectrophotometer (Thermo Scientific, Fisher Scientific UK) and diluted with ultra-pure water to 50 ng  $\mu$ l<sup>-1</sup> and 20  $\mu$ l of each sample was placed in a 1.5 ml microcentrifuge tube. All tubes were sent for single read sequencing (GATC Biotech Ltd, The London BioScience Innovation Centre, London, UK.).

### *5.2.7.3. Sequence identification*

All sequence data were analysed by searching BLAST (NCBI) microbial genomes and the searches were optimised for highly similar sequences. Identification was based on a minimum of 90% identity match. Accession numbers and % identity can be found in Appendix 8.

### *5.2.8. Qualitative analysis of DGGE gels*

#### *5.2.8.1. DGGE analysis using GelCompar software*

All DGGE gels were analysed with GelCompar II software (BioSystematica, Llandysul, Ceredigion, Wales, UK) to create normalised databases of gel band patterns. The molecular ladders were used as reference markers for band alignment and normalisation. Manual adjustment of gels was carried out to ensure that only major bands were included in the analysis.

#### *5.2.8.2. Analysis of wound flora community dynamics with Primer-E*

Presence/absence and abundance data from Gel Compar II was input into Primer-E software (Primer-E Ltd, Ivybridge, Devon, UK). For each gel, a Bray Curtis similarity matrix was constructed and plotted as an MDS plot. A RELATE<sup>12</sup> test for seriality using Primer E multivariate statistic software was carried out for each set of data.

### *5.2.9. Identification of fungal communities in chronic wounds*

In addition to identification of bacteria, the presence of fungal species in all wound samples taken prior to HBO treatment, and in a mid and an end of treatment sample for wounds 1b and 6 were investigated. Budgetary and time constraints meant that not all samples could be analysed for fungal communities.

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This compares the resemblance matrix to a model matrix of a linear temporal sequence and tests for similarities between the two. R is Rho.

### 5.2.9.1. Nested PCR using 18S rDNA fungi specific primers

Punches taken from wound sample FTA cards were used to amplify fungal DNA with a nested PCR protocol. Nested PCR was chosen because, again, a single stage of PCR gave a low yield of amplified material from wound samples. FTA card punches were prepared for PCR as previously described (Section 5.2.4.2.) and the first stage of PCR utilised the primer pair NS1/NS6 (Table 5.1) to obtain an amplicon of approximately 1400 bp. A gradient PCR cycle with a range of temperatures from 50.9 to 59.9 °C was carried out to determine the optimal annealing temperature for these primers. Second stage PCR utilised the primer pair FF1/FR1 (Table 5.1), which produced an amplicon of 630 bp. Concentrations (Table 5.14) and cycling programmes (Table 5.15) were consistent for all further fungal PCR protocols unless otherwise indicated in the text.

Table 5.14. Composition of PCR for either primer pairs. Total reaction volume = 50 µl. \*5 × buffer supplied with polymerase.

<b>Component</b>	<b>Volume</b>
5 × buffer*	10 µl
dNTPs 2 mM	5 µl
MgCl <sub>2</sub> 25 mM	3 µl
NS1 10 µM or FF1 10 µM	10 µl
NS6 10 µM or FR1 10 µM	10 µl
GoTaqFlexi DNA polymerase 5 u µl <sup>-1</sup>	0.25 µl
Template DNA	One punch (or 1 µl PCR product)
RNase free water	11.75 µl

Table 5.15. PCR cycling protocol for either primer pairs. All were run for a total of 35 cycles.

Step	Temperature	Time
Initial activation	95 °C	2 min
Denaturation	95 °C	1 min
Annealing	56 °C (NS1/NS6) 52 °C (FF1/FR1)	1 min
Extension	72 °C	1 min
Final extension	72 °C	5 min

Controls included: negative control; positive control with DNA extracted from *Saccharomyces cerevisiae*; and bacterial control using DNA extracted from *P. aeruginosa*.

#### 5.2.9.2. Confirmation of PCR products with agarose gel electrophoresis

All PCR products were screened using agarose gel electrophoresis, as previously described (Section 5.2.5.2.) In particular the absence of bands for the negative control and bacterial DNA was monitored; it was crucial that the primers did not amplify bacterial DNA.

#### 5.2.10. Cloning of fungal PCR products

##### 5.2.10.1. Preparation of fungal PCR products for cloning

PCR products from wound samples were cleaned with GenElute™ PCR Clean-Up Kit and DNA was measured with a nano-drop spectrophotometer.

##### 5.2.10.2. Fungal cloning protocol

The protocol described below is based on that produced by Promega (Promega, 2010) for use with the pGEM®-T Easy Vector System which indicates the presence of an insert in the *lacZ* gene of pGEM vector by the production of white colonies instead of blue on indicating agar plates. The product of the *lacZ* gene is  $\beta$ -galactosidase which cleaves X-gal to form the bright blue pigment 5,5'-dibromo-4,4'-dichloro-indigo; when X-gal is not hydrolysed, this indicates the presence of an insert in the *lacZ* gene. LB broth was prepared by dissolving 20 g dried medium into 1 l of water; the pH was adjusted to 7.0 with NaOH. The medium was autoclaved before use. LB plates with ampicillin, IPTG and X-Gal

were prepared as follows; 15 g of agar was dissolved in 1 l of LB medium and autoclaved, ampicillin was added to a final concentration of 100 µg ml<sup>-1</sup> when the medium had cooled to 50 °C, IPTG was added to a final concentration of 0.5 mM and X-Gal was added to a final concentration of 80 µg ml<sup>-1</sup>, 30-35 ml was poured into 85 mm Petri dishes and allowed to set. IPTG stock solution (0.1 M in water) was filter sterilised and stored at 4 °C. X-Gal solution (50 mg ml<sup>-1</sup> in *N,N'*-dimethyl-formamide) was covered in foil and stored at -20 °C. Poured agar plates were stored at 4 °C. Two plates were prepared for each ligation reaction.

### Ligation

All reagents and reaction tubes (0.5 ml) were kept on ice. The pGEM<sup>®</sup>-T Easy Vector and Control Insert DNA were centrifuged briefly prior to use. The ligation reactions were set up as detailed in Table 5.16. The Rapid Ligation Buffer was vortexed vigorously before each use.

*Table 5.16.* Components of ligation reactions. \*The calculation for determining the amount of PCR product to use is explained in the text. This table has been reproduced from the Promega protocol (Promega, 2010).

Reaction Component	Standard Reaction	Positive Control	Background Control
<b>2 × rapid Ligation Buffer, T4 DNA Ligase</b>	5 µl	5 µl	5 µl
<b>pGEM<sup>®</sup>-T Easy Vector (50 ng)</b>	1 µl	1 µl	1 µl
<b>PCR product</b>	× µl*	-	-
<b>Control Insert DNA</b>	-	2 µl	-
<b>T4 DNA Ligase (3 Weiss u µl<sup>-1</sup>)</b>	1 µl	1 µl	1 µl
<b>Nuclease free water to a final volume of</b>	10 µl	10 µl	10 µl

The equation to determine the volume of PCR product to add to the ligation reaction was:

$$\frac{50 \text{ ng vector} \times 0.63 \text{ kb PCR product}}{3.0 \text{ kb vector}} \times \frac{3}{1} = 31.5 \text{ ng PCR product}$$

All the reactions were mixed by pipetting and incubated for 1 h at room temperature.

### Transformation

All LB/ampicillin/IPTG/X-Gal plates were equilibrated to room temperature. All ligation reaction tubes were centrifuged briefly and 2 µl from each reaction was transferred to sterile 1.5 ml microfuge tubes kept on ice. Frozen JM109 High Efficiency Competent Cells ( $>10^8$  cfu  $\mu\text{g}^{-1}$ ) were thawed slowly on ice and then mixed by gently flicking the tube. Using very gentle pipetting 50 µl of cells were added to each tube, gently mixed again and then kept on ice for 20 min. The cells were heat shocked by immersing the tubes in a water bath at exactly 42 °C for 45 s. The tubes were then returned to ice for 2 min and 950 µl room temperature LB medium were added to each tube. All tubes were incubated at 37 °C, with shaking at 150 rpm, for 1.5 h. Five LB/ampicillin/IPTG/X-Gal plates were used for each reaction with 200 µl being spread onto each plate. One plate was used for each of the controls. All plates were incubated at 37 °C overnight. The following morning plates were checked for the presence of blue or white colonies; the positive control plate contained predominantly white colonies, which indicated that ligation had successfully occurred. The background control plate contained no colonies, and the wound sample reaction plates contained predominantly white colonies, which indicated that transformation and ligation of PCR products into the vector had been successful.

### 5.2.10.3. PCR amplification of cloned fungal DNA

PCR components were prepared and 25  $\mu\text{l}$  were added to 0.2 ml PCR tubes, with 11 tubes being prepared for each wound sample. Table 5.17 shows the PCR reaction composition for each tube.

Table 5.17. Composition of PCR for post-cloning colonies.

Component	Volume
5 × buffer*	5 $\mu\text{l}$
dNTPs 2 mM	2.5 $\mu\text{l}$
MgCl <sub>2</sub> 25 mM	1.5 $\mu\text{l}$
M13F 10 $\mu\text{M}$	0.5 $\mu\text{l}$
M13R 10 $\mu\text{M}$	0.5 $\mu\text{l}$
GoTaqFlexi DNA polymerase 5 u $\mu\text{l}^{-1}$	0.2 $\mu\text{l}$
RNase free water	14.8 $\mu\text{l}$

A sterile pipette tip was used to gently pick up no more than half of a single white colony from one of the plates. The tip was gently agitated in a single PCR tube containing reaction mix. This was repeated, picking 10 white colonies and 1 blue colony, for each wound sample. PCR was performed on each sample (Table 5.18).

Table 5.18. PCR cycling protocol for post-cloning colonies (30 cycles).

Step	Temperature	Time
Initial activation	94 °C	4 min
Denaturation	94 °C	1 min
Annealing	58 °C	1 min
Extension	72 °C	1 min
Final extension	72 °C	5 min

All PCR products were checked on agarose gels, as previously described, cleaned with GenElute™ PCR Clean-Up Kit and DNA was quantified using a nano-drop spectrophotometer. PCR products were diluted to 1 ng  $\mu\text{l}^{-1}$  and 30  $\mu\text{l}$  were added to PCR tubes.

### 5.2.10.4. Analysis of sequence data

Sequencing results were inserted into MEGA software (Tamura *et al.* 2011); sequences were aligned following removal of the plasmid sequence, and using

FF1 and FFR primers as motif identifiers. All sequence data were analysed by searching BLAST (NCBI) fungi genomes and searches were optimised for highly similar sequences. Identification was based on a minimum of 85% identity. Alignment and comparison of sequences from bands at different migration points were performed using the alignment tool in BLAST (bl2seq). Accession numbers and % identity can be found in Appendix 9.

#### *5.2.11. Incorporation of relevant patient data*

A database of information was collated for each wound. This included pertinent clinical information (Table 5.19), transcutaneous oximetry (TCOM) data (Table 5.20), antibiotic usage (Table 5.21) and microbiology culture reports from Derriford microbiology laboratory (Table 5.22). This information was obtained from clinical notes maintained by staff at DDRRC.

Diabetic patients in the UK have Hb<sub>A1C</sub> measured every 3-6 months; this is a measure of glycosylated haemoglobin and correlates with mean plasma glucose. The target for diabetic patients is 6.5-7.5% (Diabetes. UK). Four patients were prescribed antibiotics during HBOT (Table 5.21.). No patients were prescribed anti-fungal drugs during the study period. Each patient had TCOM performed during their HBO assessment (an example of a recording can be seen in Figure 5.3).

*Figure 5.3.* TCOM recording for patient 1. The initial rise during the first 20 min (breathing air) is due to increasing pressure as the chamber is compressed to 2.4 ATA. The subsequent rise is due to the patient breathing 100% O<sub>2</sub> at 2.4 ATA. The dip mid-cycle is due to an air break.

For clarity three values (expressed as mm Hg) are given (Table 5.20); these represent the value in the skin adjacent to the wound at sea level on air, at 2.4 ATA on air and at 2.4 ATA on 100% O<sub>2</sub>. Patient 5 also had recordings performed

69 days later; these indicated an increase in TCOM at 2.4 ATA, on 100% O<sub>2</sub> of 421%.

Table 5.19. Patient data pertinent to the study. \* Hb<sub>A1C</sub>, measure of glycosylated haemoglobin. \*\* units per week. \*\*\* See Table 5.25.

	<b>Patient 1 Wd 1a &amp; 1b</b>	<b>Patient 2 Wd 2</b>	<b>Patient 3 Wd 3</b>	<b>Patient 4 Wd 4</b>	<b>Patient 5 Wd 5</b>	<b>Patient 6 Wd 6</b>
<b>Gender</b>	Male	Male	Male	Female	Male	Male
<b>Age</b>	61 y	47 y	75 y	69 y	71 y	71 y
<b>Type of diabetes</b>	Type II	Type II	Type II	Type II	Type II	Type II
<b>Duration of diabetes</b>	7 y	5 y	3 y	16 y	Unknown	21 y
<b>Hb<sub>A1C</sub>*</b>	10.2 %	5.5 %	6.3 %	7.4 %	7.8 %	5.5 %
<b>Vascular disease</b>	Yes	No	Yes	Yes	Yes	Yes
<b>Smoker</b>	No	Yes	No	No	No	No
<b>Alcohol intake</b>	0**	4**	20-24**	0**	<1**	<20**
<b>Antibiotics</b>	Yes***	No	Yes***	Yes***	Yes***	No
<b>Subsequent amputation</b>	No	No	Yes	No	Yes	No
<b>Change in size of wound area (%)</b>	Wd 1 ↓ 14	↓ 40	No data	↑ 12.5	↑ 10	No data
	Wd 2 ↓ 49					

Table 5.20. TCOMs for all patients (Pt) in study. Measurements were taken during the first-HBO treatment. \* Measured 69 days after the first reading for pt 5 (5a).

	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5a	Pt 5b*	Pt 6
<b>Air, sea level (mm Hg)</b>	6	9	21	40	13	47	64
<b>Air, 2.4 ATA (mm Hg)</b>	129	23	31	73	40	377	405
<b>100% O<sub>2</sub>, 2.4 ATA (mm Hg)</b>	1144	663	299	922	205	1068	797

Table 5.21. Antibiotics prescribed for wound study patients. \* Indicates either pre-HBO or number of days into HBO treatment. For pre-HBO the patients were still taking antibiotics when starting HBO. For further details on the actions of antibiotics see Chapter 1.

Patient	Antibiotic	Date prescribed*	Notes
<b>1 (Wound 1a &amp; 1b)</b>	Augmentin	Pre-HBO	Prescribed for 6 weeks. Combination of amoxicillin (see below) and clavulanic acid ( $\beta$ -lactamase inhibitor).
	Amoxicillin	+ 52 days	Penicillin class, $\beta$ -lactam, broad spectrum of activity. Inhibits synthesis of bacterial cell wall.
	Levofloxacin	+ 63 days	Fluoroquinolone class, broad spectrum of activity. Inhibits DNA gyrase.
	Clindamycin	+ 63 days	Lincosamide, targets anaerobe bacteria. Inhibits protein synthesis.
	Doxycycline	+ 73 days	Tetracycline group. Inhibits protein synthesis.
<b>2</b>	Augmentin	Pre-HBO	See above
	Clindamycin	Pre-HBO	See above
	Levofloxacin	Pre-HBO	See above
<b>4</b>	Flucloxacillin	Pre-HBO	Penicillin class, narrow spectrum of activity. Inhibits synthesis of bacterial cell wall.
	Levofloxacin	Pre-HBO	See above
	Clindamycin	Pre-HBO	See above
<b>5</b>	Levofloxacin	+ 13 days	See above
	Clindamycin	+ 13 days	See above
	Augmentin	+ 20 days	See above

### *5.3. Results*

#### *5.3.1. Wound bacterial community dynamics during HBOT*

##### *5.3.1.1. Molecular methods identified aerobic and anaerobic bacteria in chronic wounds*

A diverse range of bacteria was identified in non-healing diabetic foot ulcers using molecular methods. A total of 23 genera were identified (Table 5.22). Approximately 50% of organisms identified were anaerobes though several of these were tolerant of aerobic environments. Each wound contained at least one facultative anaerobe.

