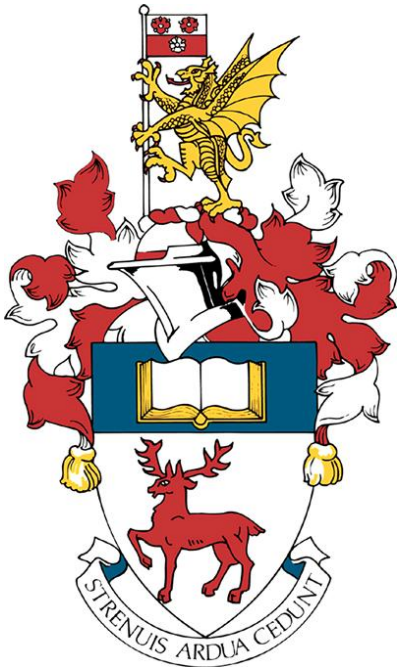

University of Southampton

Faculty of Natural and Environmental Sciences

School for Biological Sciences

Investigation into Novel Nitric Oxide Based Anti-Biofilm Strategies to Target *Pseudomonas aeruginosa* Infection in Cystic Fibrosis

by

Odel Soren

Thesis for the degree of Doctor of Philosophy

January 2019

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ABSTRACT

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Pseudomonas aeruginosa infection in the cystic fibrosis (CF) lung poses an immense therapeutic burden due the formation and tolerance of biofilms. Conventional antibiotics are not effective in tackling *P. aeruginosa* biofilm growth leading to many patients developing chronic infection which negatively impacts on quality of life and prognosis. The recent discovery of nitric oxide (NO) as a *P. aeruginosa* biofilm dispersal molecule had enabled the prospect of novel NO-based anti-biofilm treatments to be developed. The main focus of this study was to further investigate the relationship between NO and *P. aeruginosa*, with emphasis specifically on biofilms formed by clinical CF isolates, and to conduct investigations into potential new NO-based antibiofilm strategies.

By way of using a series of microbiological techniques including multiple phenotyping assays, biofilm culturing, molecular based methods, and mechanistic investigations, this study showed the response of biofilms formed by CF isolates of *P. aeruginosa* to NO is varied. In fact, opposing results were shown whereby isolates that demonstrated good *in vitro* biofilm growth were dispersed, and isolates that demonstrated poorer *in vitro* biofilm growth showed evidence of biofilm promotion with NO donor sodium nitroprusside (SNP). Interestingly, no obvious genetic adaptation to the NO signal was demonstrated following repeated pre-exposure to NO, and analysis of the c-di-GMP levels showed that levels were still reduced in an isolate that had biofilm formation promoted with NO, suggesting a more complex underlying cause and multiple factors to be in play.

Two novel approaches to tackling *P. aeruginosa* biofilms were investigated in this study, one exploring the use of a group of NO-releasing prodrugs termed cephalasporin-3'-diazeniumdiolates (C3Ds), and a second looking at the use of a novel, portable NO-generating prototype device. With the latter, although we were able to demonstrate the prototype device was effective in producing gaseous NO, biofilm dispersal was not demonstrated when utilised in an *in vitro* system, and further studies are needed to determine if this device could be utilised as part of CF management.

The C3D prodrugs were designed as a targeted pharmaceutical alternative to gaseous NO and to limit systemic exposure. Biochemical assays demonstrated the selectivity and targeted NO release from multiple C3D analogues in the presence of bacterial specific enzyme β-lactamase. Initial lead compound DEA-CP (Diethylamine NONOate Cephalosporin Prodrug) was shown to be effective in dispersing biofilms formed by clinical isolates of *P. aeruginosa* and was particularly effective in combination with colistin. Although effective as a dispersal and anti-biofilm agent, DEA-CP did not affect cell viability. Multiple newer-generation C3Ds were also investigated with the aim of developing an 'all-in-one' compound with both anti-biofilm and anti-bacterial activity. Results from antimicrobial susceptibility testing and detailed biofilm experiments using 3-dimensional microscopy, had identified one compound from the group of analogues to be particularly promising. Compound AMINOPIP2-ceftazidime was shown to be more effective than its parent antibiotic ceftazidime, both in terms of its anti-biofilm action, and anti-bacterial activity when tested against a panel of *P. aeruginosa* CF isolates, and therefore represents a potential new anti-biofilm therapy to be used in CF.

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Research Thesis: Declaration of Authorship

Print name:	Odel Soren

Title of thesis:	Investigation into Novel Nitric Oxide Based Anti-Biofilm Strategies to Target *Pseudomonas aeruginosa* Infection in Cystic Fibrosis

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

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2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
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5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
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Acknowledgements

A sincere thank you to my supervisors Jeremy Webb, Gary Connett, Robert Howlin, and Saul Faust for providing me with the opportunity to work with such an incredible research team in Southampton, and for their help and advice throughout the course of this project. Special thanks also go our colleagues at Imperial College London, for their comments and advice at our regular update meetings, and in particular to Jane Davies for organisation of the Strategic Research Centre and bringing this collaboration together. I would also like to thank the UK CF Trust for the funding of this project and for the networking opportunities and experiences provided by the conferences held by the UK Trust and European CF Society.

A huge thank you to Michael Kelso, Ardeshir Rineh and the team at the University of Wollongong, Australia, responsible for the design and synthesis of the novel compounds investigated in this thesis; to collaborate with you on this project was a pleasure and the support you provided despite being oceans away is deeply appreciated.

I am also grateful for the expert advice and support from Martin Feelisch and Diogo D'Silva with regards to the biochemical assays and use of the chemiluminescence detector. I would also like to thank Dilem Ruhuel, for the help she provided, in particular with the minimum inhibitory concentration experiments, whilst undergoing a summer internship in our lab.

To all the members of the CL2 laboratory in Southampton, I couldn't have imagined a better group of people to work with. The presence of such a large and friendly group made long working days more enjoyable, and in particular I would like to express my thanks to Yuming Cai for our incredibly beneficial late night sciencey conversations.

Special thanks to my family, in particular my parents, aunt, uncle and grandad, for their continued love and support and for always believing in my ability. To my friends, your companionship and the days and nights of fun over these past few years were invaluable and kept me sane despite all the struggles. And finally, thank you to Metin, for providing me with the much-needed emotional support that kept me going and led me to the completion of this thesis.

Definitions and Abbreviations

ABC – Adenosine triphosphate Binding Cassette

AHL – N-acyl L-Homoserine

AI - Autoinducer

AMS – Antimicrobial Susceptibility

ASL – Airway Surface Liquid

AST – Antibiotic Susceptibility Testing

c-di-GMP - bis-(3'-5')-Cyclic dimeric Guanosine Monophosphate

C3D – Cephalosporin-3'-Diazeneiumdiolate

CAMHB – Cation-Adjusted Muller Hinton Broth

CF – Cystic Fibrosis

CFTR – Cystic Fibrosis Transmembrane Regulator protein

CFU – Colony Forming Unit

CLD – Chemiluminescence Detector

CLSI – Clinical Laboratory Standards Institute

CLSM – Confocal Laser Scanning Microscopy

CO_2 – Carbon Dioxide

CV – Crystal Violet

DEA-CP – DiEthylAmine NONOate Cephalosporin Prodrug

DGC – Diguanylate Cyclase

DMSO – Dimethyl Sulfoxide

eDNA – extracellular Deoxyribonucleic acid

ENaC – Epithelial Sodium Channel

EPS – Extracellular Polymeric Substance

ETA – Endotoxin A

FDA – Food and Drug Administration

FEV1 – Forced Expiratory Volume of the first second of exhaled breath

FISH - Fluorescent *In Situ* Hybridisation

FVC – Forced Vital Capacity

gNO – gaseous Nitric Oxide

HBSS – Hanks Buffered Saline Solution

LB – Luria Bertani

LPS – Lipopolysaccharide

MIC – Minimum Inhibitory Concentration

MFP – Membrane Fusion Protein

MRSA – Methicillin Resistant *Staphylococcus aureus*

MSD – Membrane Spanning Domain

NBD – Nucleotide Binding Domain

NO – Nitric Oxide

NO_2 – Nitrogen Dioxide

NO_2^- – Nitrite

NO_3^- – Nitrate

OD – Optical Density

OM – Outer Membrane

OMF/P – Outer Membrane Factor/Protein

ORF – Open Reading Frame

PAS – Per Arnt Sim

PBP – Penicillin Binding Protein

PBS – Phosphate Buffered Saline

PCL – Periciliary Liquid

PCR – Polymerase Chain Reaction

PDE – Phosophodiesterase

PIA – *Pseudomonas* Isolation Agar

PMN – Poly Mononuclear leucocyte

PNA-FISH – Peptide Nucleic Acid Fluorescent *In Situ* Hybridisation

PPHN – Persistent Pulmonary Hypertension of Newborn

PQS – Pseudomonas Quinolone Signal

PTIO – 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (NO scavenger molecule)

QS – Quorum Sensing

RND – Resistance Nodulation-Division

ROS – Reactive Oxygen Species

rpm – Rotations Per Minute

SCV – Small Colony Variant

SNP – Sodium Nitroprusside

SP-A/D – Surfactant Protein A/D

TLR4 – Toll-like Receptor 4

TSA – Tryptone Soya Agar

TXSS – Type X Secretion System (I/II/III/IV/V/VI)

VCl_3 – Vanadium (III) Chloride

Chapter 1 Introduction: The therapeutic burden of *Pseudomonas aeruginosa* in the cystic fibrosis lung

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is the most common fatal genetic condition amongst the Caucasian population, with an incidence of approximately 1 in 2500 births (O'Sullivan and Freedman, 2009). Over 10,000 people are diagnosed with CF in the UK alone, and around 100,000 individuals are affected worldwide (Cystic Fibrosis Trust, 2017; Uchmanowicz et al., 2014). Although a multi organ condition, the most notable consequences of this disease are on the pulmonary system, causing repeated and chronic lung infections which have overwhelming impacts on morbidity and mortality (Ratjen and Döring, 2003).