Table 5.22. Summary of bacteria identified in wounds; Swab (S), Tissue (T). \* Number of wounds with organism present.

Genus	Species	O <sub>2</sub> Tolerance	Wounds*	S	T
<b>Achromobacter</b>	<i>xylosoxidans</i>	Aerobe	1	—	—
<b>Alcaligenes</b>	multiple	Obligate aerobe	1	—	
<b>Bradyrhizobium</b>	Sp 523321		1	—	
<b>Corynebacterium</b>	<i>aurimucosum</i>	Aerobe	2	☐	
	<i>diphtheriae</i>	Aerobe	1		☐
<b>Enterobacter</b>	<i>cloacae</i>	Facultative anaerobe	3	—	—
<b>Enterococcus</b>	<i>faecalis</i>	Facultative anaerobe	2	—	—
	<i>facium</i>	Facultative anaerobe	1	—	☐
<b>Fusobacterium</b>	<i>nucleatum</i>	Anaerobe	1	—	
<b>Klebsiella</b>	<i>oxytoca</i>	Anaerobe	1	☐	
<b>Lactobacillus</b>	<i>plantarum</i>	Aerotolerant	1	—	
<b>Microbacterium</b>	<i>testaceum</i>	Aerobe	1	☐	
<b>Micrococcus</b>	<i>luteus</i>	Obligate aerobe	1	—	
<b>Morganella</b>	<i>morganii</i>	Facultative anaerobe	2	—	—
<b>Porphyromonas</b>	<i>gingivalis</i>	Anaerobe	1	—	
<b>Prevotella</b>	<i>dentalis</i>		1	—	
<b>Propionibacterium</b>	<i>acnes</i>	Aerotolerant	1		—
<b>Providencia</b>	<i>stuartii</i>	Facultative anaerobe	1	—	
<b>Pseudomonas</b>	<i>multiple</i>	Facultative anaerobe	1	—	
	<i>aeruginosa</i>	Obligate aerobe	4	—	—
<b>Saccharomonospora</b>	<i>glauca</i>		1	—	
<b>Serratia</b>	<i>proteamaculans</i>	Facultative anaerobe	1	—	—
<b>Staphylococcus</b>	<i>aureus</i>	Facultative anaerobe	1	☐	—
	<i>epidermidis</i>		1		—
	<i>warneri</i>		1	☐	
<b>Stenotrophomonas</b>	<i>maltophilia</i>	Aerobe	2	—	
<b>Streptococcus</b>	<i>agalactiae</i>	Facultative anaerobe	1	—	
<b>Streptomyces</b>	multiple	Obligate aerobe	1	—	

The most common genus identified was *Pseudomonas*, found in a total of four wounds; the next most common were *Staphylococcus*, *Enterobacter* and *Enterococcus*, each found in a total of three wounds.

#### *5.3.1.2. Bacterial populations changed during HBOT*

Bacterial populations did not remain static during HBO treatment. Some DGGE bands were present throughout treatment, but some appear part way through treatment and some were absent by the end of HBO. The majority of wounds did not show a reduction in the number of bands present by the end of HBO, though four out of seven wounds showed clearance of anaerobes by the last treatment. As all DGGE profiles were unique the results for each wound will be presented individually.

### 5.3.1.2.1. Wound 1a

The DGGE profile for wound 1a (Fig. 5.4) shows a complex pattern of bands; a key point was the persistence of bands for *P. aeruginosa* throughout HBOT. The pattern of other bands were inconsistent. The MDS plot (Fig. 5.5) indicates that the similarity of bacterial populations did not correlate with the chronological order of HBOTs. Wound 1a contained *Lactobacillus plantarum* an organism not previously reported in a wound. This patients Hb<sub>A1C</sub> of 10.2% suggests poorly controlled blood glucose and antibiotics were prescribed during HBOT (Table 5.19). This wound was reduced in size by 14% at the end of HBOT and this patient did not require amputation.

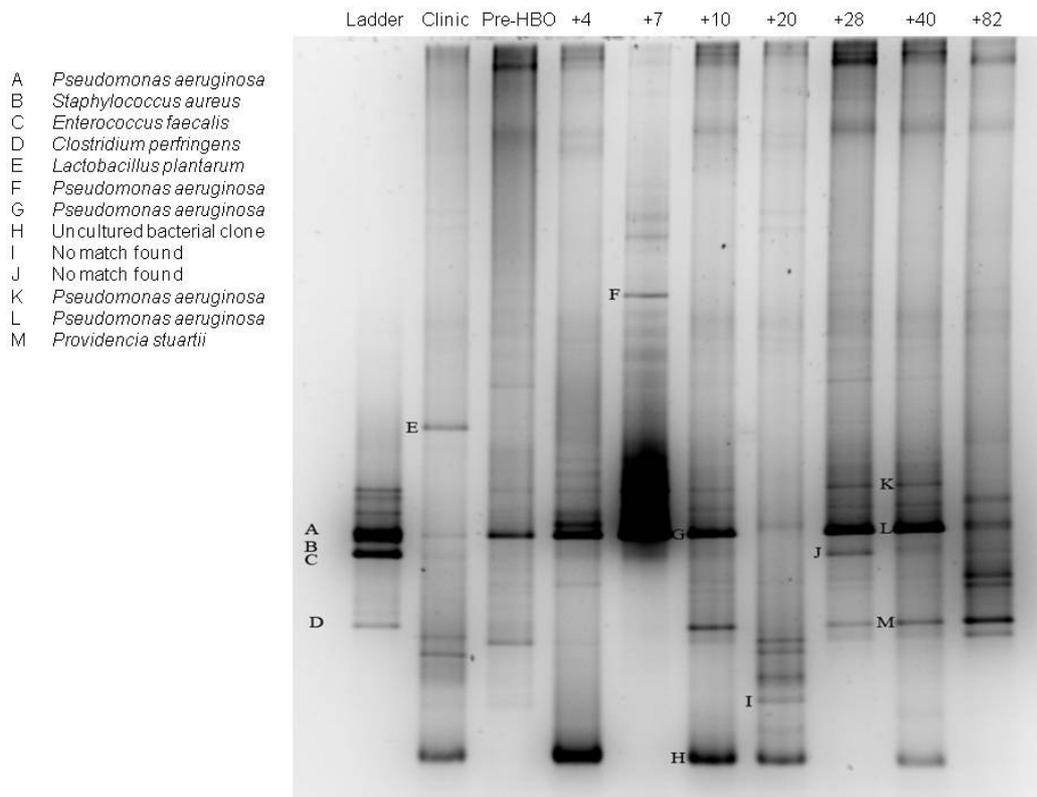


Figure 5.4. Wound 1a DGGE. +4 etc. - days into HBOT not number of treatments

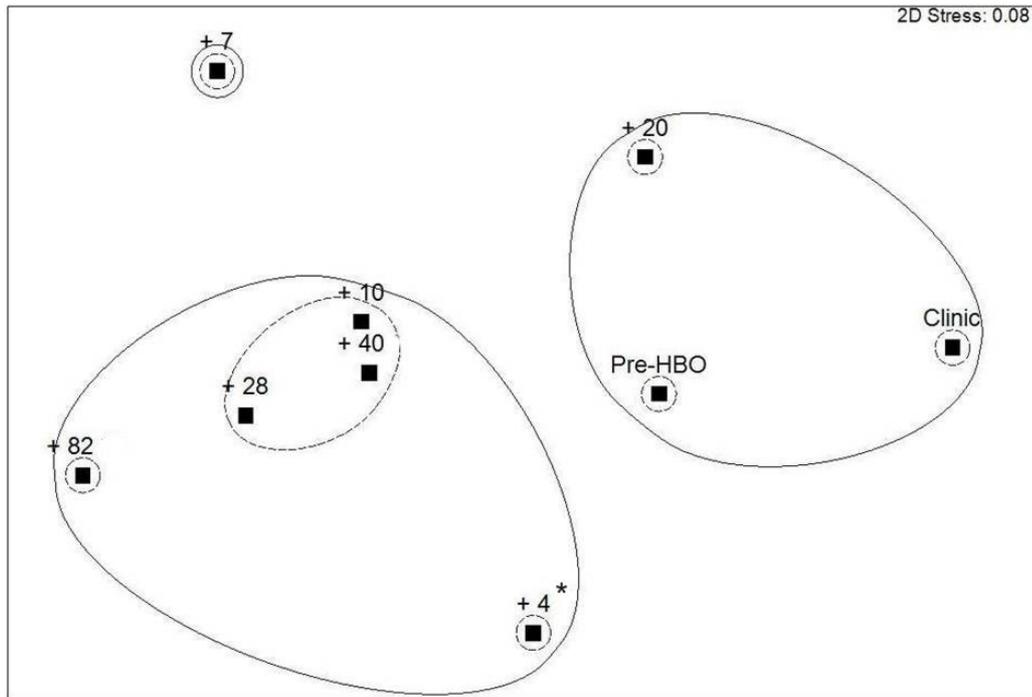


Figure 5.5. MDS plot (Bray Curtis Similarity Matrix) of presence/absence data from DGGE bands from wound1a. +4 etc. indicates days into HBOT not number of treatments. Solid line, 40% similarity, dashed line, 60% similarity. \* Antibiotics prescribed.

#### 5.3.1.2.2. Wound 1b

The DGGE profile for wound 1b (Fig. 5.6), which was on the same patient as wound 1a, indicated a loss of bacterial species by the end of HBOT, though *Pseudomonas* was again a persistent genus, still being present at the end of treatment. *M. morgani* was absent from the wound after 40 days of treatment. The MDS plot (Fig. 5.7) indicates a shift in population similarity between pre-HBOT samples and all other samples, apart from that taken during the last treatment; each group having a similarity of 60%. A RELATE test for seriality using Primer E multivariate statistic software also indicated the samples gradually changed through time ( $R = 0.36$ ,  $p = 0.045$ ). This wound was reduced in size by 49% at the end of HBOT and this patient did not require amputation.

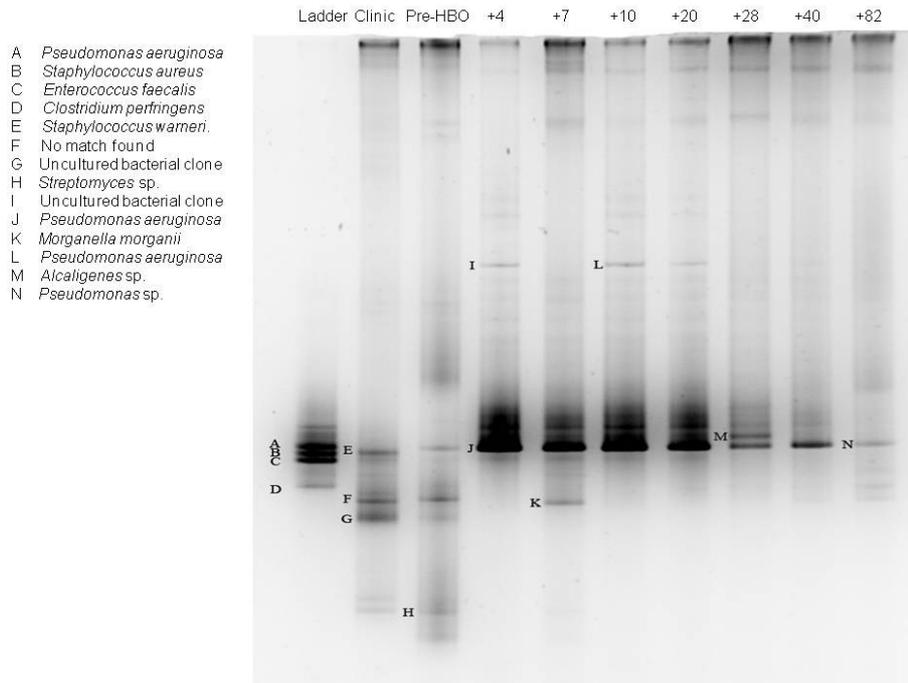


Figure 5.6. Wound 1b DGGE. +4 etc. - days into HBOT not number of treatments

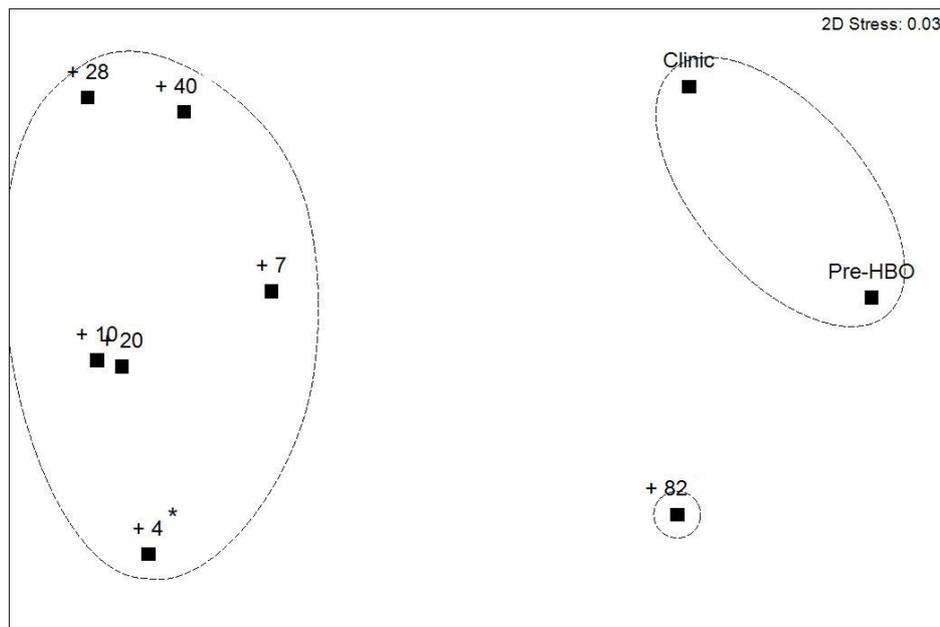


Figure 5.7. MDS plot (Bray Curtis similarity matrix) of DGGE bands from wound 1b. +4 etc. indicates days into HBOT not number of treatments. Dashed line, 60% similarity.

\* Antibiotics prescribed.

### 5.3.1.2.3. Wound 2

In wound 2 (Fig. 5.8), *Streptococcus agalactiae* was present throughout treatment, and the same appeared to be the case for *S. aureus*. *C. aurimucosum* appeared in the wound from day 22 and persisted to the end of treatment. An unidentified organism appeared at day 6, but was cleared by day 20. *P. aeruginosa* was present on day 2, but appears to have been cleared by day 3. The MDS plot indicated two distinct populations, one during the first 2 weeks of treatment and the other during the last 4 weeks (Fig. 5.9). This patient had well controlled blood glucose and was not prescribed antibiotics during HBOT. The wound had reduced in size by 40% at the end of HBOT and did not require subsequent amputation.