1.1.1 CFTR and mutations

CF is caused by mutations in the CFTR gene, which is approximately 250kb in size, contains 27 exons, and is located on chromosome 7 (q32.1). (Lubamba et al., 2012; Riordan et al., 1989). CF is an autosomal recessive condition with the highest carrier rate for a fatal disease; approximately 1 in 25 Caucasian people in the UK is a carrier (Bilton, 2008). Although more common in those of Northern European descent, CF is known to occur in individuals from multiple ethnicities and backgrounds (Cutting, 2014).

The CFTR protein is a cAMP activated ATP gated chloride channel belonging to the ATP-binding cassette (ABC) transporter family, containing 1480 amino acids (Lubamba et al., 2012). CFTR is expressed in a range of cells and is primarily located on the apical surface of polarized epithelial cells, notably including bronchial epithelium. CFTR has 2 motifs, each made up of a membrane spanning domain (MSD) and a nucleotide binding domain (NBDs). The MSD usually consists of 6 transmembrane segments, and the two motifs are joined together by a regulatory (R) domain (Sheppard and Welsh, 1999).

The primary function of CFTR is as an ion channel, allowing the transport of chloride ions via passive diffusion down an electrochemical potential gradient (Hwang et al., 2013). CFTR is unique in that it is the only ABC transporter to function as an ion channel, and also possesses enzymatic activity. Binding of ATP to the intracellular NBDs induces a conformational change, dimerization, and causes opening of the channel; subsequent hydrolysation of ATP reverses the conformation change causing disengagement, closing the channel. The gating process occurs by a complex mechanism mediated by the c-AMP dependent phosphorylation activities of protein kinase A (PKA).

With reference specifically to CFTR in the lung, Cl- is exported from the intracellular space of the bronchial epithelial cell into the extracellular space of the airway lumen (Smith et al., 1999). This creates an osmotic gradient, and water follows the movement of the Cl- ions, maintaining the hydration of the airway surface liquid (ASL) present on the extracellular surface of the epithelium. This is crucial for maintenance of regular ciliary function and therefore clearance of inhaled foreign particles and mucus (Munkholm and Mortensen, 2014; Ratjen, 2009)

CFTR also transports bicarbonate (HCO_3^-) ions. In the lung, the secretion of HNO_3^- by the epithelium maintains the alkalinity of the extracellular fluid. In addition to Cl- and HNO_3^-, a third anion known as thiocyanate (SCN^-), has been identified to be transported by CFTR (Riordan, 2008). Thiocyanate is important as it is a secreted precursor into the airway lumen and undergoes oxidation to hypothiocyanite, which has antimicrobial effects in the airway. Additionally, the antioxidant glutathione is also permeant to CFTR (Linsdell, 2014; Linsdell and Hanrahan, 1998).

Secondary functions of CFTR involve interactions with other channels. These include the epithelial sodium channels (ENaC), of which its suppression by CFTR is suggested to act as an additional regulator of airway surface hydration (Riordan, 2008). Additionally, CFTR is known to regulate or control: the outwardly rectifying chloride channel (ORCC), the Ca^{2+} activated chloride conductance (CaCC), the renal outer medullar K^+ (ROMK) channels, the sodium/proton exchanger NHE3 and an aquaporin channel (Riordan, 2008).

Around 2000 mutations of the CFTR gene have been identified, and these are divided into 6 classes (Marangi and Pistritto, 2018), described and summarised in **Figure 1**. Generally, patients with Class I to III mutations tend to have more severe clinical presentations than patients with Class IV to VI mutations (Kreindler, 2011). The most common mutation by far is the class II mutation F508del, whereby a deletion of three nucleotides (CTT) from the genetic coding region results in the loss of the 508th amino acid, phenylalanine, from the CFTR protein (Welsh and Smith, 1995). The omitted phenylalanine from the primary peptide sequence hinders normal folding of the protein, which is recognised by the cells quality control system. Instead of undergoing post translational modification in the endoplasmic reticulum and Golgi apparatus, the defective misfolded protein is proteolytically degraded and never reaches the cell surface (Ratjen, 2009). Hence, this is referred to as an intracellular trafficking defect (Welsh and Smith, 1995). The F508del mutation accounts for around 70% of all mutant alleles, however incidence varies with ethnicity, affecting approximately 82% and 32% of CF patients in Denmark and Turkey respectively (Kreindler, 2011; Ratjen and Döring, 2003).

The frequency of other CFTR mutations also varies substantially with ethnicity. For example, the N1303K mutation is known to be more common amongst ancient Mediterranean populations, with a frequency of approximately 20% in Algeria and the Middle East. In Ashkenazi Jews, the

W1282X mutation is the most common with a frequency of 48% (Bobadilla et al., 2002).

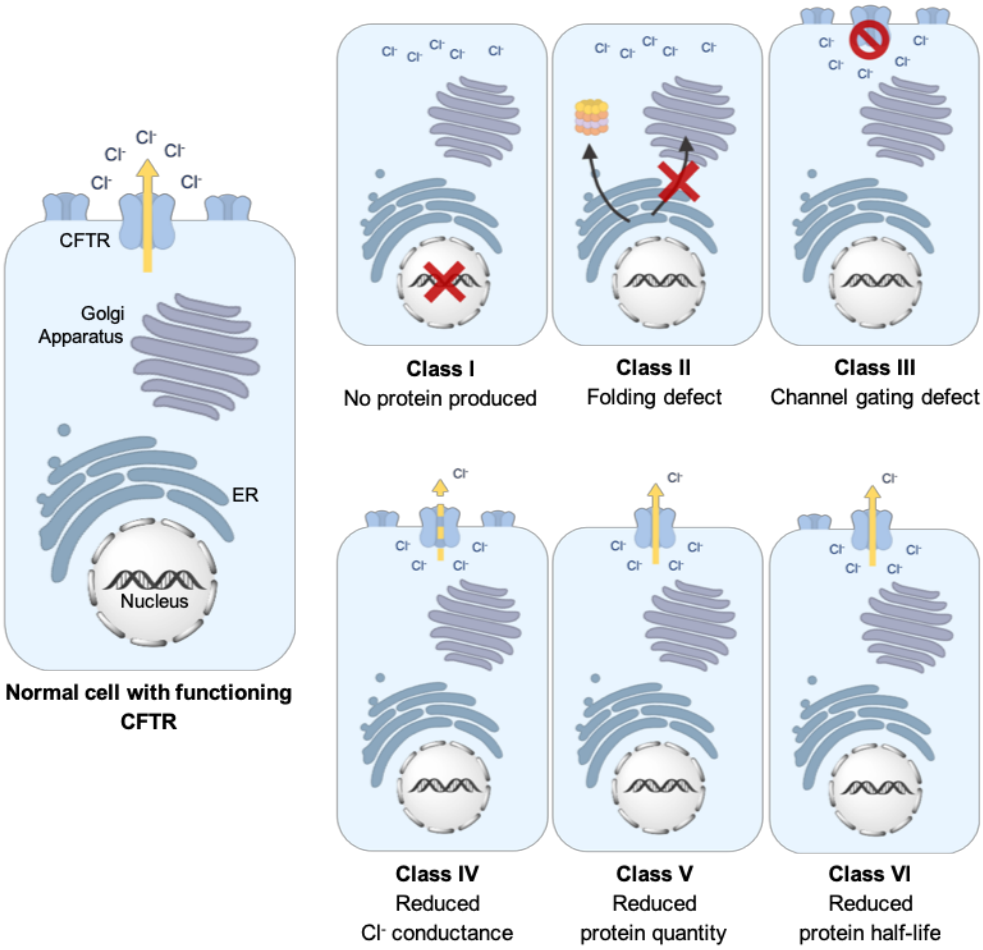


Figure 1. The six classes of CFTR mutations. In a healthy cell (left), the CFTR protein is transcribed, translated and undergoes post-translational modifications in endoplasmic reticulum (ER) and golgi apparatus before reaching its final intended destination at the epithelial apical membrane. Class I mutations are usually large deletions, frame-shift mutations, nonsense mutations or are premature stop codons; transcription does not occur and there is complete lack of CFTR protein. Similarly, with class II mutations, there is no CFTR protein at the membrane, though these are usually missense mutations causing a protein folding defect. Class III, IV, V and VI mutations are often missense mutations and result in CFTR at the membrane however functionality has been compromised. Class III mutations cause a 'gating defect' and the channel is no longer responsive to cAMP. Class IV mutations result in alterations in the CFTR pore, known as a 'conductance defect', and few chloride ions pass through. Class V mutations result in alternative splicing, and only small amounts of the normal CFTR are present at the membrane. Finally, with Class VI mutations, although normal protein is produced, it is unstable and undergoes rapid turnover, hence at any particular moment expression at the membrane is low.