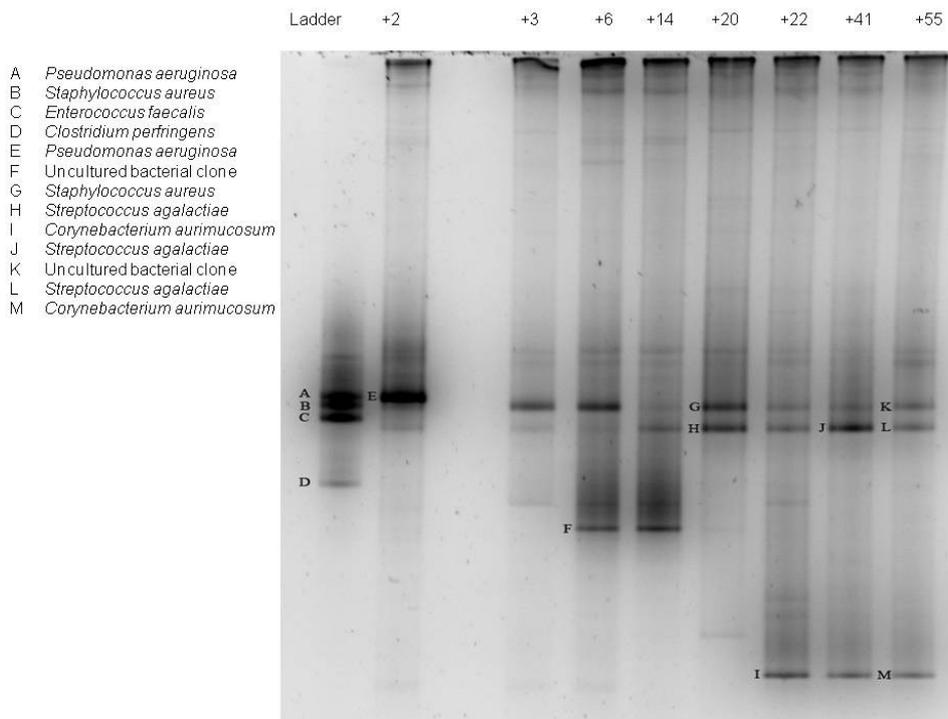


Figure 5.8. Wound 2 DGGE. +2 etc. - days into HBOT not number of treatments.

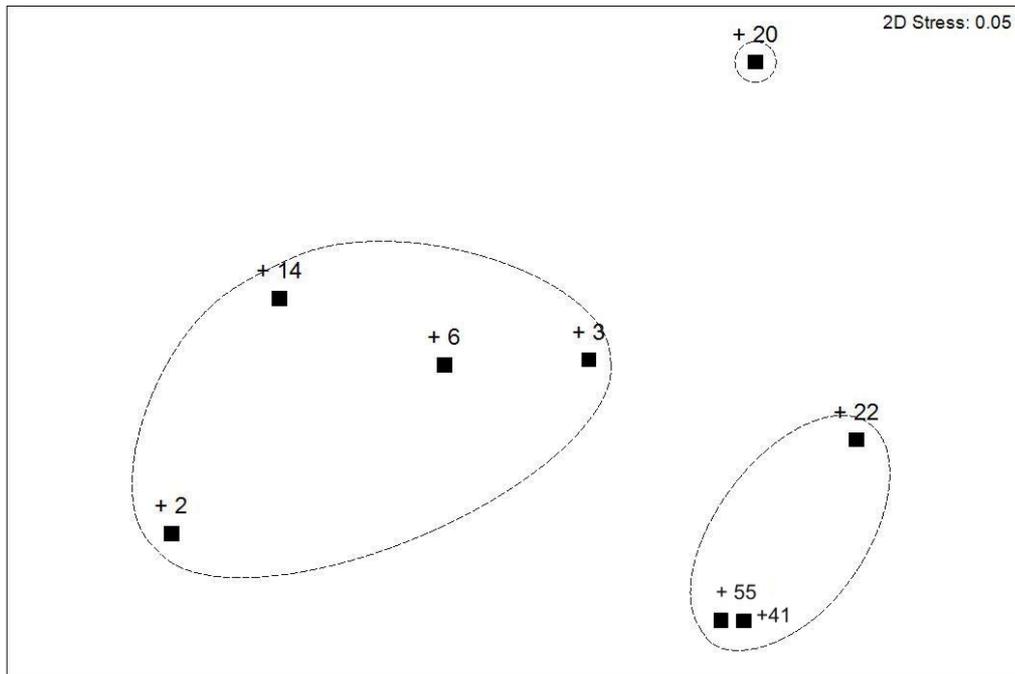


Figure 5.9. MDS plot (Bray Curtis similarity matrix) of DGGE bands from wound 2. +2 etc. indicates days into HBOT not number of treatments. Dashed line, 60% similarity.

#### 5.3.1.2.4. Wound 3

Wound 2 showed a distinct change in bacterial population community after the first week of treatment; initially the wound was colonised by *Prevotella dentalis*, which was replaced by *Serratia proteamaculans* after day 8, clearing the wound by day 47 (Fig. 5.10). The 2 communities, which each showed 60% similarity, can be seen in the MDS plot (Fig. 5.11). Similar to wound 1b, a RELATE test for seriality also indicated the wound samples varied linearly through time ( $R = 0.826$ ,  $p = 0.002$ ). This patient had well controlled blood glucose and was prescribed antibiotics during HBOT (Table 5.19). No data was available concerning a change in wound size but this patient did require subsequent amputation.

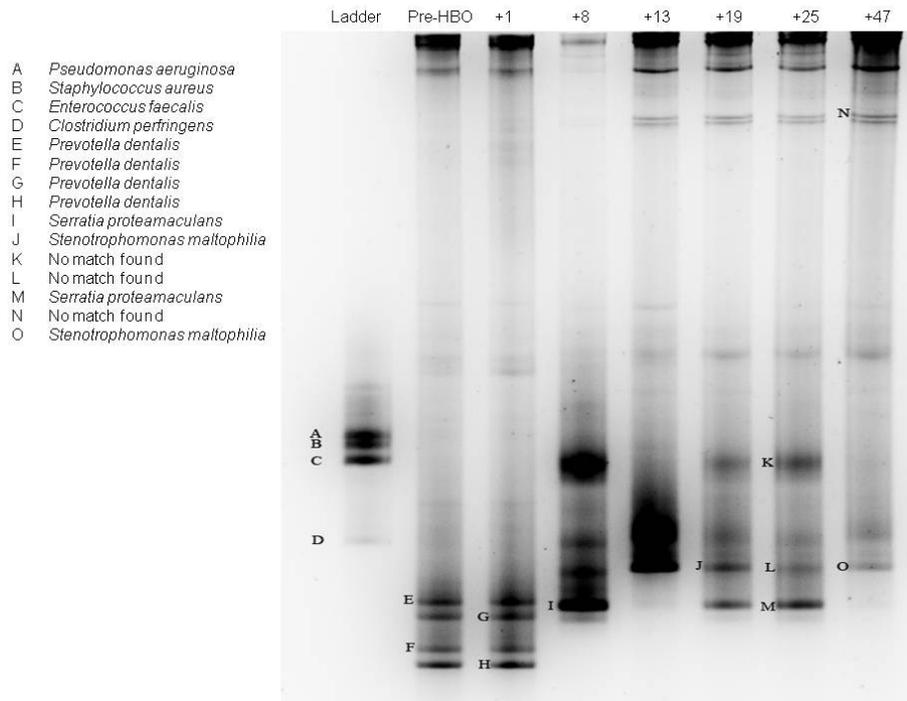


Figure 5.10. Wound 3 DGGE. +1 etc. - days into HBOT not number of treatments.

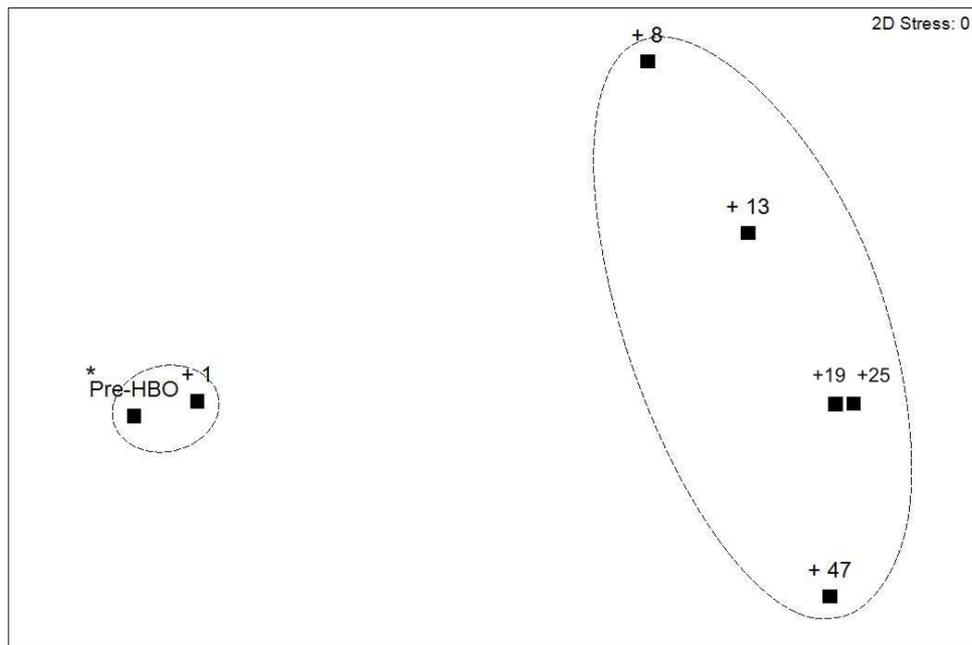


Figure 5.11. MDS plot (Bray Curtis similarity matrix) of DGGE bands from wound 3. +1 etc. indicates days into HBOT not number of treatments. Dashed line, 60% similarity.

\* Antibiotics prescribed.

### 5.3.1.2.5. Wound 4

Wound 4 contained a variety of bacteria (Fig 5.12) that resulted in a complex community dynamic (Fig. 5.13). This wound contained *Saccharomonospora glauca* a species not previously reported in a chronic wound. This patient had well controlled blood glucose and was prescribed antibiotics during HBOT (Table 5.19). The wound area increased in size by 12.5% and subsequent amputation was required.

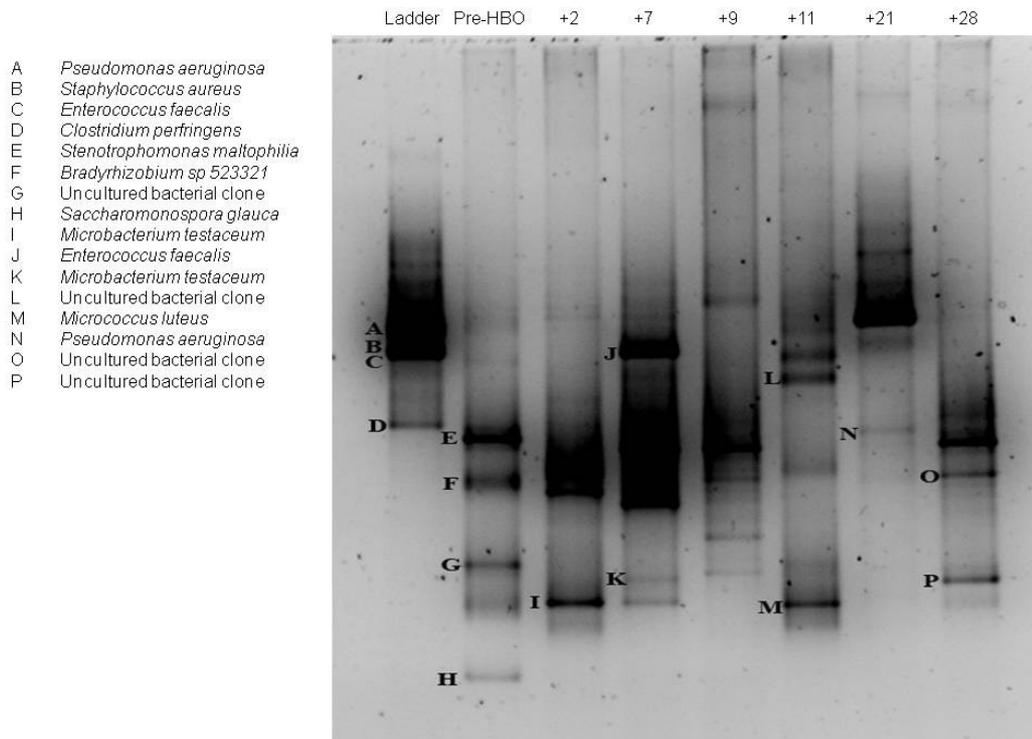


Figure 5.12. Wound 4 DGGE. +2 etc. - days into HBOT not number of treatments.

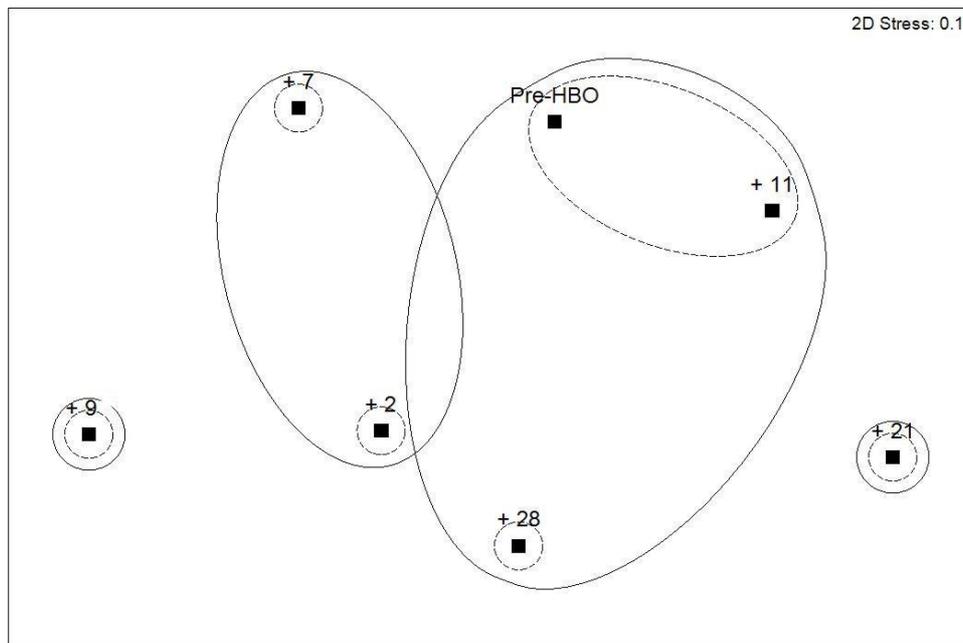


Figure 5.13. MDS plot (Bray Curtis similarity matrix) of DGGE bands from wound 4. +2 etc. indicates days into HBOT not number of treatments. Solid line, 40% similarity, dashed line, 60% similarity.

#### 5.3.1.2.6. Wound 5

In wound 5 (Fig. 5.14), *Enterobacter cloacae* was present before HBOT but was absent by the start of treatment; *E. faecium* and *Achromobacter xylosoxidans* appeared in the wound during the second week of HBOT. By the end of treatment *E. faecium* had been cleared whilst *Achromobacter xylosoxidans* persisted. The MDS plot (Fig. 5.15) indicated three clear population changes during treatment; during the first 10 days, half way through treatment, and during the last 4-5 weeks, and there was significant linearity to these changes through time (RELATE  $R = 0.635$ ,  $p = 0.001$ ). This patient had fairly well controlled blood glucose and was prescribed antibiotics during HBOT (Table 5.19). The wound area had increased in size by 10% at the end of HBOT and subsequent amputation was required.

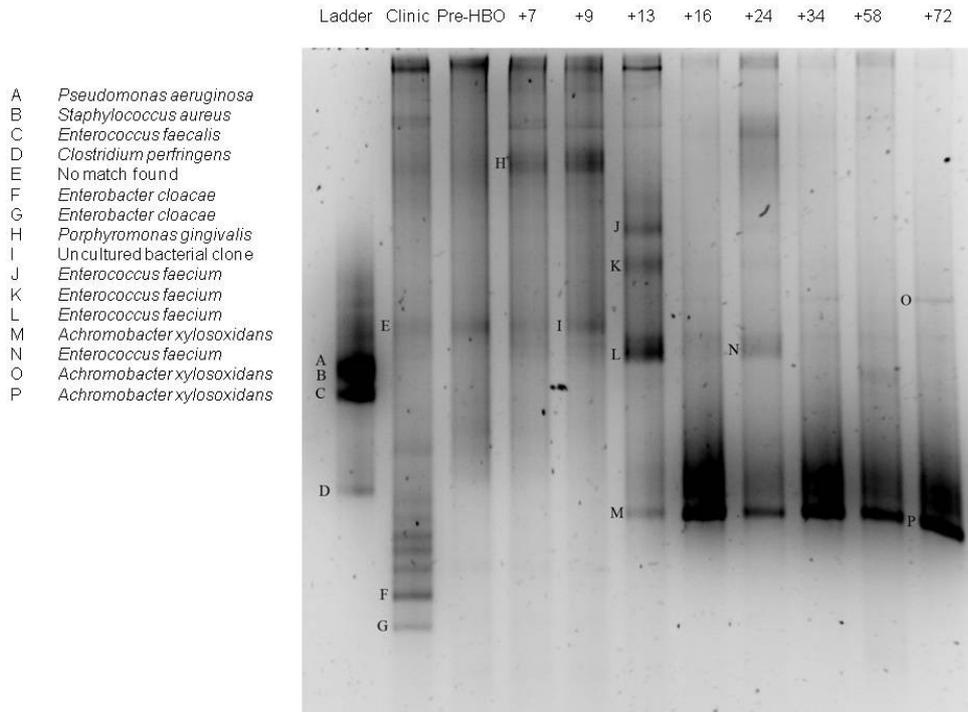


Figure 5.14. Wound 5 DGGE. +7 etc. - days into HBOT not number of treatments.

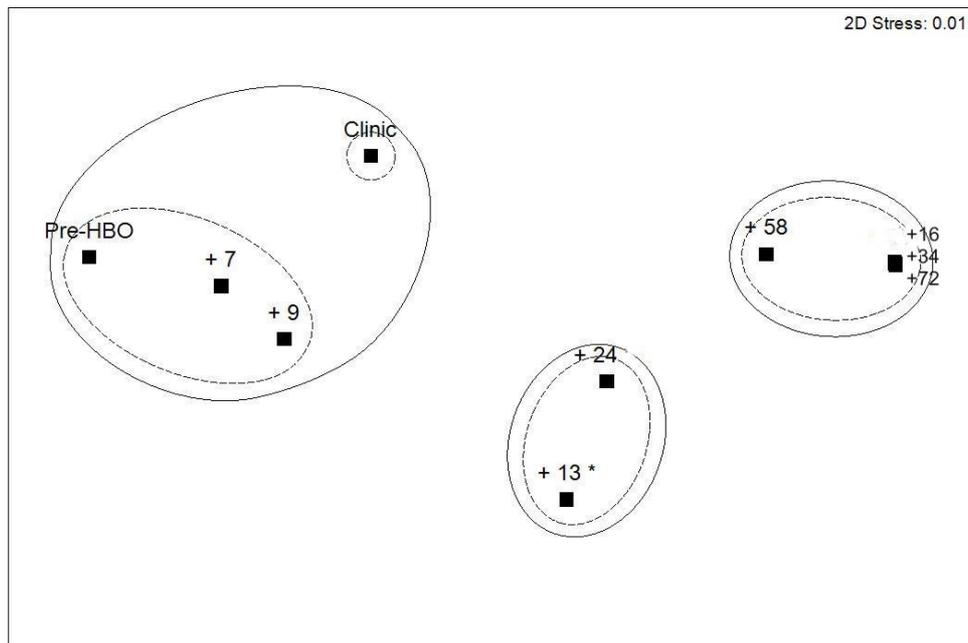


Figure 5.15. MDS plot (Bray Curtis similarity matrix) of DGGE bands from wound 5. +7 etc. indicates days into HBOT not number of treatments. Solid line, 40% similarity, dashed line, 60% similarity. \* Antibiotics prescribed.

### 5.3.1.2.7. Wound 6

In wound 6 (Fig. 5.16), *Fusobacterium nucleatum*, present before HBOT, appears to have cleared by day 18; *C. aurimucosum* and *Enterobacter cloacae* persisted in the wound throughout treatment. Taking all DGGE bands into consideration community changes were more complex (Fig. 5.17). This patient had well controlled blood glucose and was not prescribed antibiotics during HBOT (Table 5.19). There was no data for wound size change available though subsequent amputation was not required.

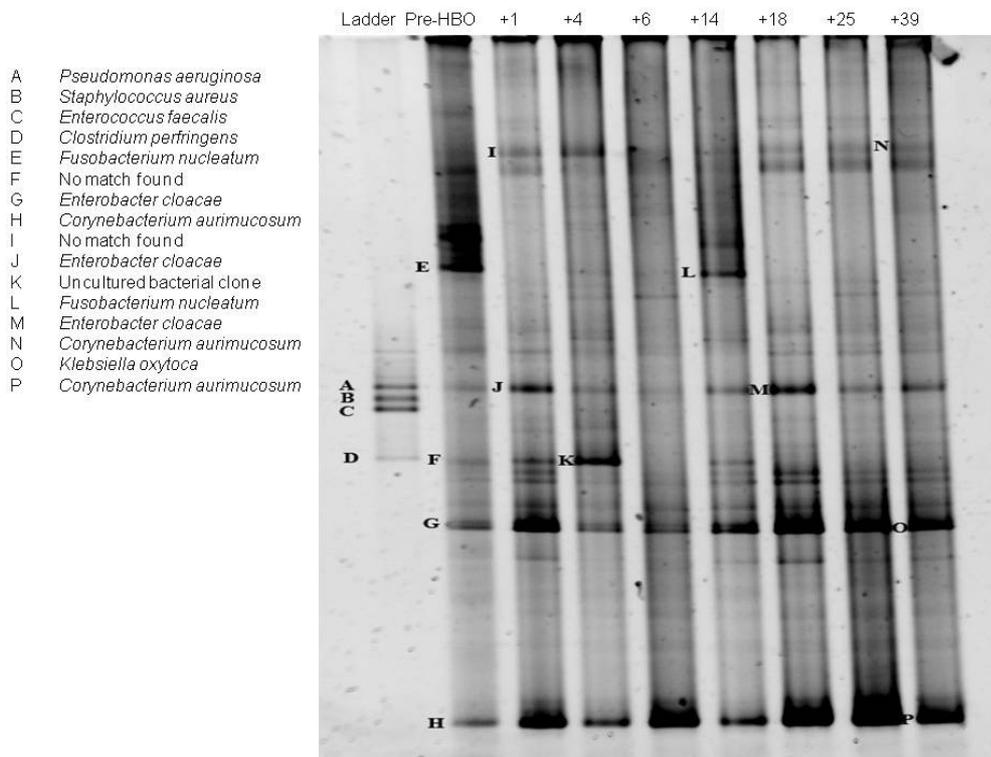


Figure 5.16. Wound 6 DGGE. +1 etc. - days into HBOT not number of treatments.

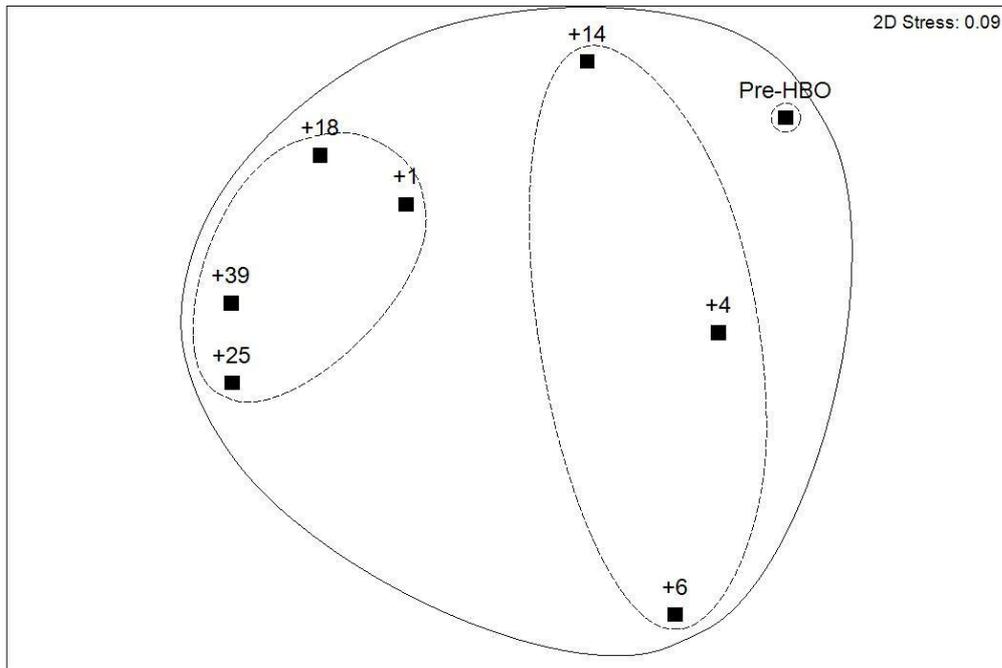


Figure 5.17. MDS plot (Bray Curtis similarity matrix) of DGGE bands from wound 6. +1 etc. indicates days into HBOT not number of treatments. Solid line, 40% similarity, dashed line, 60% similarity.

**5.3.1.3. Similarity of sequences of bands at different migration points were identified as the same species.**

In wound 1a, sequences from bands G, K and L were aligned for similarity; the sequence for band K (*P. aeruginosa*) had 99% similarity to bands G and L (Fig. 5.18). Bands G and L had the same migration points and were 100% similar. The difference with band K was 3 bp, though the notation for band K indicated that the 3 nucleotides were undetermined. In wound 1b, aligned sequences from bands J and L (both *P. aeruginosa*) showed 100% similarity. In wound 3, there were 4 bands (E, F, G and H) identified as *Prevotella dentalis*; bands G and H showed 100% similarity, E and F, E and G, F and G, and F and H showed 99% similarity. The differences with band E were due to undetermined nucleotides and with band F, a substitution of a single thymine in place of a cytosine in bands G and H. Alignments not included below can be found in Appendix 10.

```

K 1  GCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAG 60
    |
L 3  GCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAG 62

K 61 TAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGC 120
    |
L 63 TAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGC 122

K 121 CAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC 180
    |
L 123 CAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC 182

K 181 GCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCC 240
    |
L 183 GCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCC 242

K 241 AAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCCTGTGTAGCGGTGAAATG 300
    |
L 243 AAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCCTGTGTAGCGGTGAAATG 302

K 301 CGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTG 360
    |
L 303 CGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTG 362

K 361 AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG 420
    |
L 363 AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG 422

K 421 ATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGCGCAGCTAACGCGATAAGTCGA 480
    |
L 423 ATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGCGCAGCTAACGCGATAAGTCGA 482

K 481 CCGCNNNGGAGTACGGCCGCAAGGTTAAAACT 513
    |
L 483 CCGCCTGGGGAGTACGGCCGCAAGGTTAAAACT 515

```

*Figure 5.18.* Alignment of sequences from DGGE bands K and L from wound 1a. Both bands are from *P. aeruginosa*.

### 5.3.2. Wound fungal community dynamics during HBOT

#### 5.3.2.1. Wounds were colonised by fungal species

All wounds in this study contained at least one species of fungi prior to starting HBOT (Table 5.23); the mean number of organisms identified per wound was 2.1. The most common genus present was *Aspergillus* (Table 5.23. Wounds 2 and 7 were also investigated for the presence of fungi at two points during HBOT, mid-treatment and at the end of HBO. A summary of the results can be seen in Table 3.22. All wounds demonstrated communities of co-existing bacteria and fungi.

Table 5.23. Fungal species identified in each wound pre-HBOT.

Organism	1a	1b	2	3	4	5	6
<i>Aspergillus spp.</i>	+	+			+	+	+
<i>Aspergillus penicillioides</i>				+			
<i>Candida parapsilosis</i>							+
<i>Cladosporium cladosporioides</i>				+			
<i>Microdochium bolleyi</i>	+						
<i>Microdochium nivale</i>	+						
<i>Penicillium argillaceum</i>	+						+
<i>Phoma herbarum</i>					+		
<i>Saccharomyces cerevisiae</i>		+					
<i>Sporobolomyces roseus</i>			+				
<b>Total</b>	<b>4</b>	<b>2</b>	<b>1</b>	<b>2</b>	<b>2</b>	<b>1</b>	<b>3</b>

Table 5.24. Frequency of fungal species in pre-HBO wounds.

Fungal species	Number of wounds
<i>Aspergillus spp.</i>	6
<i>Candida sp.</i>	1
<i>Cladosporium sp.</i>	1
<i>Microdochium spp.</i>	1
<i>Penicillium sp.</i>	2
<i>Phoma sp.</i>	1
<i>Saccharomyces sp.</i>	1
<i>Sporobolomyces sp.</i>	1

Table 5.25. Fungi identified at three points during the wound study in wounds 1b and 6  
 \* No PCR bands were detected post cloning.

	Wound 1b	Wound 6
<b>Pre-HBO</b>	<i>Aspergillus spp.</i>	<i>Aspergillus spp.</i>
	<i>Saccharomyces cerevisiae</i>	<i>Candida parapsilosis</i>
<b>Mid-HBO</b>	<i>Aspergillus spp.</i>	<i>Penicillium spp.</i> <i>Aspergillus spp.</i>
	<i>Chaetomium elatum</i>	
	<i>Fusarium oxysporum</i>	
	<i>Phoma herbarum</i>	
<b>End of HBO</b>	<i>Sporobolomyces roseus</i> <i>Aspergillus spp.</i>	None*
	<i>Phoma herbarum</i>	

### 5.3.3. A comparison of cultural and molecular techniques for bacteria

There were 16 occasions when duplicate swabs were analysed; one in Derriford microbiology laboratory (see Section 1.3.1) and the other with molecular methods (PCR-DGGE, sequencing); of those, three samples showed no bacterial growth using cultural methods; however, molecular analysis detected the presence of at least one organism in each swab. The number of bands in the DGGE however, indicated that there may have been several more (Table 5.26). None of the organisms identified with molecular analysis were concurrently identified with cultural analysis of the duplicate swab.

Table 5.26. Organisms detected in wound swabs (at least 1 from each wound) with cultural and molecular analysis. \* Named organisms are those identified with sequencing; approximate number of bands on DGGE gel for that swab. \*\* moderate numbers, \*\*\* large numbers.