The conservation of these mutations across hundreds of generations, along with the current high carrier rate, strongly suggests an evolutionary carrier advantage. Previous theories have suggested increased protection against cholera and secretory diarrhoea to explain the mutations' persistence in the human species (Poolman and Galvani, 2007). Newer theories propose the heterozygous genotype provides a degree of resistance against tuberculosis, which is supported by molecular, clinical and epidemiological geographical evidence (Bosch et al., 2017).

1.1.2 CF lung pathophysiology

CF as a disease was first described by Dorothy Anderson and her team in 1939 (Kreindler, 2011). However, it was not until the early 1980's that the role of ion transport was linked to CF pathogenesis. In 1989, Tsui and Riordan discovered and cloned the CFTR gene and identified the F508del mutation (Riordan et al., 1989). However, the precise pathophysiological processes by which the genetic defect manifests to result in pulmonary infections is still unclear. Molecular and cellular investigations have been the primary methods of making headway. One reason for this lack of clarity has been lack of an appropriate animal model. CFTR-null and CFTR-F508del mice do not develop profound infection and inflammation in the lungs, and rather pathology of the intestines is the dominating feature (Fisher, Zhang, and Engelhardt, 2011). Mice overexpressing βeNaC (epithelial sodium channels) show evidence of lung disease similar to humans however, also fail to spontaneously develop lung infections (Frizzell and Pilewski, 2004). In recent years, CFTR-null and CFTR-F508del pigs have been developed which spontaneously develop lung disease in a similar manner to human CF sufferers, with hopes that this will aid in understanding more about CF pathogenesis (Stoltz et al., 2010; Welsh et al., 2009).

The most commonly accepted theory for CF pathogenesis is known as the "low volume hypothesis" (Boucher, 2004, 2007). This suggests that without proper functioning CFTR on the epithelial surface of respiratory epithelium, Cl- ions are not secreted, and Na+ ions are reabsorbed without regulation due to the loss of CFTR's inhibitory effect on ENaC. Subsequent alteration of the osmotic gradient causes dehydration of the ASL, which consists of a mucus layer above a periciliary liquid (PCL) layer. This dehydration impairs the normal actions of the muco-ciliary escalator, which in healthy lungs is an essential innate defence mechanism present to maintain sterility of the lower airways (Moskwa et al., 2007). In normal circumstances, the mucus layer traps inhaled particles and pathogens and the ciliated epithelium remove the mucus and trapped matter from the lower airways and up towards the pharynx (Hart and Winstanley, 2002). In CF, dehydration of the ASL not only causes mucus to become more vicious and difficult to move but the depletion of the PCL layer causes the mucus to compress the cilia under it and obstruct their normal beating movement (Lyczak, Cannon, and Pier, 2002), as shown in **Figure 2**. Hence, inhaled micro-organisms are not removed and are able to colonize the lungs; the inability to clear these

infections leads to a vicious cycle of airway infection, mucus plugs and inflammation, resulting in irreversible lung damage (Bilton, 2008).

As a result of the pulmonary infections, there is an intense and excessive inflammatory response, reasons for which are not entirely clear (Davies, Alton, and Bush, 2007). High levels of macrophages, T-lymphocytes, tumour necrosis factor-α (TNF), interleukin-1 (IL-1), and IL-8 (Ciofu et al., 2014; Hart and Winstanley, 2002) are characteristic. The accumulation of inflammatory cells (alive and dead) further contributes to the mucus plugging via an increase in cell content. In particular, degraded neutrophil DNA contributes to around 10% of sputum viscosity (Balfour-Lynn and Dinwiddie, 1996). Additionally, the neutrophil release of α-defensins, reactive oxidants, and proteases induces oxidative stress, further worsening the issue of defective muco-ciliary clearance and promotes destruction and damage of lung tissue (Hart and Winstanley, 2002). It is generally accepted that chronic inflammation causes much of the damage to the CF lungs and is mediated by the antibody response to *P. aeruginosa* antigens, in addition to leucocyte enzymes, myeloperoxidase and oxygen radicals (Govan and Nelson, 1993).

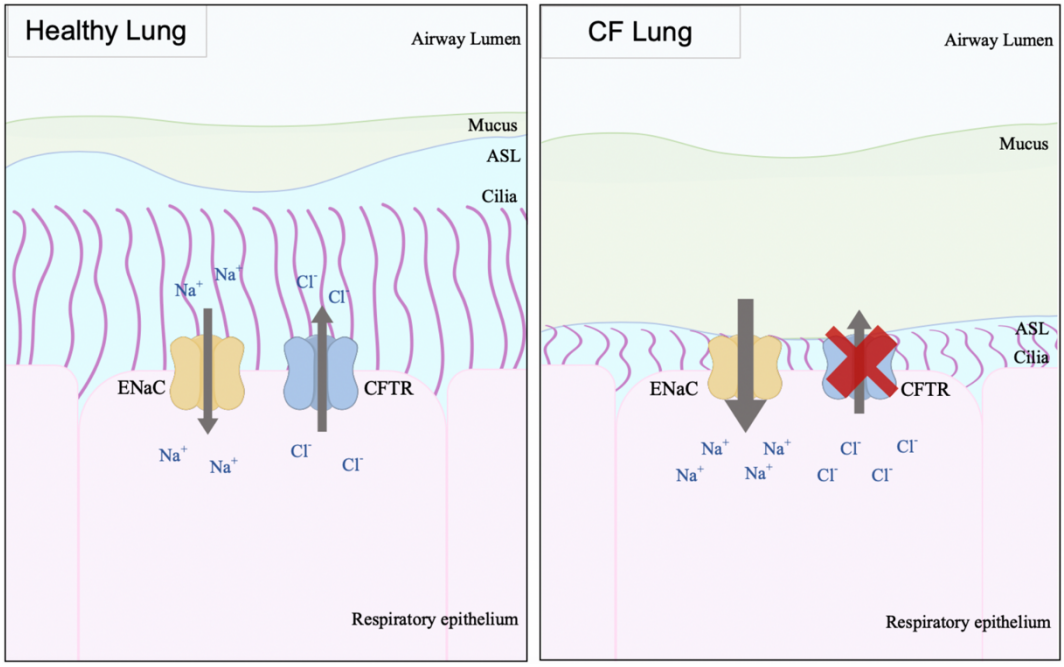


Figure 2. Pathophysiology of the CF lung. The respiratory epithelium is a ciliated pseudostratified columnar type epithelium and consists of multiple cell types including mucocilliary cells. In a healthy lung (left), functioning CFTR maintains hydration of the airway surface liquid (ASL), allowing normal mucocilliary transport. In the CF lung (right), lack of CFTR function and loss of the inhibitory effect on ENaC causes depletion of the ASL and compression of the cilia by the mucus layer, resulting in defective mucocilliary transport and the failure to clear inhaled pathogens.

1.1.3 Diagnosis and clinical manifestations

In the UK, CF is most frequently diagnosed within the first few weeks of life, following the newborn screening test (Jacob, 2015; Lim et al., 2014). Meconium ileus (bowel obstruction) occurs in 5 to 10% of newborns with CF, and is also a diagnostic indication (Cutting, 2014; Davies et al., 2007). The appearance of classic symptoms and a 'sweat test' can be used to diagnosis individuals post the newborn stage, as CF suffers have elevated sodium chloride levels in their sweat (Ratjen and Döring, 2003).

Symptoms including a chronic productive cough with increased sputum production occurs in patients of all ages with lung infections (Ratjen and Döring, 2003). Haemoptysis, sinus disease, and development of nasal polyps which affects breathing during sleep can also occur, generally affecting adolescents and adults (Cystic Fibrosis Trust UK, 2015). An additional, but less common, respiratory complication is pneumothorax. Chronic pulmonary infection results in irreversible lung tissue damage and bronchiectasis. FEV1 values (the forced expiratory volume of air in the first second of an exhaled breath) are monitored and indicate lung function. A FEV1 value of >85% indicates near-normal lung health, whilst at a value of <50%, patients struggle with everyday life (Cystic Fibrosis Trust UK, 2015). Ultimately, patients experience a progressive decline in lung function and respiratory failure, which is the cause of death in over 90% of CF sufferers (Cystic Fibrosis Trust UK, 2015; Lyczak et al., 2002; Welsh and Smith, 1995).

CF patients will inevitably experience pulmonary exacerbations; these are important clinical events, characterised as recurrent episodes of worsening pulmonary symptoms (Goss and Burns, 2007). Typically, the clinical presentation manifests as increased cough and sputum volume, dyspnoea, decreased energy level and fatigue, loss of appetite, weight loss, and decreased spirometric parameters (Goss and Burns, 2007). Exacerbations do however vary greatly in their clinical presentation and a wide spectrum of severity exists. Generally, mild exacerbations can treated on an outpatient basis or at home, whereas more severe exacerbations will require hospital admission (Bhatt, 2013; Goss and Burns, 2007; Smyth and Elborn, 2008). Exacerbations are more common with adolescent and adult patients and multiple episodes can occur in a single year (Ferkol, Rosenfeld, and Milla, 2006). Multiple exacerbation events are unfortunately directly linked to a negative impact on life and reduced survival (Bhatt, 2013).