Wound and day of swabbing	Culture	Molecular analysis*
Wd 1a, Clinic	No bacterial growth	<i>Lactobacillus plantarum</i> >6 bands
Wd 1a, +10	Mixed bacterial growth	<i>Pseudomonas aeruginosa</i> >5 bands
Wd 1b, +20	Coliform bacilli *** <i>Pseudomonas aeruginosa</i> ***	No bands sequenced >6 bands
Wd 2, +2	Mixed bacterial growth Diphtheroid bacilli ** Coliform bacilli ** <i>Staphylococcus aureus</i> **	<i>P. aeruginosa</i> >1 band
Wd 2, +14	Diphtheroid bacilli *** Coliform bacilli *** <i>Staphylococcus aureus</i> **	No bands sequenced >4 bands
Wd 2, +55	Mixed bacterial growth <i>Staphylococcus aureus</i> **	<i>Streptococcus agalactiae</i> <i>Corynebacterium aurimucosum</i> >5 bands
Wd 3, Pre-HBO	Mixed bacterial growth	<i>Prevotella dentalis</i> >4 bands
Wd 3, +3	Mixed bacterial growth ***	No bands sequenced >1 bands
Wd 3, +47	No bacterial growth	<i>Stenotrophomonas maltophilia</i> >3 bands
Wd 4, Pre-HBO	No bacterial growth	<i>Stenotrophomonas maltophilia</i> <i>Streptococcus agalactiae</i> <i>Bradyrhizobium</i> sp 523321 <i>Saccharomonospora glauca</i> >5 bands
Wd 4, +11	Mixed skin flora	<i>Micrococcus luteus</i> >3 bands
Wd 5, Clinic	Mixed bacterial growth	<i>Enterobacter cloacae</i> >7 bands
Wd 5, Pre-HBO	<i>Staphylococcus aureus</i> <i>Enterococcus</i> sp.	No bands sequenced >2 bands
Wd 5, +16	Mixed skin flora <i>Enterococcus</i> sp.	No bands sequenced >1 bands
Wd 6, Clinic	Coliform bacilli <i>Staphylococcus</i> sp. <i>Enterococcus</i> sp. Diphtheroid bacilli Mixed anaerobes	<i>Fusobacterium nucleatum</i> <i>Enterobacter cloacae</i> <i>Corynebacterium aurimucosum</i> >6 bands
Wd 6, +14	Coliform bacilli <i>Staphylococcus</i> sp. <i>Enterococcus</i> sp. Diphtheroid bacilli <i>Bacteroides</i> sp.	<i>Fusobacterium nucleatum</i> >7 bands

#### 5.4. Discussion

This study examined for the first time changes in bacterial and fungal community population dynamics during HBO treatment. The polymicrobial nature of non-healing chronic wounds has already been extensively reported (Redkar *et al.* 2000; Hill *et al.* 2003; Davies *et al.* 2004; Gjødsbøl *et al.* 2006; Dowd *et al.* 2008; Frank *et al.* 2008). The species identified in this study support that body of work, though two species, *Saccharomonospora glauca* and *Lactobacillus plantarum*, are to our knowledge, so far unreported in chronic wounds.

The first major observation from this study is that the bacterial community during a course of HBO is complex and dynamic. It is disappointing that we were unable to recruit patients to act as non-HBO controls, as an audit of wound microflora over time is lacking in published work. Gjødsbøl *et al.* (2006) identified resident bacteria (species present in “all or all but one” of the sampling times) in chronic venous ulcers over a period of 8 weeks but did not present the bacterial community for each wound at each time point, so apart from reporting that 76% of wounds had two or more resident species, it is not known how stable the rest of the community was over time. *S. aureus* and *P. aeruginosa* were two of the most common resident species in the study of Gjødsbøl *et al.* and, interestingly, they observed that *P. aeruginosa* was resident in ulcers that increased in size, but not resident in ulcers that reduced in size. In our study *P. aeruginosa* was present in four HBO treated wounds and band patterns suggested that they do persist over time; however, 3 of those 4 wounds were reduced in size by the end of HBO (Table 5.19), despite the presence of this organism. The small number of wounds included in this study negates any firm conclusions concerning this observation, but it is useful to consider whether HBO is able to modify the toxicity of organisms in some manner, to facilitate

healing, even though the organism is still present. A point that must also be considered is that DGGE is a tool that indicates the presence of bacterial genetic material in a wound, not the presence of live organisms. Wounds are cleaned as a part of routine care but it cannot be assumed that all dead material is removed.

It has been conjectured that the presence of anaerobic bacteria in chronic wounds may be a factor in non-healing (Section 1.3.1) and indeed, approximately 50% of organisms found in our study were facultative anaerobes (Table 5.22); the majority of those were tolerant to oxygen to varying degrees meaning that they would be less vulnerable to the direct toxicity of a high oxygen environment afforded by HBO. However, anaerobes were cleared from four of the wounds by the end of treatment. There does not appear to be any correlation between the clearance of anaerobes and either a reduction in wound size or the level of tissue oxygenation achieved during HBO, supporting the observation that wound healing is multifactorial and not exacerbated by any single component.

The MDS plots of species suggested that bacterial populations during HBOT do undergo distinct changes in 5 out of 7 of the wounds (wounds 1b, 2, 3, 5 and 6). For example, wound 3 had two populations with 60% similarity, one consisting of 2 samples at the beginning of HBOT, and the other consisting of 5 samples taken during the remainder of the treatment period, indicating a shift in population after only a few treatments. Early shifts in population similarity was seen in 3 wounds and suggested that bacterial species were responding to the modified oxygen environment; wounds 2 and 6 were on patients not taking antibiotics, which ruled that out as a factor for change and wound 3 saw a loss of anaerobic bacteria even though this patient was not prescribed antibiotics

that target anaerobes. Wound 5 demonstrated a shift in population similarity from day 13 of treatment and as antibiotics were not prescribed until day 13, this change must have been the result of some other factor.

An interesting observation was that the 2 patients (wounds 3 and 5) with the lowest TCOMs at 100% O<sub>2</sub> at 2.4 ATA, went on to require amputations; at the pre-HBO assessment they just achieved the minimum required to indicate that HBO might be effective (299 and 205 mm Hg, respectively). The TCOM for wound 5 did show significant improvement after 69 days of HBOT (Section 5.2.11), indicating that oxygen delivery to the wound had increased, perhaps due to neo-angiogenesis. However, this wound still increased in size by 10% and required amputation. It is difficult to arrive at any firm conclusions as to why this wound failed to heal, but it highlights the point that despite an improvement in tissue oxygenation and all the concurrent physiological benefits, some wounds will not heal. This wound demonstrated shifts in bacterial population but it could not be determined whether these changes were beneficial or detrimental to healing, despite the end result. There were many other factors that could have affected this wound; poor blood glucose control, poor immune response and other health problems, may have contributed significantly to the lack of healing.

The identification of fungi in wounds is of increasing interest as evidence suggests that members of this group may play a role in the non-healing state; Heald *et al.* (2001) identified fungal species, predominantly *Candida spp.*, in all 17 of the diabetic foot ulcers that they examined and observed that 15 wounds healed or improved when anti-fungal therapy was implemented, following a period of non-response to standard antibiotic therapy. A later survey of fungal infections in chronic wounds (Dowd *et al.* 2010) found that 41% of DFUs were

fungal positive, again with *Candida spp.* being the dominant species present. This study adds to this work in finding that 100% of the wounds contained fungal species, including 6 species that to our knowledge have been previously unreported in DFUs. Of interest, though not significant due to the small sample size, a single *Candida* species, *Candida parapsilosis*, was found in one wound only; the most frequent species identified were *Aspergillus spp.* Why *Candida spp.* were not found more frequently in this study is unclear; hospital clinics and the home environment could potentially increase the diversity of contamination. DDRC may have a specific environmental pool of fungal species that would limit exposure, but patients only attended for 3-4 h each day and unless being re-dressed the wounds were always covered.

Also previously unreported is an audit of fungal species present in DFUs before, during and at the end of HBOT. Due to time and financial constraints only two wounds were examined, which showed different patterns of infection (Table 5.25). Definitive conclusions cannot be drawn from such a small sample size but two points are of interest; (i) the patient with the higher fungal load had less well controlled blood glucose than the patient with less fungal species present (Table 5.25) and (ii) the patient with the higher fungal load was prescribed antibiotics. It is now widely accepted that diabetic patients are more likely to experience fungal foot infections than their non-diabetic counterparts (Rosseeuw *et al.* 1999), and Alteras *et al.* (1979) found a correlation between blood glucose and the presence of fungi in the toe webs and toe nails of diabetic patients. It has also been suggested that patients taking antibiotics are also more likely to have fungal infections; a study investigating the development of nosocomial candidemia found that taking two or more antibiotics was a major risk factor (Bross, *et al.* 1989). It is possible that antibiotics, in reducing the

bacterial load, provide a less competitive environment for fungi to proliferate. This may explain why the fungal load of wound 1b was greater than wound 6, but not why wound 6 was cleared of fungi during HBOT but wound 1b was not. A possible explanation is that improved neutrophil function, due to increased oxygen availability, was able to clear the lower burden of wound 6 but not that of wound 1b.

The use of DGGE as a tool, to examine environmental and medical bacterial populations, has become very popular and has facilitated the identification of viable but non-culturable species. Particularly in the medical field, routine culture is limited by not what is there but what is looked for, and often what is looked for is determined by the clinical symptoms of infection; anaerobes and fungi generally being under-reported (Bowler and Davies, 1999). However, the results of DGGE need to be examined with some caution; bias can occur at several points: choice of primers, PCR programme parameters, nested PCR, denaturing gradients etc. Protocol optimisation will hopefully reduce these effects and result in accurate gel resolution of PCR products; however, this will depend to some degree on the sensitivity required. It could be argued that DGGE is too sensitive a tool if the study is concerned with identifying to species level the population under examination; and pre-determining this question is important when analysing banding patterns. This study used GelCompar software which makes the assumption that every band in a single column of gel is a different species with its own migration position. However, as DGGE can be sensitive to single bp differences, it is likely that more than one band in a column is from the same species of bacteria. In fact, this is what this study observed as sequencing results from bands at different migration points on the gel were identified as belonging to the same species. Aligning the sequence

data from such bands indicated that some of the differences were indeed only 2-3 bp (Section 5.3.1.3), so it is important to ascertain whether this level of sensitivity is a help or a hindrance when leading on to band analysis and comparison of population similarity. Most bacteria have multiple copies of the 16S gene, for example *P. aeruginosa* has 4 copies of this gene (<http://rrndb.mmg.msu.edu/search.php>), which could account for the different migration points of the PCR products seen in this study. For this reason, gel banding analysis may give an impression of greater diversity than is actually present.

The final protocol for PCR-DGGE in this study was arrived at after several months of preparatory work optimising conditions. However, compromises had to be made and sometimes this resulted in sub-optimal gels. For example, initial dilution of the PCR products gave very dense bands that were poorly resolved (Figure 5.4. column +7), but further dilution resulted in clearer bands but the loss of fainter bands. Altering the denaturant gradient to separate dense bands into what could have been different species again resulted in the loss of some bands. Others have found that single bands can contain multiple species (Gafan & Spratt, 2005) which still do not migrate to different points in the gel with very narrow gradients, which may indicate that species diversity is greater than that calculated. In conclusion, DGGE, though having limitations regarding the precise resolution of different species, does provide an excellent means of monitoring any changes in bacterial populations over time, providing that the protocol design addresses and limits intra-gel variability. If identification of species present is required the production of a clone library would give a more detailed picture. A summary of the pros and cons for using DGGE or cloning as tools in assessing wound microflora communities is presented in Table 5.21. In

this study the decision to use cloning for fungi was influenced by the lack of time available to optimise DGGE, which was crucial to avoid some of the cons of this technique (Table 5.21), and the decision to analyse a fewer number of samples, making cloning a more direct means of determining identification of fungal species. Ideally, a combination of the two techniques for bacteria and fungi would have been optimal.

Table 5.21. Pros and cons of DGGE and cloning as techniques for analysing microflora

Technique	Pros	Cons
<b>DGGE</b>	A large number of samples can be analysed together	May overestimate diversity
	An excellent tool for monitoring changes in community	It is not quantitative
	Once optimised the technique is quick	Limit on amplicon size may reduce accurate identification following sequencing
	Allows rapid visualisation of sample community	Co-migration of species
<b>Cloning</b>		PCR artifacts may show as bands
		Standardisation of gels can be a problem due to inconsistent gradients
		Choice of bands for sequencing is subjective
	A direct technique with minimal electrophoresis	Very labour intensive for a large number of samples
	Larger size of insert allows greater accuracy of identification following sequencing	It is not quantitative

This study has provided a valuable snapshot of bacterial and, to a limited extent, fungal, community dynamics during a course of HBOT; future work could expand on this with a greater number of participating patients. This could be achieved by a multi-centre collaboration, with common procedural and analytical protocols. Ensuring that non-HBOT patients were also included would provide a detailed temporal analysis of wound microflora during the healing/non-healing process. A more detailed picture of wound microflora over time might help physicians construct more effective antibiotic regimens, including the informed use of anti-fungals. DGGE could be used to monitor population changes over time and cloning could provide accurate identification of species in order to determine if the presence of specific organisms was instrumental in the non-healing process. This study has highlighted the need for

further investigation into the population dynamics of bacteria and fungi during the treatment process. It has also reiterated the differences in the qualitative aspect of information gleaned between culture methods and molecular methods, and how the latter can provide a more diverse, detailed and ultimately valuable analysis of wound microflora; the importance of which is discussed further in Chapter 6.

## Chapter 6

### Final Discussion

#### *6.1. Summary of the findings of this study*

This study has provided evidence of oxidative damage to an antibiotic degrading enzyme (penicillinase) and to bacterial QS signalling molecules (AHLs) that resulted in reduced activity. It has demonstrated that oxidants in conjunction with antibiotics reduced the number of live cells in pre-formed bacterial biofilms to a greater extent than either agent alone. The audit of microflora in a non-healing diabetic foot ulcer during HBOT has provided a picture of microbial community changes over time.

This final discussion will bring together these findings to illustrate how a modified oxygen environment may affect mechanisms of antibiotic resistance during a course of HBOT and how this may facilitate wound healing.

#### *6.2. A possible scenario of HBOT mediated changes to a non-healing wound*

A chronic, non-healing wound is a wound that has become locked into a cycle of infection and inflammation. Periodic antibiotics may give temporary relief from some of the symptoms of bacterial contamination, but eventually the wound deteriorates and, in the case of DFUs, the final solution is usually to amputate a proportion of the limb back to healthy tissue. Evidence is accumulating that HBOT provides a viable alternative for some wounds. Until now it has not been known whether or how improved tissue oxygenation may modify antibiotic resistance in a non-healing wound environment but this study has provided evidence that suggests possible means whereby this could occur.

In formulating this scenario two assumptions have been made: (i) HBOT increases tissue oxygenation to at least normoxic levels in the wound area and (ii) increased oxygen availability facilitates oxidant production by phagocytes.

Chapter 1 discussed the positive effects of increasing the  $pO_2$  of a non-healing wound and one of those effects was the formation of new blood vessels (neovascularisation). One outcome of increased tissue vascularisation would be an improvement of the delivery of systemic antibiotics, carried in blood plasma. Maintaining effective bactericidal/bacteriostatic concentrations of antibiotics in blood is futile if the drugs cannot be delivered to the area contaminated with microorganisms, and lengthy courses of antibiotics may result in poor patient compliance if side-effects are being experienced. HBOT, delivered as an adjunct to antibiotic therapy early in the treatment process, might result in improved healing by facilitating antibiotic delivery to the wound. Unfortunately, HBOT is often regarded as a last resort option when all other therapies have been tried, by which time the wound will have severely deteriorated.

Many  $\beta$ -lactamase producing bacteria secrete these enzymes into the extracellular milieu where they degrade  $\beta$ -lactam antibiotics before they enter bacterial cells (Ciofu *et al.* 2000). In a wound environment containing ROS/RNS such as  $H_2O_2$ , HOCl and  $ONOO^-$  a significant proportion of  $\beta$ -lactamases could be modified allowing antibiotics to enter cells undamaged. This work has provided evidence that serine and metallo- $\beta$ -lactamases are vulnerable to damage by  $H_2O_2$ , HOCl and  $ONOO^-$  (Chapter 2), a situation particularly valuable regarding the latter group due to the paucity of drugs effective against them. The concentration and stability of  $ONOO^-$  in a chronic wound is unknown so it cannot be assumed that the concentration used in this study would be as effective *in vivo*. However, it is feasible that the concentrations of  $H_2O_2$  and HOCl used could occur in wounds and that they could significantly reduce the activity of  $\beta$ -lactamases. The increased vulnerability of bacteria should result in a

subsequent reduction in bacterial load that could facilitate bacterial clearance by the immune system.

Chapter 3 provided evidence that HOCl was capable of damaging AHLs, depending on chain length and substitution, *in vitro*. The implication of this *in vivo* would depend on the presence and abundance of bacterial species and whether vulnerable AHLs were secreted. In the case of *P. aeruginosa*, O-C12-HSL could be damaged but the C4-HSL would not, however with a reduction in available O-C12-HSL it is possible that only background levels of C4 would be produced, which could be insufficient to drive a response. Organisms that don't produce vulnerable AHLs but do respond to them could be affected in some way, though whether this would be a positive or negative effect regarding wound pathology would depend on the modified outcome.

The *in vivo* results did not reflect the *in vitro* results of AHL damage and an explanation for this was presented in Section 3.4. These results may also predict those that would be seen for  $\beta$ -lactamases in a wound environment, however HBOT extends for several weeks, allowing for the possibility that bacterial antioxidant defences may become depleted and less effective over time which may allow damage to occur. As AHLs and  $\beta$ -lactamases are secreted into the extracellular environment, the antioxidant status of the patient should also be considered. In general, levels of human extracellular antioxidants, i.e. GSH and SOD, are low and extracellular fluids contain virtually no catalase activity (Halliwell & Gutteridge, 2007). Studies that have investigated antioxidant capacity following repeated HBOT have shown reduction or no change to levels of endogenous antioxidants (Benedetti *et al.* 2004, Eken *et al.* 2005, Gurdol *et al.* 2008, Groger *et al.* 2009). However, oxidative damage to selected markers was still detected, which would suggest

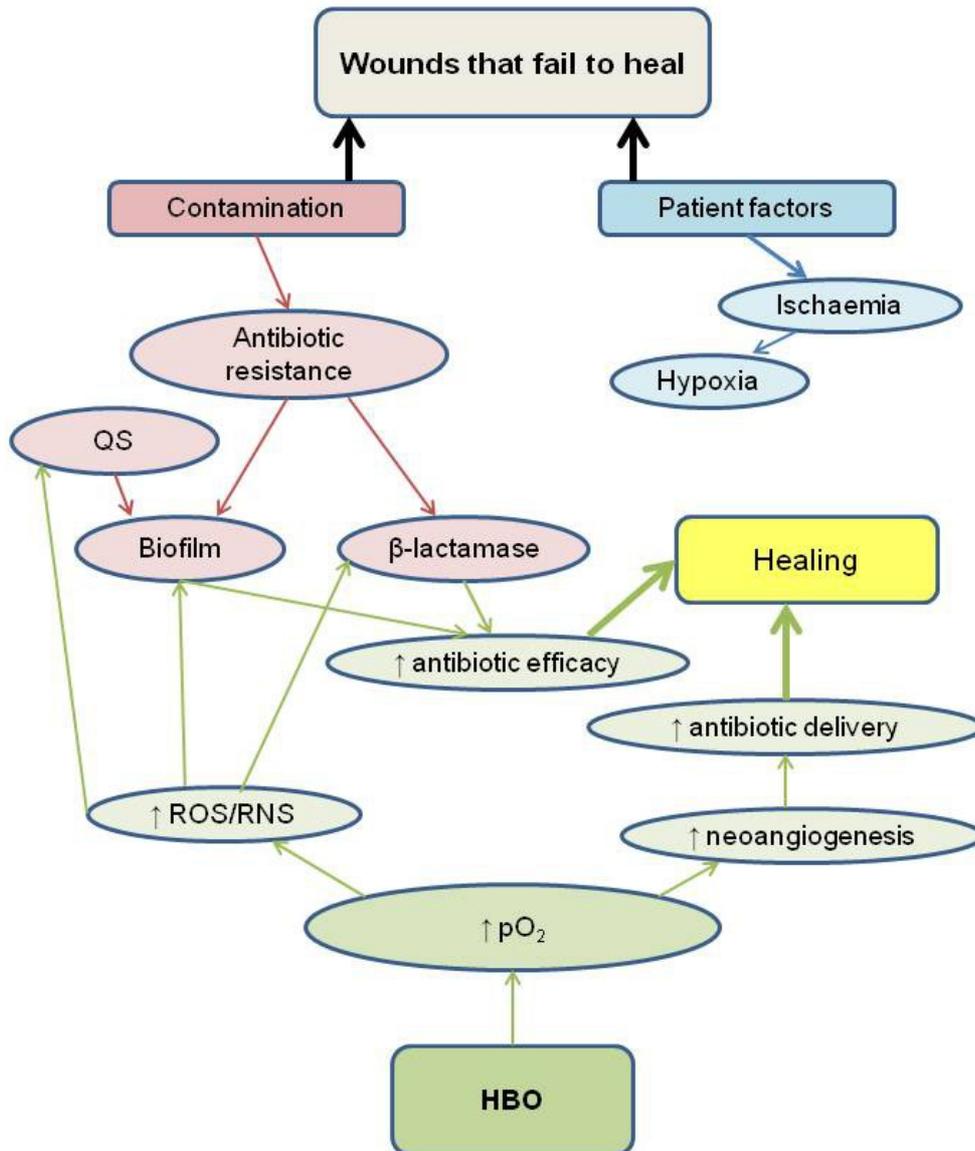
that, with prolonged HBOT, antioxidant defences, at already low levels extracellularly, could be insufficient to prevent oxidative damage to molecules secreted by bacteria. There are several non-enzyme antioxidant defences found in human plasma, e.g. erythrocytes, albumin, urate, (Halliwell & Gutteridge, 2007), that could play a role in scavenging ROS in a wound environment. Ultimately, whether damage occurs or not will depend on the balance between levels of ROS and antioxidant defences.

If AHLs, instrumental in the formation of biofilms in wounds, were to be damaged by extracellular HOCl then it is feasible that HBOT could reduce the biofilm load of a wound. However, as discussed in Chapter 1, QS and biofilm formation is complex and a more comprehensive picture might be devised when more is known about the effects of QS interference in multi-organism biofilms. The lack of an effect of oxidants on AHLs, *in vivo*, from this study may indicate that antioxidant defences are effectively quenching the ROS, as discussed above, and this has implications for biofilm formation in wounds undergoing HBOT. Again, HBOT may overwhelm antioxidant defences rendering AHLs, and other QS signalling molecules vulnerable to damage. This study did not produce *P. aeruginosa* biofilms of sufficient quality and reproducibility to investigate the effects of damaged AHLs on biofilm formation.

The investigation of *S. aureus* biofilms in this study suggested that HBOT could facilitate the reduction of biofilm mass by the additive effects of oxidant production and antibiotics on the reduction of live cells in biofilms (Chapter 4). It was not determined whether the effects of the ROS were directly lethal to biofilm cells or whether they modified bacterial defences that made them more vulnerable to antibiotics. The kinetic properties of ROS generating enzymes in humans, as discussed in Section 4.4. relating to oxygen concentration and

enzyme saturation, may mean that HBOT will not result in increased ROS production. However, HBOT, in returning wounds to, at least, a normoxic environment should still facilitate ROS production.

A schematic representation of how HBOT might aid wound healing, from the perspective of contamination, is presented in Figure 6.1. HBOT increases  $pO_2$  in the wound environment which results in the formation of new blood vessels (neoangiogenesis). Improved vascularisation means that the blood supply to the damaged tissues is improved allowing better delivery of plasma-borne antibiotics. This should allow for the accumulation of target concentrations to achieve bactericidal or bacteriostatic outcomes. Secondary to this, increased  $pO_2$  might lead to improved immune cell function by providing increased oxygen, a substrate for the production of ROS/RNS. As well as improved microorganism clearance an increase in extracellular concentrations of ROS/RNS might occur; this would provide a deleterious environment for secreted AHLs and  $\beta$ -lactamases. QS interference may hinder the formation of biofilm in the wound and the combination of oxidants and improved antibiotic delivery might result in improved killing of live cells within any biofilm that does form. Taken together this improved antibiotic efficacy would result in increased wound healing.



*Figure 6.1.* Schematic representation of how HBO could facilitate wound healing by the modification of antibiotic delivery and efficacy. HBO increases pO<sub>2</sub> which results in increased neoangiogenesis, facilitating improved delivery of antibiotics to the wound. Increased pO<sub>2</sub> also facilitates ROS/RNS production by immune cells which results in damage to the QS signalling molecules that influence biofilm formation. The combination of ROS and antibiotics results in increased killing of biofilm cells and ROS/RNS damages β-lactamase enzymes. Together these effects increase antibiotic efficacy, which, along with improved delivery, leads to improved wound healing.

One of the critical components in the treatment regimens of DFUs is the prescription of antibiotics. It is crucial that the correct drugs are prescribed, at optimal doses, at the correct time and herein lies a potential hindrance to wound healing. This study, along with others has highlighted the drawbacks of relying on conventional culture methods to identify microorganisms in non-healing wounds. Antibiotic prescribing protocols (Appendix 7) therefore, are based on providing broad coverage rather than targeting key species and the lack of evidence supporting antibiotic therapy for the treatment of non-healing wounds is of concern (O'Meara *et al.* 2000). It is possible that this approach clears wound flora that have no significant detrimental role, removing competition which then allows the proliferation of pathological species. Wound ischaemia might result in wounds receiving only sub-inhibitory concentrations of antibiotics which may be conducive to biofilm formation rather than detrimental (Hoffman *et al.* 2005). HBOT, by improving antibiotic delivery, might reduce this possibility. There are wound treatment centres that now utilise a different approach to the prescribing of antibiotics to treat non-healing wounds. Molecular methods are used to optimise the identification of wound microflora so that antibiotic regimens can be formulated that are specific to each individual wound, targeting key species. These changes have resulted in improved healing rates (62.4% compared to 48.5%) in wounds that had antibiotic prescriptions based on personalised molecular diagnostics (Dowd *et al.* 2011).

The audit of wound microflora during HBOT in this study (Chapter 3) highlighted the complexity of changing bacterial and fungal communities over time and supported the focused approach achievable through accurate identification by molecular methods. It was not possible to draw any definitive conclusions regarding wound microflora and non-healing as no clear correlations were

detected. Bacterial population changes did occur over time but whether these were due to HBOT or other factors, such as antibiotic use, could not be determined. One of the key observations from this study was that chronic DFUs are colonised by a broad range of bacteria and fungi and the challenge is to identify which species are pathological in this environment. It is to be expected that certain species, ubiquitous in the environment around us, are found in wounds; whether they delay healing in that wound needs to be determined. A wound is a breach in one of our defences against infection and this allows the opportunity for commensal and commonplace organisms to become potentially harmful. Fungi, in particular, have been largely ignored in chronic wounds and this study has reiterated that fungi do colonise these wounds. As discussed in Section 5.4., there is evidence to suggest that the presence of fungi does delay healing (Heald *et al.* 2001). So, perhaps it is time for clinicians to reassess their approach to the investigation and treatment of infected non-healing wounds, to encompass a molecular approach to diagnostics and to include fungi in their search for colonising organisms.

### 6.3. *Summary of recommended further work*

It would be valuable to separate the two  $\beta$ -lactamases in penicillinase and investigate further the differential activity following exposure to ROS. The site of ROS damage/modification could be determined with mass spectroscopy. An improved protocol for the synthesis of ONOO<sup>-</sup>, yielding sufficient quantity and concentration of product would allow further investigation into the effects of this RS on penicillinase. This work from this study could be extended by investigating a range of  $\beta$ -lactamases from other classes and from different bacterial species.

Future work could examine the effects of other ROS/RNS, such as ONOO<sup>-</sup>, on AHLs, and to investigate the relationship between damaged AHLs and the formation of biofilms. For example, mutant strains of *P. aeruginosa* that do not produce endogenous AHLs could be incubated in media containing HOCl exposed synthetic AHLs, in conditions that allow quantification of biofilm mass. This work could also be extended to include QS signalling molecules utilised by Gram-positive bacteria. Performing MS-MS on AHLs exposed to H<sub>2</sub>O<sub>2</sub> could confirm whether oxidation was taking place.

In relation to biofilms, further work could investigate the effect of ROS and antibiotics on the biofilms of a greater diversity of bacteria, in both mono-species and multiple-species biofilms, including both antibiotic sensitive and resistant strains. It would also be useful to include a pressure component to the oxygen environment, equivalent to that experienced by wound tissue during a session of HBOT. Carrying out microscopy on forming and pre-formed biofilms would be valuable to visualise changes in biofilm architecture following exposure to damaged AHLs or ROS/antibiotic combinations. Using selective stains could ascertain whether changes are occurring to live cells or matrix components. Including fungal species in the work discussed above would be beneficial and could include mixed-species biofilms of bacteria and fungi.

Continuing to investigate wound microflora population dynamics, with molecular techniques, over time would be valuable, which may include a course of HBOT or with conventional treatment only, to further determine how these populations change and whether there are any correlations with healing. In the case of HBOT, a greater number of participating patients, possibly via a multi-centre collaboration, would provide more statistically relevant data. Every attempt should be made to include non-HBOT control wounds. It would be valuable to

include analysis of fungal species present at every sampling point, as well as bacteria to build a more detailed picture of fungal colonisation of non-healing wounds over time. Wound biopsies would be valuable for the visualisation of *in situ* biofilms using microscopy techniques. Screening of wound exudates for the presence and identification of AHLs would also be beneficial.

## Appendix 1

Calculation to determine catalase activity:

$$\text{Catalase activity} = \frac{\Delta A_{240} \text{ min}^{-1}}{40 \times \text{mg enzyme ml}^{-1} \text{ reaction mixture}}$$

$$\epsilon \text{ of H}_2\text{O}_2 = 40 \text{ mM}^{-1} \text{ cm}^{-1}$$

## Appendix 2

Initial rate of penicillinase activity

$$\text{Initial rate} = b \times (a + y^0 - c)$$

Where  $c$  = absorbance of CENTA only.