The cause of these pulmonary exacerbations is far from understood. However, it is likely that these recurrent episodes of worsening of symptoms are related to the complex relationship that exists between the microbiology of the airways and the host defence system, and slight changes that may alter the dynamics of this relationship (Goss and Burns, 2007). Acquisition of new bacterial strains were originally thought to be responsible for pulmonary exacerbations. However, in a study by Aaron et al. (2004), only 2 of the 80 patients in the study had new *P. aeruginosa* strains cultured at times of exacerbation compared with cultures from a time of clinical stability. Additionally,

Stressman *et al.* (2011), using polymerase chain reaction (PCR) methods, showed that sputum cultures immediately preceding exacerbation did not show significant increase in bacterial cell density. It is now clear that the more likely responsible event is the clonal expansion of existing strains (Bhatt, 2013; Ferkol et al., 2006). Independent viral infections, or those triggering bacterial infections, are also recognised as a cause of pulmonary exacerbations; influenza, rhinoviruses, and respiratory syncytial virus (RSV) are amongst those implicated (Bhatt, 2013; Goss and Burns, 2007).

1.1.4 Management of pulmonary disease

Patients often undergo care at specialist CF centres, with the help of a multidisciplinary team (Davies et al., 2007). Sputum or cough swab samples should be taken and cultures grown at every point of medical contact to monitor the organisms present in the lung, as recommended by the UK CF Trust (UK Cystic Fibrosis Trust Antibiotic Working Group, 2009). Bacterial lung infections are treated vigorously with antimicrobial agents in nebulised, inhaled powder, IV or oral form, depending on the likely infecting organism, confirmed by microbiological laboratory testing.

CF patients are administered antibiotics throughout their entire life, however there is still no universal agreement concerning specificity of treatment. As a result, treatment duration, dosage, route of administration and choice of agents can vary patient to patient. However, it is recognised that intense and rapid escalation of antimicrobial therapy is necessary in response to a sudden decline in lung function or pulmonary exacerbation in an effort to maintain health (Smyth et al., 2014). More aggressive therapy does not completely clear the infection, but does appear to alleviate the symptoms surrounding an exacerbation, further indicating its association with the complex microbiota of the CF infected lungs (Smyth and Elborn, 2008).

Despite the numerous antimicrobial agents' available as part of the clinician's armamentarium and their heavy use, the beneficial actions of antibiotic treatment are substantially limited. Antibiotic agents can be effective in treating early transient infections, often termed as 'eradication therapy', and is thought to delay progressive lung damage and the development of chronic infection (Döring et al., 2000). However, if treatment is successful in clearing an acute infection, the lung damage caused makes re-infection more likely and hence repeated infections occur. It is estimated that eradication therapy for *P. aeruginosa* fails in 10-40% of new cases, and if successfully eradicated, re-infection occurs within 1 year (Tramper-Stranders et al., 2010).

Furthermore, although the development of chronic infection can be delayed by antibiotics, it cannot be prevented. Establishment of chronic *P. aeruginosa* infection is common in many patients, and once this occurs, no antimicrobial therapeutic regime is able to eradicate the organism from the lungs (Hurley and Smyth, 2012). Patients will still continue to receive antibiotic treatment as part of 'chronic suppressive therapy', however the aim then becomes to maintain lung function for as

long as possible and reduce the severity of the pulmonary symptoms in the patient's final years (Hurley and Smyth, 2012).

1.1.4.1 Anti-pseudomonal antimicrobial chemotherapy

Typically, anti-pseudomonal therapy consists of a combination of at least two antimicrobial agents with different modes of action. Tobramycin and ceftazidime are the most commonly used aminoglycoside and β-lactam antibiotics in the UK, though antibiotics including cefepime, meropenem, ciprofloxacin, colistin and aztreonam are also used. **Table 1** lists antibiotics used in CF and details relating to their mechanism of action and clinical use. The European Cystic Fibrosis Society Standards of Care documents states that no one regimen has been shown to be superior in treating *P. aeruginosa* infection, though suggest one option as a combination of tobramycin solution for inhalation, with nebulised colistin and oral ciprofloxacin (Smyth et al., 2014).

1.1.4.2 Other management options and novel emerging therapies

Alongside antimicrobial chemotherapy, physiotherapy is always used to help with the extrusion of mucus by way of "chest percussion", involving gentle pounding of the chest (Welsh and Smith, 1995). Mucolytic agent dornase alfa, non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, and corticosteroids can also be used to relieve pulmonary symptoms (Bell and Robinson, 2007; Davies et al., 2007).

Novel and emerging CF treatments can be divided into two themes: correction of the basic defect, or alleviation of the symptomatic consequences of the disease. In theory, correction of the basic defect could result in a cure for CF. Gene therapy considers the use of liposomes or viral vectors to insert a normal copy of the CFTR gene into the cells of the patients lung (Griesenbach, Pytel, and Alton, 2015). Clinical trials have shown modest effects of gene therapy, with small improvements in FEV1 compared to placebo, though no gene therapy is currently FDA approved (Alton et al., 2015, 2016; Cooney, Mccray, and Sinn, 2018). Another approach has been to develop CFTR modulating drugs; these can be further subdivided into potentiators, correctors and amplifiers. Ivacaftor, a CFTR potentiator approved for clinical use in 2012, was shown to restore some CFTR activity and improve FEV1 (Barry, Donaldson, and Jones, 2018). However, ivacaftor is only effective in patients with the G551D mutation, which accounts for 5% of the CF population. In 2015, a combination of ivacaftor and lumacaftor, a CFTR corrector, gained clinical approval and was used in patients with the F508del mutation, though the effects on lung function were not as positive compared to ivacaftor and G551D patients (Cholon et al., 2016). More recently in 2018, another CFTR corrector in combination with ivacaftor, tezacaftor, received clinical approval and further similar drugs are currently being investigated with the focus moving towards use of a "triple combination therapy" of CFTR modulators.

The second theme of alleviating symptoms, places emphasis on targeting the bacterial infections to limit the destruction of lung tissue. Potential novel emerging therapies include drugs that have been shown to have anti-biofilm effects such a alginate oligosaccharide derivate OligoG, which is currently under investigation in clinical trials (Powell et al., 2018). The use of bacteriophages and immunotherapies to target infecting bacteria, as well as simple thiol compound cysteamine which has been shown to have anti-biofilm and mucolytic actions are also subject of investigation though are further behind in development (Smith et al., 2017) .

Unfortunately, despite the during the late stages of the disease, FEV1 values are often below 30% and patients experience end stage respiratory failure. Supplemental oxygen may be required and hypercapnia with increased pulmonary hypertension can occur (Shah et al., 1995). During this point, the only treatment available to prevent death is a bilateral lung transplant (Cystic Fibrosis Trust UK, 2015). Unfortunately, not all patients are suitable transplant candidates and donor lungs are in short supply. In 2017, less than 56 CF patients in the UK underwent the procedure (Cystic Fibrosis Trust, 2017). Following transplant, the 8 year survival rate is approximately 70% (Bech et al., 2004).

Table 1. Antimicrobial chemotherapy agents used in CF

Agent	**Mechanism of Action**	**Route of Administration**	**Other Notes/ Adverse Effects**
Aminoglycoside: tobramycin[a]	Bactericidal via irreversibly binding to the 30S and/or the 50S subunits of bacterial ribosomes to inhibit cell wall protein synthesis	Intravenously or via inhalation of dry powder or solution	Ototoxic and nephrotoxic, though slightly lower toxicity and better penetration into bronchial secretions than other aminoglycosides
Cephalosporins: ceftazidime and cefepime[b]	Bactericidal via inhibition of cell wall biosynthesis	Intravenously, usually via intermittent dosing regimes	Generally well tolerated, though hypersensitivity reactions can occur
Carbapenems: imipenem, meropenem and doripenem[c]	Bactericidal via inhibition of cell wall biosynthesis	Parenterally, imipenem administered with cilastatin (metabolic enzyme renal dehydropeptidase I inhibitor)	Hypersensitivity reactions, nausea, vomiting and seizures can occur
Semi-synthetic β-lactam: piperacillin[d]	Bactericidal via inhibition of cell wall biosynthesis	Administered intravenously with β-lactamase inhibitor tazobactam.	Generally well tolerated, though hypersensitivity reactions and serum-sickness can occur
Monobactam: Aztreonam[e]	Bactericidal via inhibition of cell wall biosynthesis	Parenterally or via inhalation	Phlebitis, diarrhoea, nausea, vomiting, and rash
Fluoroquinolones: ciprofloxacin (2^{nd} gen) and levofloxacin (3^{rd} gen)[f]	Bactericidal via inhibition of DNA gyrase and topoisomerase IV, regulatory enzymes involved in DNA replication and repair	Intravenous or oral; oral ciprofloxacin commonly prescribed in combination with nebulised tobramycin and/or colistin	Well tolerated; light-headedness, insomnia and nausea occasionally reported

Agent	Mechanism of Action	Route of Administration	Other Notes/ Adverse Effects
Polymyxin: colistin[g]	Bactericidal via disruption of outer membrane of Gram-negative bacteria leading to loss of cell envelope integrity and leakage of intracellular contents	Via nebuliser to minimise toxic effects	Nephrotoxicity, and neurotoxicity resulting in dizziness, mental confusion, muscle weakness, ataxia, blurred vision, and in most severe cases respiratory paralysis
Fosfomycin; chemically synthesised phosphonic acid derivate, unrelated to all other antibiotics[h]	Bactericidal via inhibition of cell wall biosynthesis	Taken orally. Evaluations underway to determine if an inhaled fosfomycin and tobramycin combination is an effective therapy in CF patients.	May be used when other agents not suitable. Adverse effects mainly gastrointestinal.
Macrolide: azithromycin[i]	Bacteriostatic via inhibition bacterial protein synthesis by binding to the 23S rRNA in the 50S ribosomal subunit, though no direct killing activity against *P. aeruginosa*.	Taken orally; Cochrane review meta-analysis showed mild improvement with oral azithromycin compared with a placebo. Recommendation is a 6 month regime.	Off-label use in CF; reported benefits including limiting virulence factor synthesis, inhibiting alginate production, and increasing the susceptibility of *P. aeruginosa* to other antibiotics. Side effects of the drug are limited to gastrointestinal symptoms.

[a](Döring et al., 2000; Lam, Vaughan, and Parkins, 2013; Whitehead et al., 2002; Young et al., 2013d); [b](Young et al., 2013b); [c](Keynan et al., 1995; Young et al., 2013a); [d](Young et al., 2013b); [e](Young et al., 2013a); [f](Hurley and Smyth, 2012; Young et al., 2013c); [g](Beringer, 2001; Claus et al., 2015); [h](Raz, 2012; Trapnell et al., 2012); [i](Imperi, Leoni, and Visca, 2014; Principi, Blasi, and Esposito, 2015; Tateda et al., 2001)

1.2 Microbiology of the CF lung

Pathogens known to affect the CF lungs are diverse and complex microbiotas develop with a range of Gram-negative and Gram-positive bacterium occurs. The most notable and 'typical' CF pathogens are *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia* species and *P. aeruginosa* (Sherrard, Tunney, and Elborn, 2014). In recent years, culture-independent methods have been increasingly utilised to try and understand more about the dynamics of the CF airway microbiome, and have demonstrated that the microbiome is even more diverse than originally thought (Rogers et al., 2017). Organisms including *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Streptococcus*, *Prevotella*, *Ralstonia* and *Pandoraea* species are being increasingly detected in the CF airways via molecular based or anaerobic-culture based analyses (Flight et al., 2015; Mahenthiralingam, 2014; Rogers et al., 2017). Whilst bacterial infections are usually the most clinically significant, CF patients are also susceptible to viral and fungal lung infections with organisms such as respiratory syncytial virus (RSV), and *Candida* and *Aspergillosis* species (Cohen-Cymberknoh et al., 2013).

Though infections in CF are known to be polymicrobial, *P. aeruginosa* is a highly competitive organism, capable of lysing bacterium of other species, and often becomes the dominating organism (Palmer et al., 2005). A review of 16 studies and 507 CF patients demonstrated that whilst *P. aeruginosa* is indeed a prevalent micro-organism in CF, it was the dominant pathogen in 42% of patients, with 'dominant' colonisation defined as accounting for over 50% of the total reads from culture-independent 16S rRNA gene analysis (O'Toole, 2018). Whilst this value is somewhat lower than traditionally thought, *P. aeruginosa* colonisation and abundance varies immensely with patient age and disease severity. Younger CF patients harbour diverse bacterial communities, but diversity is lost as patient age increases and this is associated with a decrease in pulmonary function (Cox et al., 2010). This was also demonstrated by analysis of explanted lungs from adult CF patients with end stage disease showing a reduction in microbial diversity and frequent dominance with *P. aeruginosa* (Goddard et al., 2012).

1.2.1 *Pseudomonas aeruginosa*

P. aeruginosa is an aerobic Gram-negative bacilli bacterium of the *Pseudomonadaceae* family and a particularly debilitating pathogen in CF (Jayaseelan, Ramaswamy, and Dharmaraj, 2014; El Solh and Alhajhusain, 2009). This bacterium is ubiquitous in nature, found in almost every environment including terrestrial, aquatic, animal, human and plant associated habitats (Hogardt and Heesemann, 2010). *P. aeruginosa* is able to colonize not only in normal atmospheric conditions but also in hypoxic and artificial environments (Baron, 1996; Jayaseelan et al., 2014). It is known as an opportunistic pathogen, however can be isolated from the throat, skin and stool of healthy individuals (Baron, 1996), and the bacterium itself measures 0.5 - 0.8 μm by 1.5 - 3 μm (Jayaseelan

et al., 2014). As well as being a common CF pathogen, *P. aeruginosa* is thought to account for 10 – 20% of all nosocomial infections (Arora, 2014).

The prevalence of *P. aeruginosa* infection in CF increases with age (**Figure 3**), generally affecting up to 60% of adult CF patients (Cystic Fibrosis Trust, 2017). Acquisition is most common from the environment, with the route of entry through to be via the nose or mouth (Döring et al., 2000). Following *P. aeruginosa* infection, patients typically experience a worsening of symptoms and hospitalisation rates are increased (Kosorok et al., 2001; Levy et al., 2008). Acquisition of *P. aeruginosa* is also known to contribute to a worse prognosis (Emerson et al., 2002).

P. aeruginosa is a particularly hazardous organism in CF as it is: i) highly virulent, ii) resistant to many antibiotics, and iii) can adapt to the CF lung environment via both genetic and phenotypic alterations.

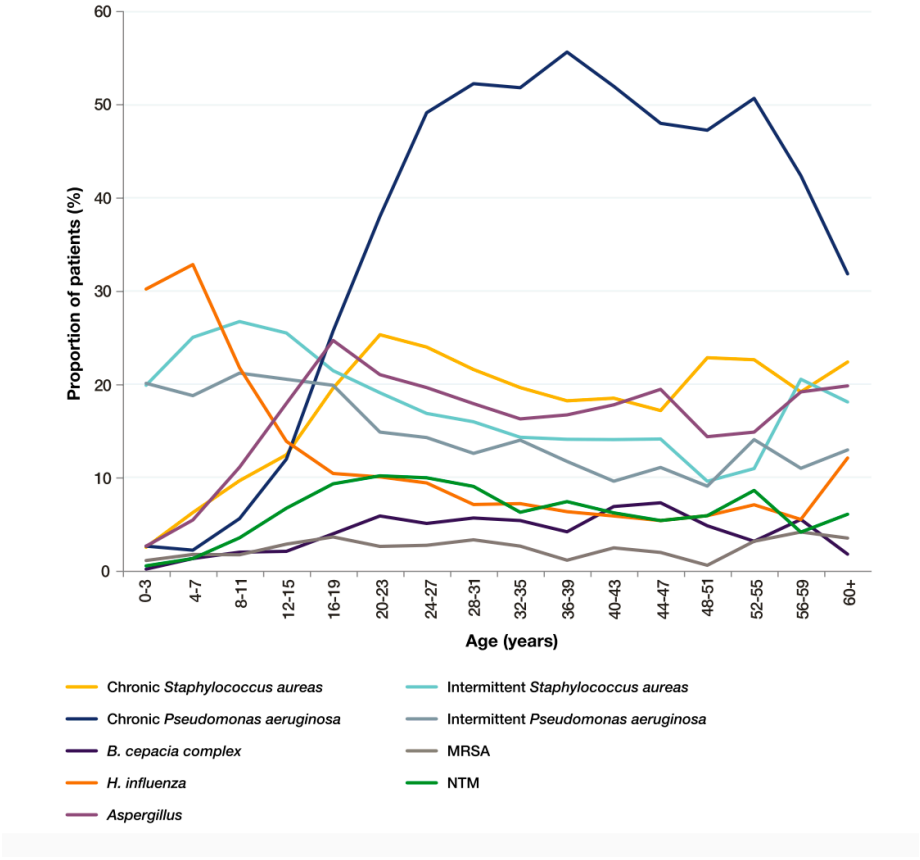


Figure 3. *P. aeruginosa* infection frequency in CF. Graphical representation of the main pathogens affecting the CF lungs in different age groups. Chronic *P. aeruginosa* infection is by far the most prevalent and the proportion of patients affected increases with age. Chronic infection defined as three or more growths of the organism from patient samples in one year (n=9887). Image reproduced with permission from the UK CF Registry Annual Data Report 2017.

1.2.1.1 Virulence factors

The numerous virulence factors of *P. aeruginosa* enable the bacterium to evade the host defence mechanisms, and invade and cause damage to host lung tissue. During acute or chronic infection, different virulence factors may be upregulated or down-regulated dependant on the mode of growth (Strateva and Mitov, 2011). Virulence factors can be divided into cell associated and extracellular/secreted factors, the latter controlled by a cell-cell communication system known as quorum sensing.