### Appendix 3

Calculation to determine LPO activity:

$$\text{LPO activity} = \frac{\Delta A_{350} \text{ min}^{-1}}{26 \times \text{mg enzyme ml}^{-1} \text{ reaction mixture}}$$

$$\epsilon \text{ of triiodide} = 26 \text{ mM}^{-1} \text{ cm}^{-1}$$

## Appendix 4

Concentrations of HOCl were calculated from the standard curve using the following equation:

$$\text{Concentration of HOCl} = \frac{y-a}{b}$$

$y$  = absorbance

$a$  =  $y$  intercept

$b$  = slope

## Appendix 5

### Amino acid sequences

#### Beta-lactamase I

1 milknkrmlk igicvgilgl sitsleaftg eslqveakek tgqvkhnqa thkefsqlek  
61 kfdarlgvya idtgnqtis yrpnerfafa stykalaagv llqqnsidsl nevitytked  
121 lvdyspvték hvdgtmklge iaeaavrssd ntagnilfnk iggpkyeka lrhmgdritm  
181 snrfetelne aipgdirtds takaiatnlk aftvgnalpa ekrkiltewm kgnatgdkli  
241 ragiptdwvv gdksgagsyg trndiavvwp pnsapiivli sskdekeaiy ndqliaeatk  
301 vivkgs

#### Beta-lactamase II

1 mkkntllkvg lcvllgtiq fvstissvqa sqkvektvik netgtisisq lnknvwwhte  
61 lgsfngeavp snglvntsk glvldsswd dkltkeliem vekkfqkrvt dviithahad  
121 riggiktlke rgikahstal taelakkngy eepglqlqv tnlkfgnmkv etfypgkght  
181 ednivwwlpq ynilyggclv kstsakdlgn vadayvnews tsienvlkry rninavvpgh  
241 gevgdkglll htldllk

**Application for NHS Research and Development  
Approval**

**Changes in Diabetic Foot Ulcer Wound  
Microbiology in Patients Undergoing  
Hyperbaric Oxygen Therapy**

## Study Group

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# Changes in Diabetic Foot Ulcer Wound Microbiology in Patients Undergoing Hyperbaric Oxygen Therapy

## Aims

The primary aim is to qualitatively characterise the change in wound microbiology in chronic diabetic foot ulcers as a result of hyperbaric oxygen therapy. The secondary aim is to compare the microbial community in chronic diabetic foot ulcers, identified by standard cultural analysis, with that identified by molecular analysis.

## Background

Chronic wounds are those which are slow to heal within a normal time span or fail to heal entirely. They remain open, are characterised by persistent inflammation, and do not progress to fully healed wounds. Evidence suggests that underlying pathophysiological conditions, e.g. diabetes [1] and the presence of microbial infection [2] both contribute to a non-healing wound. However, exactly what physiological processes are at work and how they interact with the effects of microbial infection have been poorly defined. Hence, a full and detailed understanding of the microbial communities present in chronic wounds is a valuable tool in deciding which treatment is the most appropriate.

Investigation of the microflora of wounds has been carried out previously, but mainly using traditional culture based techniques (reviewed by Bowler, [3]). Commonly held indicators of infection include a bacterial load of  $10^5$  colony forming units (cfu) per gram of tissue, regardless of species, and identification of key pathogenic organisms [3, 4]. The most commonly isolated organisms from infected wounds are cited as staphylococci, pseudomonads, and beta-haemolytic streptococci. However, this may be due to the ease of culturing these bacteria over fastidious anaerobes, and those in the VBNC<sup>13</sup> state. Indeed, many studies indicate that the anaerobic and difficult to culture wound microbes are prevalent in wounds, and short incubation periods contribute to their being overlooked [3, 5, 6]. However, anaerobes are still often treated as one group, as exemplified by a recent study [2]; a clear hindrance to understanding wound microbiology. Further, differences in methods of sampling wounds may lead to variability in results i.e. swabbing versus tissue samples, and differences in swabbing technique. For example, it is thought that different microbial populations inhabit the upper and lower layers of a wound, and hence tissue biopsies have been used as reference standards when assessing swabbing techniques for accuracy [6, 7, 8]. Further to the identification of key organisms is the appreciation of the interaction between bacterial species and with their environment, rather than the singular effects of individual species. For example, Mayrand and McBride [9] provide evidence of bacterial synergy, with the dependence of *Porphyromonas asaccharolytica* upon *Klebsiella pneumoniae* to cause infection. Further, Trengove *et al.* [10] concluded that the presence of four or more bacterial species was associated with delayed healing in leg ulcers. Trengove also highlighted the dynamic nature of the wound environment; additional bacterial species were detected over time.

Recently, studies of wound microbiology have benefited from the advent of molecular techniques which can overcome the difficulties of culture based analysis: the DNA of the bacteria is identified and sequenced directly, avoiding the need to culture to identify individual species [5]. Further, in a comparison of culture and molecular techniques, molecular methods identified key organisms that resisted identification by culturing [11, 12]. Therefore, use of molecular and culture based techniques in tandem should provide a more comprehensive analysis of wound microbiology.

In treating chronic wounds, evidence suggests that simultaneous treatment with both antibiotics (AB) and hyperbaric oxygen therapy (HBO)<sup>14</sup> is beneficial (reviewed by Roeckl-Wiedmann *et al.* [13]), resulting in a higher incidence of healing, and a reduced rate of amputation for patients with lower limb chronic wounds. It is known that HBO can increase tissue oxygen gradients [14], promote blood vessel proliferation [15], facilitate increased phagocytic killing in hypoxic tissues [16], and inactivate at least one microbial toxin (*Clostridium perfringens* alpha-toxin) [14]. However, the direct (e.g. increased oxygen tensions) and indirect (e.g. increased phagocytic killing) effects of HBO upon the microbial community have not been investigated *in vivo*. Using molecular and culture techniques for the analysis of samples from wound swabs and tissue samples, we hope to identify bacterial species present, and monitor the change in community composition throughout HBO therapy. This information may throw some light onto why a sub-group of patients undergoing HBO do not experience satisfactory wound healing. Further, we hope to determine the accuracy with which culture techniques are able to characterise the microbial population of chronic wounds, compared to molecular techniques. It is realised that the taking of tissue samples is contentious; several studies have previously highlighted the greater range of bacteria found in deeper tissues, compared to surface swabs, however, the goal is usually that of deciding on a cost-effective technique for routine sampling. One of the aims of this study is to characterise, as comprehensively as possible, the bacterial population of chronic wounds, and how composition of that population changes over time, in healing, and non-healing wounds. It is possible that prescribed broad-spectrum antibiotics, which form a component of the treatment regime, are selecting for antibiotic resistance, and failing to target clinically important bacteria.

## Patient Inclusion/Exclusion and Identification

### Patient Inclusion Criteria

- At least one diabetic foot ulcer, regardless of infection status (see Medical History below)
- Over 18 years of age

### Patient Exclusion Criteria

- None

### Patient Identification

Patients attending the Diabetic Foot Clinic (DFC) are referred from the Podiatry Clinic (PC) by Mr. Graham Bruce. Patients will therefore be identified at the PC where they will be provided with a Patient Information Sheet (PIS) and letter asking them to join the study by Mr. Graham Bruce. Patients wishing to join the study will be consented on their first visit to the DFC, by Dr Patrick English, Mr Graham Bruce, or a member of DDRC clinical staff (Ms. Alexandra Bishop or Dr Philip Bryson). Patients will therefore have between 1-3 days to consider joining the study, depending on when their first appointment at the DFC is. No PCT staff will be involved in patient recruitment or consenting.

We aim to recruit approximately 20 patients, with ca. 25% eventually referred for HBO after their initial DFC treatment, providing a test group of 5 patients.

### Medical history

In order to interpret the data obtained from this study in the correct context we will collect specific information from the patients' DDRC medical notes. A number of factors may influence the microbiology of the patient's wound, but as this is a prospective study it would be

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HBO is a therapy whereby a patient intermittently breathes 100% oxygen whilst in a chamber at a pressure greater than that at sea level.

impractical and in certain cases unethical to control them. Hence, to account for these variables we will obtain information about each patient's:

- Underlying medical condition (e.g. diabetes, hypertension, osteomyelitis)
- Recent treatments (e.g. AB or other drugs, wound dressing, wound debridement, prescribed footwear, repeat HBO)
- Recent blood glucose (Hb<sub>A1C</sub>, see *Protocol: Blood Glucose Monitoring* below)
- Social factors (e.g. mobility, smoking)
- Transcutaneous oxygen monitoring (TCOM)<sup>15</sup> measurements

## Protocol

### Wound Sampling

Wounds will be sampled using both swabs and tissue sampling. Swabbing will be carried out by the DDRC Research Nurse at the DFC. Swabbing at the DDRC will be carried out by the DDRC Research Nurse or by other DDRC nurses charged with caring for the patient. Swabbing will be carried out using the Levine technique (6), as this technique provides swabs most representative of the wound microflora and will ensure all swab samples are taken in the same manner.

Tissue sampling involves removing a small amount of wound bed material with a scalpel. This is a normal method of wound sampling, and is usually carried out during routine wound debridement<sup>16</sup>. The DDRC Research Nurse, other DDRC nurses, and Mr. Graham Bruce are competent in this technique. The wound tissue sample will only be taken if it is appropriate to do so<sup>17</sup>.

Swabbing will take place at named 'swabbing events' (see below and Fig 1). At specified swabbing events, two swabs will be taken; one will be sent to Derriford Microbiology Laboratory for standard cultural analysis, the other will be frozen at -80 °C and stored at the DDRC to await molecular analysis (see below, *Wound Sample Analysis*). Where only one swab is taken, this will be used for molecular analysis alone. At the points indicated (Fig 1) one tissue sample will be taken, which will be divided in two, one piece of tissue going to Derriford Microbiology Laboratory for cultural analysis, the other to await molecular analysis.

At all swabbing events, the state and size of the wound will be recorded using Visitrak<sup>®</sup> (Smith and Nephew), currently used as standard by staff at the DDRC. Visitrak<sup>®</sup> is an acetate tracing system which records the 2D wound surface area.

### Patient Treatment and Sampling Schedule

Patients participating in this study will follow their normal treatment regimen, as set out by their doctor. Whether a patient is referred for HBO or not is a clinical decision independent from participation in this study. Importantly, the treatment and sampling schedule set out below and in Fig 1 are a guideline only; patients differ in their treatment (e.g. type of antibiotic/dressing, length of treatment) and so the following schedule will be used as a model, with a degree of flexibility.

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A non-invasive means of measuring tissue oxygenation

16

The removal of devitalised and dead tissue from the wound, which would otherwise impair healing.

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As with any treatment, the sampling of a wound is implicitly dependent upon its healing state; we will not sample a wound where doing so will cause unnecessary harm to the patient. For tissue sampling, this may be where the previous tissue sampling site has not healed, or where there are clear signs of granulation tissue and wound healing (e.g. a significant reduction in wound size), or if the wound is an awkward shape (e.g. too deep and narrow). Ultimately, this decision is the responsibility of the clinical staff taking the sample.

Following agreement to take part in this study, two swabs and a tissue sample will be taken at the DFC on the patient's initial visit, and they will begin their treatment regime. Treatment prescribed by the DFC may consist of dressings and antibiotic therapy, the length of which is usually around four weeks. After a further four weeks, the patient returns to the DFC for assessment. Here, another swab and tissue sample will be taken. At this point, assessment of the patient's wound by the DFC doctor determines whether the patient would benefit from HBO. Hence, the patients divide into two separate groups for the purposes of the study.

Non-HBO referred patients, or those choosing not to receive HBO therapy, will continue with their prescribed treatment regimes, and will have two wound swabs taken after three weeks or when they return to the DFC. Further swabs and tissue samples will then be taken every 2-4 weeks at times convenient to the patient, i.e. when they make a routine visit to the DFC. Treatments for patients in this group may involve a change in antibiotic therapy, vascular surgery, or non-intervention. The latter group will form the most applicable control group for HBO treated patients.

Patients are referred for HBO at the DDRC as a final attempt to heal the wound. Each HBO-referred patient will serve as their own control from all swabs and tissue samples prior to beginning HBO. Upon referral, patients visit the DDRC for assessment (usually around one week after referral). HBO treatment begins the following week. Two swabs and a tissue sample will be obtained immediately prior to the first treatment. Patients usually receive 40 HBO treatments over eight weeks (one per weekday). Consequently, swab samples will be taken after treatment 2, 5, 7, 10, 15, 20, 30, and 40, with two swabs being taken after treatment 10 and 40, if appropriate. Tissue samples will be taken after treatments 5 and 10. Sampling is clustered around the first four weeks of HBO treatment to reveal any immediate changes in wound microflora as a result of the treatment.

In total, each patient referred for HBO will undergo at least 11 swabbing sessions consisting of 14 swabs and 5 tissue samples; each non-referred patient will undergo at least 3 swabbing sessions consisting of 5 swabs and 2 tissue samples. Swab sampling will therefore provide a time-course for changes in the microbial community, both prior to, and during subsequent treatment regimes. Tissue samples will provide a comparative and hopefully, more comprehensive audit of lower limb wound microbiology.

### Wound Sample Analysis

Molecular analysis of the samples will be by Polymerase Chain Reaction DNA amplification and Denaturing Gradient Gel Electrophoresis (PCR-DGGE) using universal primers for rDNA, producing a DNA fingerprint of the wound. Corresponding isolates from each swab-culture will also be run alongside PCR products from the same swab to aid in bacterial identification (they will provide a ladder). Unmatched bands will be identified by sequence analysis e.g. a sequenced band on an initial gel identified as *Pseudomonas aeruginosa* can be matched up to a corresponding band on another gel by virtue of its position, without having to sequence it. Hence, a time course of DNA fingerprints will be generated, comparing the microbial community before and during HBO treatment in each patient.

Comparisons in the microbial community will be made between the healing and non-healing patients in the periods prior to, and during, HBO treatment. Further, the community elucidated from the DNA fingerprinting will be compared to that identified by hospital standard culture methods.

Formal statistical analysis is not feasible for this study due to the low patient numbers involved (please see enclosed letter). Descriptive statistics will be included in the final analysis.

### Blood Glucose Monitoring

As part of the normal treatment plan of DFC patients, an Hb<sub>A1C</sub> test is carried out (through a blood sample). This provides a good indication of the patient's blood glucose levels over the past three months. Blood glucose levels could have an impact upon the microbiology of wounds, especially in diabetic patients, therefore, each patient will have their Hb<sub>A1C</sub> taken at the beginning (by Mr Graham Bruce as part of their regular treatment at the PC), and at the

end of the study, (by the DDRC Research Nurse). However, if the patient has had a recent Hb<sub>A1C</sub> test, they will only have one taken at the end.

### **TCOM**

Patients have TCOM measurements recorded routinely during the first HBO treatment to assess vascular competence. Measurements will be taken every two weeks during HBO treatment to quantify changes to tissue oxygenation in the area around the wound. It will not be practical to perform TCOM in non-HBO patients. TCOM will be performed during compression, if possible, as this will provide the most accurate assessment of tissue oxygenation levels achieved during treatment sessions.

### **Patient Impact**

No data from this study will impact upon the patient's care, other than data normally obtained throughout the course of the patient's treatment (typically this would include an initial Hb<sub>A1C</sub> test and swabs and tissue samples throughout the patient's care). All molecular analysis of samples will be carried out after the sampling period of the study is finished. Further, all clinical decisions will remain independent of the study.

### **Research Governance**

This research will be a joint project between the DDRC, University of Plymouth, and Plymouth Hospitals NHS Trust. Patients participating in this study are initially under the care of the NHS trust whilst at the PC and DFC, but during HBO they are under the care of the DDRC. Hence, the PHNT will be responsible for patient care at the PC and DFC, and for the scientific review process. The DDRC will be responsible for collecting the samples and patient information throughout the study and the University of Plymouth will be responsible for sample and data analysis, and scientific knowledge.

### **Funding**

The DDRC and the University of Plymouth are jointly responsible for funding of the Ph.D. as the thesis is supervised by both organisations. Total funding for the Ph.D. is £50,000 over three years. Funding for this specific project will be provided by DDRC. Costs incurred by this project and the organisation liable are proposed below:

- DDRC Research nurse time (consenting, swabbing, travelling), *DDRC* - £1,400
- Swabs, scalpels, tissue containers and other disposables, *DDRC* - £400
- Microbiological analysis of swabs at Derriford Microbiology Laboratory, *DDRC* - £1,300
- Molecular analysis of swabs at the university, DDRC and University of Plymouth - £3,000

All patients participating in this study will be undergoing normal therapies, as prescribed by their doctor, and as such the DFC or Plymouth Hospitals NHS Trust will accrue no extra costs associated with this study.