1.2.1.1.1 Cell associated

Cell associated virulence factors include type IV pili and flagellum, which are required for cell motility; pili for 'twitching' motility and flagella for 'swimming' motility (Strateva and Mitov, 2011). Both are involved in triggering an innate immune response in the body (Gellatly and Hancock, 2013). Pili aid in the initial colonization of *P. aeruginosa*, and act as adhesins binding to specific pilus receptors on the surface of epithelial cells (Strateva and Mitov, 2011). Soluble lectins, for example LecA and LecB, are also present on the Pseudomonas outer membrane, aiding in adhesion activities and exerting cytotoxic effects on lung tissue (Chemani et al., 2009).

Lipopolysaccharides (LPS) are notable virulence factors, and a crucial component to the bacterial cell wall and maintains its stability. The LPS is a pathogen associated pattern molecule (PAMP) recognised by host Toll-like receptor 4 (TLR4) and initiates a host-pathogen interaction and an immune response. Recognition of *P. aeruginosa* LPS by TLR4 in the lung stimulates the production of cytokines and chemokines to recruit immune cells such as alveolar macrophages to the site of detection (Raoust et al., 2009). Interestingly, CF isolates of *P. aeruginosa* have been shown to possess a unique hexa-acylated LPS structure in contrast to the pent-acylated type found in non-CF isolates; human TLR4 are able to detect this variation and lower concentration of the hexa-acylated LPS is required to illict the same response (Ernst et al., 1999; Hajjar et al., 2002). Further modifications noted in LPS of *P. aeruginosa* CF isolates are the addition of ethanolamine, aminoarabinose and palmitate, which promote resistance against host antimicrobial peptides and antibiotics (Ernst et al., 1999, 2007; Moskowitz and Ernst, 2010).

1.2.1.1.2 Extracellular factors and secretion systems

P. aeruginosa has 5 of the 6 secretion systems found in Gram-negative bacteria, designed to transport virulence factors from their production site in the cytoplasm and release them into the extracellular environment (Bleves et al., 2010).

Alkaline protease (AprA), a type 1 zinc metalloprotease, is the principle factor secreted by the type I secretion system (T1SS) (Bleves et al., 2010). AprA degrades multiple host immune molecules including immunoglobulins, cytokines and complement proteins C1q, C2 and C3, blocking

phagocytosis and the killing of *P. aeruginosa* by neutrophils (Ballok and O'Toole, 2013; Davies, 2003). AprA also cleaves transferrin to facilitate iron acquisition by siderophores and cleaves flagellin monomers to prevent immune recognition by the host, promoting infection persistence of *P. aeruginosa* in the lung (Ballok and O'Toole, 2013).

Protease IV, LasA, LasB, exotoxin A, and PlcH are secreted via the T2SS (Bleves et al., 2010). Protease IV, a serine protease, degrades complements proteins and immunoglobulins, fibrinogen and pulmonary surfactant proteins A (SP-A) and D (SP-D) (Ballok and O'Toole, 2013). Elastases LasA and LasB, in particular the latter, have high detrimental effect in the lung, as they break down elastin, a protein that comprises 28% of lung tissue and is required for the organs elasticity and retractability (Gellatly and Hancock, 2013; Pereira et al., 2014). Elastin is also a prominent component of blood vessels and its breakdown causes haemorrhagic lesions, likely contributing to haemoptysis (Strateva and Mitov, 2011). LasB also breaks down SP-A and SP-D. SP-A and SP-D ease the effort of breathing, have important mediatory effects on innate immune mechanisms such a phagocytosis by alveolar macrophages, and aids in infection resolution (Kuang et al., 2011; Strateva and Mitov, 2011). Exotoxin A (ETA), a ADP-ribosylase, is the most lethal and cytotoxic *P. aeruginosa* virulence factor. ETA inhibits host cell elongation factor 2, thereby inhibiting protein synthesis, causing cell death of the host tissues, and downregulates the tight cell associated proteins ZO-1 and ZO-2 (Bentzmann et al., 2000; El-zaim et al., 1998; Gellatly and Hancock, 2013)

The T3SS plays an important role in the acute stages of infection, with toxins being injected directly onto host cells. In *P. aeruginosa*, there are four T3SS effectors, ExoS, ExoT, ExoU, and ExoY. Two effectors are commonly expressed in any particular strain, and each one has varied actions. ExoS inhibits endocytosis of bacteria by epithelial and phagocytic cells, inhibits cell migration and phagocytic cell motility, disrupts the actin cytoskeleton, inhibits vesicular trafficking and is cytotoxic to poly-mononuclear leucocytes (PMNs) and macrophages (Engel and Balachandran, 2009; Gellatly and Hancock, 2013; Lyczak et al., 2002). ExoT also inhibits cell migration, immune cell functions and bacterial endocytosis, however it also inhibits cell division and disrupts cell adhesions to induce cell death (Engel and Balachandran, 2009). ExoU, possessing phospholipase A2-like activity, is an extremely potent cytotoxin and causes the rapid death of host cells via the loss of eukaryotic plasma membrane integrity (Engel and Balachandran, 2009). ExoY's function remains unclear but it thought to be an adenylate cyclase that disrupts the actin cytoskeleton and increases membrane permeability of host cells. Together, these effectors of the T3SS aid in acute *P. aeruginosa* infection by inhibiting wound repair and pathogen clearance, propagating tissue injury and promoting susceptibility to further injury and colonization (Ballok and O'Toole, 2013; Engel and Balachandran, 2009).

EstA and LepA are enzymes exported by the T5SS. EstA, an outer membrance bound lipolytic enzyme, is thought to be involved in the production of rhamolipids, and is required for motility and

full virulence of *P. aeruginosa* (Rosenau and Jaeger, 2000; Wilhelm et al., 2007). LepA, an exoprotease, cleaves receptors of transcription factor NF-κβ, involved in the host inflammatory and immune response to infection, and results in increased inflammation (Ballok and O'Toole, 2013). Interestingly, LepA production is only noted with clinical isolates and not in laboratory strain PAO1 (Bleves et al., 2010).

Hcp1 (haem carrier protein 1) is the only protein associated with the most recently described T6SS (Bleves et al., 2010). Hcp1 is related to nanotube formation, though Hcp1 in CF sputum and Hcp1 specific antibiotics in CF patient serum suggest a role in virulence. (Mougous et al., 2009).

1.2.1.1.3 Other extracellular factors

1.2.1.1.3.1 Pyocyanin

P. aeruginosa secretes multiple pigments, most notably the cytotoxic redox active phenazine derivative pyocyanin, responsible for the bacterium's distinctive blue-green colour (Chai et al., 2013). Pyocyanin is known to affect several cellular functions. These include electron transport, cellular respiration, energy metabolism, gene expression, and innate immune mechanisms, all of which enable the bacterium to establish and maintain chronic infection (Rada and Leto, 2013). In the CF lung, pyocyanin is regarded to have major implications in the pathogenesis of lung disease and a prominent role in tissue damage. Experiments performed on murine airways indicate that it is crucial for virulence and chronic infection. Comparison of wild type and pyocyanin deficient mutant strains showed the latter were less able to maintain infection and were able to be cleared by the host (Lau et al., 2004). Investigations into the interactions between pyocyanin and the respiratory epithelium have noted that pyocyanin, which is not produced by any other Gram-negative non-fermenting bacteria, inactivates nitric oxide (NO) produced by macrophages and epithelial cells, causes the production of reactive oxygen species (ROS) and superoxide anions (Rada and Leto, 2013). Furthermore, inhibition of catalase, an enzyme that protects cells from oxidative stress, ensures tissue damage. *In vitro* investigations have also shown that the pigment depletes glutathionine, intracellular cAMP and ATP; inhibits CFTR; and inhibits the beating of cilia of bronchial epithelial cells further compromising muco-cilliary clearance (Kanthakumar et al., 1993; Schwarzer et al., 2008; Wilson et al., 1987). Pyocyanin's actions as a pro-inflammatory mediator, promoting the expression and recruitment of immune cells, further contribute to deleterious actions on lung tissue. Unfortunately, pyocyanin is able to cause overexpression of itself and other virulence factors of *P. aeruginosa* such as LasA. Interestingly, pyocyanin has been shown to have antimicrobial effects against other bacterial and fungal species such as *S. aureus, E. coli*, *Klebsiella pneumoniae* and *Aspergillus fumigatus*; this is consistent with *P. aeruginosa*'s ability to persist as the dominating pathogen in a polymicrobial environment such as the CF lung (Jayaseelan et al., 2014). High prevalence of pyocyanin is found in the CF sputum and levels

appear to correlate with lung disease severity and declining lung function in CF (Rada and Leto, 2013).