### **Patient Data Handling**

The DDRC Research Nurse will be responsible for collecting all patient information required for the study. All patient information and wound samples will be stored in an anonymised form, using a DDRC patient identification number. Samples will only be able to be matched up to patients by the DDRC nurse. Researchers will only be able to identify samples by the DDRC patient identification number. Anonymised data will be stored by the Chief Investigator for five years.

### **Health and Safety**

Risk assessments will be carried out at the appropriate stages of the study. All DNA extractions will be performed at DDRC where there is hospital-grade, clinical waste disposal facilities.

### **Publishing**

It is the intention of the Research Group to publish the data obtained from this study. It will also form part of a Ph.D. thesis.

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## Appendix 7

A summary of antibiotic prescribing criteria for diabetic foot ulcers, taken from Infection Therapy ([www.plymouthdiabetes.org](http://www.plymouthdiabetes.org)). IV-intravenous.

<b>Grade of infection</b>	<b>First antibiotic choice</b>	<b>Duration</b>
Mild	Oral Flucloxacillin	1-2 weeks
Moderate	Oral Levofloxacin Oral Clindamycin	2-4 weeks
Severe	IV Teicoplanin Oral/IV Rifampicin Oral/IV Levofloxacin Metronidazole or Piperacillin	2-4 weeks
Bone and joint	Oral Levofloxacin Oral Clindamycin	2-12 weeks depending on viable/non-viable bone

## **Appendix 8**

Accession numbers and identity similarity for bacteria found in this study.

<b>Genus</b>	<b>Species</b>	<b>Accession number</b>	<b>Maximum identity (%)</b>
<b><i>Achromobacter</i></b>	<i>xylosoxidans</i>	NC 014640.1	99
<b><i>Alcaligenes</i></b>	multiple	multiple	86-89
<b><i>Bradyrhizobium</i></b>	<i>Sp 523321</i>	NC 017082.1	99
<b><i>Corynebacterium</i></b>	<i>aurimucosum</i>	NC 012590.1	98
	<i>diphtheriae</i>	NC 016802.1	96
<b><i>Enterobacter</i></b>	<i>cloacae</i>	NC 018405.1	97-99
<b><i>Enterococcus</i></b>	<i>faecalis</i>	FP 929040.1 NC 019770.1	98-99 99
	<i>faecium</i>	NC 020207.1	99-100
<b><i>Fusobacterium</i></b>	<i>nucleatum</i>	NC 003454.1	97
<b><i>Klebsiella</i></b>	<i>oxytoca</i>	NC 018106.1	92
<b><i>Lactobacillus</i></b>	<i>plantarum</i>	NC 020229.1	99
<b><i>Microbacterium</i></b>	<i>testaceum</i>	NC 015125.1	99
<b><i>Micrococcus</i></b>	<i>luteus</i>	NC 012803.1	99
<b><i>Morganella</i></b>	<i>morganii</i>	NC 020418.1	99
<b><i>Porphyromonas</i></b>	<i>gingivalis</i>	NC 015571.1	85
<b><i>Prevotella</i></b>	<i>dentalis</i>	NC 019968.1	90
<b><i>Propionibacterium</i></b>	<i>acnes</i>	NC 018707.1 or 006085.1	99
<b><i>Providencia</i></b>	<i>stuartii</i>	NC 017731.1	98
<b><i>Pseudomonas</i></b>	<i>multiple</i>	multiple	83-100
	<i>aeruginosa</i>	NC 018080.1	97-100
<b><i>Saccharomonospora</i></b>	<i>glauca</i>	CM 001484.1	100
<b><i>Serratia</i></b>	<i>proteamaculans</i>	NC 009832.1	98-99
<b><i>Staphylococcus</i></b>	<i>aureus</i>	NC 009632.1	91
		multiple	99
	<i>epidermidis</i>	multiple	98
	<i>warneri</i>	NC 020164.1	93
<b><i>Stenotrophomonas</i></b>	<i>maltophilia</i>	NC 015947.1	95-99
<b><i>Streptococcus</i></b>	<i>agalactiae</i>	NC 019048.1	99
<b><i>Streptomyces</i></b>	multiple	multiple	100

## Appendix 9

Accession numbers and identity similarity for fungi found in this study

<b>Genus</b>	<b>Species</b>	<b>Accession number</b>	<b>Maximum identity</b>
<b><i>Aspergillus</i></b>	Multiple	Multiple	99-100
	<i>penicillinoides</i>	gi 12862455 AB002078.1	99
<b><i>Candida</i></b>	<i>parapsilosis</i>	Multiple strains	100
<b><i>Chaetomium</i></b>	<i>elatum</i>	Multiple strains	99
<b><i>Cladosporium</i></b>	<i>cladosporioides</i>	gi 408474862 JX470338.1	99
		or gi 408474860 JX470336.1	99
<b><i>Fusarium</i></b>	<i>oxysporum</i>	Multiple strains	99
<b><i>Microdochium</i></b>	<i>bolleyi</i>	gi 296410172 HM216190.1	99
	<i>nivale</i>	gi 33334391 AF548077.1	100
<b><i>Penicillium</i></b>	Multiple	Multiple	99
	<i>argillaceum</i>	gi 19909869 AB033534.1	97
<b><i>Phoma</i></b>	<i>herbarum</i>	or gi 15822823 AB032120.1	97
		Multiple strains	99
<b><i>Saccharomyces</i></b>	<i>cerevisiae</i>	gi 120971643 EF153845.1	99
<b><i>Sporobolomyces</i></b>	<i>roseus</i>	gi 111283898 DQ832235.1	99
		or gi 340536084	
		HQ913900.1	

## Appendix 10

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G 5  GCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAG 64
    |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K 1  GCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAG 60

G 65 TAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGC 124
    |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K 61 TAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGC 120

G 125 CAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC 184
    |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K 121 CAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC 180

G 185 GCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCC 244
    |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K 181 GCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCC 240

G 245 AAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCCTGTGTAGCGGTGAAATG 304
    |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K 241 AAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCCTGTGTAGCGGTGAAATG 300

G 305 CGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTG 364
    |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K 301 CGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTG 360

G 365 AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG 424
    |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K 361 AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG 420

G 425 ATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGA 484
    |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
K 421 ATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGA 480

G 485 CCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTC 518
    ||||  |||||||||||||||||||||||||||
K 481 CCGCENNNGGAGTACGGCCGCAAGGTTAAAACTC 514

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Alignment of sequences from DGGE bands G and K from wound 1a.

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G 1  CGTGCAGGATGACGGCCCTATGGGTTGTAAACTGCTTTTATGCGGGGATAAAGTGCGCGA 60
    |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
E 5  CGTGCAGGNGACGGCCCTATGGGTTGTAAACTGCTTTTATGCGGGGATAAAGTGCGCGA 64

G 61 CGTGTCGTGCATTGCAGGTACCGCATGAATAAGGACCGGCTAATTCCGTGCCAGCAGCCG 120
    |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
E 65 CGTGTCGTGCATTGCAGGTACCGCATGAATAAGGACCGGCTAATTCCGTGCCAGCAGCCG 124

G 121 CGGTAATACGGAAGGTCCGGGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGGCC 180
    |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
E 125 CGGTAATACGGAAGGTCCGGGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGGCC 184

G 181 GCCAGGTAAGCGTGTTGTGAAATGTACCGGCTCAACCGGTGAATTGCAGCGCGAACTGTC 40
    ||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
E 185 GCNAGGTAAGCGTGTTGTGAAATGTACCGGCTCAACCGGTGAATTGCAGCGCGAACTGTC 244

G 241 TGGCTTGAGTGCACGGTAAGCAGGCGGAATTCATGGTGTAGCGGTGAAATGCTTAGATAT 00
    |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
E 245 TGGCTTGAGTGCACGGTAAGCAGGCGGAATTCATGGTGTAGCGGTGAAATGCTTAGATAT 304

G 301 CATGAAGAACTCCGATTGCGAAGGCAGCTTGCTGCAGTGC GACTGACGCTGATGCTCGAA 360
    |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
E 305 CATGAAGAACTCCGATTGCGAAGGCAGCTTGCTGCAGTGC GACTGACGCTGATGCTCGAA 364

G 361 GGTGCGGGTATCAAACAGGATTAGATACCCTGGTAGTCCGCACGGTAAACGATGGATGCC 420
    |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
E 65  GGTGCGGGTATCAAACAGGATTAGATACCCTGGTAGTCCGCACGGTAAACGATGGATGCC 424

G 421 CGCTGTCCGCCCTTTGTGGCGGGTGGCCAAGCGAAAGCGTTAAGCATCCCACCTGGGGA 480
    |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
E 425 CGCTGTCCGCCCTTTGTGGCGGGTGGCCAAGCGAAAGCGTTAAGCATCCCACCTGGGGA 484

G 481 GTACGCCGGCAACGGTGAAACTCAA 505
    |||||||  |||||||  |||||||  |||||||  |||||||
E 485 GTACGCCGGCAACGGTGAAACTCAA 509

```

Alignment of sequences from DGGE bands G and E from wound 3.

```

G 1  CGTGCAGGATGACGGCCCTATGGGTTGTAAACTGCTTTTATGCGGGGATAAAAGTGCGCGA 60
|
F 12 CGTGCAGGATGACGGCCCTATGGGTTGTAAACTGCTTTTATGCGGGGATAAAAGTGTGCGA 71
|
G 61 CGTGTCGTGCATTGCAGGTACCGCATGAATAAGGACCGGCTAATTCCGTGCCAGCAGCCG 120
|
F 72 CGTGTCGTGCATTGCAGGTACCGCATGAATAAGGACCGGCTAATTCCGTGCCAGCAGCCG 31
|
G 121 CGGTAATACGGAAGGTCCGGGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGGCC 180
|
F 132 CGGTAATACGGAAGGTCCGGGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGGCC 191
|
G 181 GCCAGGTAAGCGTGTTGTGAAATGTACCGGCTCAACCGGTGAATTGCAGCGCGAACTGTC 240
|
F 192 GCCAGGTAAGCGTGTTGTGAAATGTACCGGCTCAACCGGTGAATTGCAGCGCGAACTGTC 251
|
G 241 TGGCTTGAGTGCACGGTAAGCAGGCGGAATTCATGGTGTAGCGGTGAAATGCTTAGATAT 300
|
F 252 TGGCTTGAGTGCACGGTAAGCAGGCGGAATTCATGGTGTAGCGGTGAAATGCTTAGATAT 311
|
G 301 CATGAAGAACTCCGATTGCGAAGGCAGCTTGCTGCAGTGC GACTGACGCTGATGCTCGAA 360
|
F 312 CATGAAGAACTCCGATTGCGAAGGCAGCTTGCTGCAGTGC GACTGACGCTGATGCTCGAA 371
|
G 361 GGTGCGGGTATCAAACAGGATTAGATACCCTGGTAGTCCGCACGGTAAACGATGGATGCC 20
|
F 372 GGTGCGGGTATCAAACAGGATTAGATACCCTGGTAGTCCGCACGGTAAACGATGGATGCC 431
|
G 421 CGCTGTCCGCCCTTTGTGGCGGGTGGCCAAGCGTAAAGCATCCCACCTGGGGGA 480
|
F 432 CGCTGTCCGCCCTTTGTGGCGGGTGGCCAAGCGTAAAGCATCCCACCTGGGGGA 491
|
G 481 GTACGCCGGCAACGGTGAAACTCAAG 506
|
F 492 GTACGCCGGCAACGGTGAAACTCAAG 517

```

Alignment of sequences from DGGE bands G and F from wound 3.

```

H 6  CGTGCAGGATGACGGCCCTATGGGTTGTAAACTGCTTTTATGCGGGGATAAAGTGCGCGA 65
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
F 12  CGTGCAGGATGACGGCCCTATGGGTTGTAAACTGCTTTTATGCGGGGATAAAGTGTGCGA 71

H 66  CGTGTTCGTGCATTGCAGGTACCGCATGAATAAGGACCGGCTAATTCCGTGCCAGCAGCCG
125
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
F 72  CGTGTTCGTGCATTGCAGGTACCGCATGAATAAGGACCGGCTAATTCCGTGCCAGCAGCCG 31

H 26  CCGTAATACGGAAGGTCCGGGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGGCC 185
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
F 132  CCGTAATACGGAAGGTCCGGGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGGCC 91

H 186  GCCAGGTAAGCGTGTTGTGAAATGTACCGGCTCAACCGGTGAATTGCAGCGCGAACTGTC 245
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
F 192  GCCAGGTAAGCGTGTTGTGAAATGTACCGGCTCAACCGGTGAATTGCAGCGCGAACTGTC 251

H 246  TGGCTTGAGTGACCGGTAAGCAGGCGGAATTCATGGTGTAGCGGTGAAATGCTTAGATAT 305
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
F 252  TGGCTTGAGTGACCGGTAAGCAGGCGGAATTCATGGTGTAGCGGTGAAATGCTTAGATAT 311

H 306  CATGAAGAACTCCGATTGCGAAGGCAGCTTGCTGCAGTGC GACTGACGCTGATGCTCGAA 365
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
F 312  CATGAAGAACTCCGATTGCGAAGGCAGCTTGCTGCAGTGC GACTGACGCTGATGCTCGAA 371

H 366  GGTGCGGGTATCAAACAGGATTAGATACCCTGGTAGTCCGCACGGTAAACGATGGATGCC 425
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
F 372  GGTGCGGGTATCAAACAGGATTAGATACCCTGGTAGTCCGCACGGTAAACGATGGATGCC 431

H 426  CGCTGTCCGCCCTTTGTGGCGGGTGGCCAAGCGAAAGCGTTAAGCATCCCACCTGGGGA 485
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
F 432  CGCTGTCCGCCCTTTGTGGCGGGTGGCCAAGCGAAAGCGTTAAGCATCCCACCTGGGGA 91

H 486  GTACGCCGGCAACGGTGAAACTCAA 510
      ||||||||||||||||||||||||||||
F 492  GTACGCCGGCAACGGTGAAACTCAA 516

```

Alignment of sequences from DGGE bands H and F from wound 3.

## Appendix 11

Equation to calculate mean % reduction in population growth for MICs:

$$\text{Mean \% reduction} = [1 - ((A_T - A_{T0}) / (A_G - A_{G0}))] \times 100$$

$A_T$  = mean absorbance of test wells at the end of incubation

$A_{T0}$  = mean absorbance of test wells before incubation

$A_G$  = mean absorbance of growth wells at the end of incubation

$A_{G0}$  = mean absorbance of growth wells before incubation

## Appendix 12

Equation to calculate the mean % reduction in live cells in biofilms:

$$\text{Mean \% reduction}^* = (1 - ((A_{T570} - (A_{T600} \times R_0)) / (A_{G570} - (A_{G600} \times R_0)))) \times 100$$

$A_{T570}$  = mean absorbance of test wells at 570 nm

$A_{T600}$  = mean absorbance of test wells at 600 nm

$A_{G570}$  = mean absorbance of growth wells at 570 nm

$A_{G600}$  = mean absorbance of growth wells at 600 nm

$$R_0 = AO_{570} / AO_{600}$$

$AO_{570}$  = absorbance of oxidised resazurin plus medium at 570 nm minus the absorbance of medium only at 570 nm

$AO_{600}$  = absorbance of oxidised resazurin plus medium at 600 nm minus the absorbance of medium only at 600 nm

\* reduction in this context refers to the reduction in absorbance as an indirect measure of live cell population not to the redox state of resazurin

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