1.2.1.1.3.2 Haemolysins

P. aeruginosa produces two haemolysins, rhamnolipids and haemolytic phospholipase C (PLC-HR), important in both initial and chronic stages of infection (May et al., 1991). The two appear to act synergistically and break down phospholipids, preferentially those with quaternary ammonium groups primarily found in eukaryotic cell membranes and lung surfactants such as lecithin (May et al., 1991; Strateva and Mitov, 2011). In particular their cytotoxic effects are noted on lymphocytes and neutrophils; the degranulation and cell death of neutrophils acts as a bacterial protection mechanism and also leads to further lung damage (Hart and Winstanley, 2002; Strateva and Mitov, 2011). Murine and ovine studies demonstrate injection of purified PLC-HR causes hepatic necrosis, renal tubular necrosis and superficial inflammatory lesions (Terada et al., 1999; Wargo et al., 2011). Antibodies to PLC are present in the serum of CF patients with *P. aeruginosa*, and the antibody level rises as chronic infection progresses. (Terada et al., 1999)

Rhamnolipids are rhamnose-containing glycolipid biosurfactants, generally with one or two L-rhamnose sugars and one or two β hydroxyl fatty acids. (Laabei et al., 2014; Maier and Soberón-Chávez, 2000; Schmidberger et al., 2014). Genes encoding for enzymes involved in the biosynthesis, RhlA and B, are known to be regulated by quorum sensing (bacterial communication system) and more specifically by the regulator protein RlhR (Schmidberger et al., 2014). Rhamnolipids are thought to have a detergent like effect to solubilise phospholipids and aid PLC-HR to cleave them (Strateva and Mitov, 2011). In addition, rhamnolipids inhibit the mucociliary actions of human epithelium (Read et al., 1992; Strateva and Mitov, 2011). Zulianello et al. (2006) also demonstrated that rhamnolipids promote the epithelial tissue destruction and paracellular invasion of *P. aeruginosa*. Perhaps due to its association with quorum sensing, rhamnolipid production is heavily implicated in *P. aeruginosa* biofilm formation (Dusane et al., 2010).

1.2.1.1.3.3 Iron sequestration

Crucial to the survival of many bacteria including *P. aeruginosa* is the acquisition of the micronutrient iron. However, in the host, iron availability is limited having already been sequestered by molecules such as transferrin and lactoferrin (Gellatly and Hancock, 2013). Hence Pseudomonas species produce iron scavenging siderophores such as pyoverdine, pyochelin and quinolobactin (Visca, Imperi, and Lamont, 2007). Of these, pyoverdine is the primary iron uptake system and several *in vitro* and *in vivo* models have shown it to be necessary for infection. Aside from iron chelating, pyoverdine acts as a signalling molecule and leads to the upregulation of its own production and that of other virulence factors such as exotoxin A (Gellatly and Hancock, 2013). Pyoverdine is also a fluorescent, yellow-green pigment of *P. aeruginosa* (Meyer, 2000).

Siderophores are particularly important in the role of biofilm formation (Ciofu et al., 2014; Harrison, 2007). When iron availability is critically limited, *P. aeruginosa* can secrete the haemophore HasAP via the (T1SS) (Bleves et al., 2010; Strateva and Mitov, 2011). HasAP binds to the haem of haemoglobin, and the complex is then addressed to an outer membrane receptor HasR, and internalised by the bacterium (Bleves et al., 2010).

1.2.1.2 Resistance

P. aeruginosa has a high level of intrinsic (or innate) resistance, and is susceptible to fewer antibiotics compared with other Gram-negative organisms such as those from of the Enterobacteriaceae family (Delcour, 2009). This is due to a combination of low cell envelope permeability and to chromosomally encoded resistance mechanisms such as efflux pump systems and antibiotic-hydrolysing enzymes, acting synergistically to prevent antibiotic concentrations reaching lethal intracellular levels (Delcour, 2009).

Acquired resistance is a serious global issue and is ever increasing with all bacterial species. *P. aeruginosa* is one of the 6 'ESKAPE' pathogens (the others being *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *Acinetobacter baummani*, and *Enterobacter* species); named so due to their ability to 'escape' antibiotic treatment (Kanj and Kanafani, 2011). These pathogens have been identified to pose the highest threat with regards to their increasing levels of resistance.

Acquired antibiotic resistance is caused by the misuse and overuse of antibiotics (Wellington et al., 2013). Such examples of this include unnecessary prescribing by clinicians, patients' disregard of instructions to finish the full course of treatment, and overuse of antibiotics in the agricultural and farming industry to maximise yield. This misuse causes selective pressure and subpopulations of mutant bacteria which can withstand the actions of the agents to survive and thrive. Furthermore, bacteria can undergo horizontal gene transfer; mobile genetic elements such as plasmids, transposons, integrons, prophages and resistance islands can harbour genes encoding for resistance factors and can be passed not only between bacteria of the same species but between other species (Breidenstein, de la Fuente-Núñez, and Hancock, 2011).

Generally acquired resistance occurs by one of three main mechanisms (Lambert, 2002). Firstly, the antibiotics accessibility to its target may be comprised, usually by reduced cell envelope permeability and/or extrusion of the antibiotic extracellularly. Secondly, the antibiotic may be inactivated, typically by bacterial enzymatic production. And thirdly, mutations can result in modification of the antibiotic target, rendering the agent ineffective. Resistance can occur by one or multiple mechanisms. β-lactams infamously demonstrate resistance via all three main mechanisms; in addition, reduced permeability and extrusion by efflux pump systems, production of β-lactamases are common and alterations in the target penicillin binding proteins (PBPs) can infer resistance to these agents. Conversely, resistance to colistin can only occur if the target is altered, more specifically by addition of aminoarabinose to the lipid A of the LPS (Johansen et al., 2008).

1.2.1.2.1 Outer membrane permeability

The outer membrane (OM), an asymmetric bilayer of phospholipids and lipopolysaccharides, acts as a selective barrier, with pore-forming porins controlling the entry of molecules into the cell (Hancock and Speert, 2000). Low permeability is accounted for by few general diffusion porins, as *P. aeruginosa* expresses dedicated specific porins for nutrient uptake (Delcour, 2009). By comparison, the OM of *P. aeruginosa* is approximately 10 to 100 fold less permeable than that of *E. coli'*s (Hancock and Speert, 2000).

1.2.1.2.2 Efflux pump systems

The presence of efflux pump systems is a major resistance mechanism of *P. aeruginosa*. Five families of efflux systems are known to exist in *P. aeruginosa*, however the most well characterised and clinically significant are those belong to the Resistance-Nodulation-Division (RND) superfamily (Schweizer, 2003). These efflux pump systems are comprised of three parts: an antiporter of the RND family in the cytoplasmic membrane, a channel forming protein in the outer membrane also known as an outer membrane factor (OMF), and a periplasmic membrane fusion protein (MFP) coupling the previous two. The RND antiporter (MexB, D, F or Y) and MFP (MexA, C, E or X) proteins are able to isolate antimicrobial agents present in the periplasmic space, cytoplasmic membrane and cytoplasmic space, whilst the OMF (OprM, J or N) is responsible for the final stage of exportation across the outer membrane and out of the cell (Aeschlimann, 2003). The removal of antibiotics and biocide molecules is an active process, so these pump systems use proton motive force as an energy source (Avrain, Mertens, and Bambeke, 2013).

Twelve efflux pump systems of the RND family have been characterised in *P. aeruginosa* (Avrain et al., 2013). Two of these, MexAB-OprM and MexXY-OprM, are of particular clinical importance and are constitutively expressed at a basal level in all strains contributing to the intrinsic resistance of many antibiotics (Poole, 2001). MexAB-OprM has the broadest activity of the efflux systems (Aeschlimann, 2003), and its substrates include fluoroquinolones, β-lactams, tetracycline, macrolides, chloramphenicol, trimethoprim and sulphonamides (Poole, 2001). The MexXY-OprM efflux pump system can export fewer antibiotics, however its substrates include clinically used antipseudomonal antibiotics including cefepime and ciprofloxacin. Like MexAB-OprM, MexXY-OprM is also involved in both intrinsic and acquired resistance. Strains lacking MexXY displayed increased susceptibility to aminoglycosides, tetracycline and erythromycin indicating a degree of intrinsic resistance to these agents. However, resistance is dependent on induction in wild type strains by the agents (Poole, 2001), and low substrate concentrations causes overproduction and acquired resistance to aminoglycosides (Aeschlimann, 2003). CF *P. aeruginosa* strains in particular show higher aminoglycoside resistance mediated by MexXY-OprM, as production is upregulated in response to ROS which are prevalent in the CF lung.

1.2.1.2.3 Chromosomal β-lactamase edit to make sense on mechanism.

All *P. aeruginosa* strains possess the inducible *ampC* gene, which encodes for a Class I β-lactamase able to inactivate most penicillins, cephalosporins, and β-lactamase inhibitors particularly clavulanate (Jacoby, 2009; Lister, Wolter, and Hanson, 2009). AmpC regulation is particularly complex in *P. aeruginosa*. Expression of the enzyme is partially controlled by regulatory factor, AmpR, which represses transcription ensuring only a low basal level of enzyme is produced in wild type strains (Lister et al., 2009). The induction process results in considerably higher levels of AmpC enzyme production and requires two additional proteins, AmpG permease and AmpD amidase, which are involved in cell wall biosynthesis and recycling (Wolter et al., 2007).

The induction of increased levels of AmpC expression can be triggered by antibiotics; antibiotics binding to their target PBPs which are involved in cell wall biosynthesis and peptidoglycan polymerisation (Lister et al., 2009). As the peptidoglycan layer is under constant turnover, inhibition of PBPs leads to a build-up of cell wall component 1,6-anhydromuropeptides. These peptides are normally transported across the cell (inner) membrane by AmpG and cleaved into UDP-MurNAc-pentapeptides by AmpD in the cytoplasm. However, AmpD is unable to cleave all of the 1,6-anhydromuropeptides when high levels are produced. These uncleaved peptides are inducing proteins and replace UDP-MurNAc-pentapeptides, which are repressing proteins, bound to the AmpR. This triggers a conformational change, converting AmpR into a transcription factor and increasing the production of AmpC. AmpC is then able to cross the cell (inner) membrane and outer membrane to hydrolyse the β-lactam ring of antibiotic in the periplasmic space and extracellularly.

Induction is traditionally a transient state and so AmpC production usually returns to low basal levels once the inducing β-lactam is no longer present. However, spontaneous chromosomal mutations, commonly within *ampD* or *ampR,* are responsible for non-transient AmpC derepression and overproduction (Lister et al., 2009). Analysis of CF *P. aeruginosa* strains show the majority exhibit stable partial derepression of AmpC, producing moderately higher basal amounts of AmpC which is inducible to an even higher level (Høiby et al., 2010; Lister et al., 2009). This is a major cause of β-lactam resistance *in vivo* (Giwercman et al., 1990).

Multiple β-lactam antibiotics are able to induce the production of β-lactamase, and do so to variable capabilities. For example, imipenem is a strong inducer and weak substrate, whilst ceftazidime and cefepime are weak inducers and weak substrates (Jacoby, 2009). However, spontaneous chromosomal mutations in the AmpR gene can result in AmpC hyperproduction, in which case ceftazidime and cefepime can be hydrolysed if sufficient amounts of enzyme is produced (Jacoby, 2009; Lambert, 2002). In a study by Giwercman et al. (1992), β-lactamase production increased in the sputum samples of CF patients treated with imipenem, piperacillin,

ceftazidime and cefsulodin, however was dramatically decreased in patients treated with aztreonam. This can be explained by aztreonam also acting as a competitive β-lactamase inhibitor.

1.2.1.2.4 Genetically acquired antibiotic inactivating enzymes

Other antibiotic inactivating enzymes produced by *P. aeruginosa* are a result of acquired resistance and are typically due to horizontal gene transfer. Unfortunately, β-lactams are a class particularly targeted by enzymatic hydrolysis and many different β-lactamases exist. Plasmids can confer genes encoding for extended spectrum β-lactamases (ESBLs) and metallo- β -lactamases (MβLs) which can inactivate penicillin's, cephalosporins and the latter can even hydrolyse carbapenems (Breidenstein et al., 2011). Genes encoding for aminoglycoside modifying enzymes are located in various mobile genetic elements; though aminoglycoside resistance can also be a result of reduced permeability and uptake by the cell, which has often been found to be the most common cause of aminoglycoside resistance in CF isolates of *P. aeruginosa* (Poole, 2005)

1.2.1.3 Predisposition to *P. aeruginosa* infection in the CF lung

The question as to why *P. aeruginosa* is the most prevalent infecting pathogen in the CF lung remains unanswered. Another unanswered question includes how infections are limited to the lungs, as bacteraemia rarely transpires in CF patients despite chronic infections lasting many years. (Goldberg and Pier, 2000). In patients with primary ciliary dyskinesia or non CF bronchiectasis, *P. aeruginosa* is not a common pathogen, further indicating a unique interaction between this Gram-negative pathogen and the environment of the CF lung (Davies, 2003). Though there are multiple theories for the *P. aeruginosa* predisposition, the supporting evidence is either insufficient or conflicting.

For example, it was previously suggested that asialo-GM1 (asialo ganglio-N-tetraosylceramide), a glycosphingolipid on bronchial epithelial cells, acts as a receptor and binds to the pili of *P. aeruginosa*, as initially demonstrated by Saiman and Prince (1993). Further studies showed an exoproduct of *P. aeruginosa*, neuraminidase, increased asialo-GM1 expression indicating potentiation and persistence of infection (Davies et al., 1999). Unfortunately, results were not replicated with clinical CF isolates of *P. aeruginosa*, and the commericially acquired asialo-GM1 antibodies used in previous studies were found to be not specific (Schroeder et al. (2001)

Other suggested receptor systems involve the pili, LPS of *P. aeruginosa* or mutant CFTR. In normal tissue, CFTR has been shown to act as a pattern recognition molecule (PRM) and bind to *P. aeruginosa* LPS to internalise the bacterium and clear the infection (Pier and colleagues in 2002, (Schroeder et al., 2002). Hence, the absence of functioning CFTR in CF would result in lack of binding and bacterial clearance. This is corroborated by previous work by Baltimore et al. (1989) whereby histological analysis showed lack of *P. aeruginosa* infiltration in epithelial tissue of post mortem CF lung tissue and the appearance of microcolonies in the lumen rather than attached to the

epithelial cell surface. Baltimore's findings also therefore discredit theories such as the asialo GM-1 receptor system as this is reliant on the assumption of direct contact between the bacterium and epithelial cells. However, it can be argued that post mortem samples are only representative of end stage lung disease, and may not correspond to events in the initial or early stages of infection.

1.2.1.4 Genomics

In 2000, the genome of *P. aeruginosa* strain PA01, at 6.3 million base pairs (Mbps), was sequenced and was one of the largest bacterial genomes to have been sequenced at the time (Stover et al., 2000). The sequence was uncovered by a straightforward implementation of whole-genome-shotgun sampling. *P. aeruginosa* was shown to have a high level of genome complexity likely accounting for the bacteria's ability to adapt and thrive in many niches and diverse environments including the CF lungs (El Solh and Alhajhusain, 2009)**.** Analysis showed *P. aeruginosa* PAO1 had 5,570 predicted open reading frames (ORFs) and lacking evidence of gene duplication; by comparison *E. coli* has around 4300 ORFs (Saka et al., 2005) and *S. aureus* around 2,600 predicated ORFs (Kuroda et al., 2001). The genome sequence also provided a great deal of information regarding *P. aeruginosa* pathogenicity, resistance capabilities and metabolic versatility.

P. aeruginosa was also shown to have a very high proportion of regulatory genes at around 9.4% of the genome (transcriptional regulators accounting for 403 ORFs and two-component regulatory systems for 118) (Stover et al., 2000). This is almost double the figure of *E. coli* where only 5.8% of genes were regulatory, and triple that of the 3% in *Mycobacterium tuberculosis*. Other notable findings showed a very high number of genes predicated to encode OMP's, around 150. Furthermore, *P. aeruginosa* appeared to have one of the most complex chemosensing and chemotaxis systems compared with other bacterial genomes, with an estimated 26 ORFs encoding chemotaxis sensory transducer proteins and 4 chemosensory systems.

Following on from PAO1, other strains of *P. aeruginosa* including CF clinical isolates were sequenced and analysed. In 2006, Lee et al. (2006) sequenced the genome of the more virulent *P. aeruginosa* strain PA14, which had a slightly larger genome of around 6.5 Mbps. Conserved genetic sequences of *P. aeruginosa* amongst all strains are often described as the 'core genome', whilst the 'accessory genome' are a group of genes which may be present or absent dependent on strain (Silby et al., 2011). In recent years, more and more strains of *P. aeruginosa* have been sequenced, and analysis has allowed for rough identification of the conserved genome common to all strains. For example, comparison of five *P. aeruginosa* strain genomes demonstrated 5021 conserved genes (Silby et al., 2011). As more strains are sequenced the true value of the number of ORFs in the conserved genome will become clearer until eventually a plateau is reached.

CF patients may be infected with different strains of *P. aeruginosa*. However, genomic differences have also been noted from coeval paired isolates from patients (Chung et al., 2012). One patient

revealed paired isolates to have differences of 54 SNPs and 38 insertions/deletions. Paired isolates from another CF patient revealed hypermutator phenotype and genomic differences due to 344 SNPs and 93 insertions/deletions. This study demonstrated genomic heterogeneity at every level of resolution between coeval paired *P. aeruginosa* isolates.

1.2.1.5 Mucoidy and alginate production

A key genetic adaptation of *P. aeruginosa* to the CF lung includes conversion from the non-mucoid to mucoid phenotype (Hogardt and Heesemann, 2010). The mucoid phenotype is characterised by overproduction of biofilm matrix component alginate, a high molecular weight, linear polyanionic exopolysaccharide composed of uronic acids (non-repetitive monomers of B-1-4 linked L-guluronic and D-mannuronic acids) (Ghafoor, Hay, and Rehm, 2011; Ryder, Byrd, and Wozniak, 2007). Generally, environmental *P. aeruginosa* strains are non-mucoid and are responsible for the initial *P. aeruginosa* infection in CF patients. However, analysis of sputum sample cultures have shown that with disease progression, mucoid variants of *P. aeruginosa* emerge and become the dominating microorganisms (Hassett et al., 2010). Alginate protects the bacteria against host immune and inflammatory cell attack: reducing the chemotaxis of PMNs, inhibiting activation of the complement system, scavenging free radicals and hypochlorite produced by phagocytes and preventing bacterial phagocytic clearance (Lyczak et al., 2002; Ryder et al., 2007). Hence, the mucoid phenotype acts as a defensive mechanism and provides the bacteria with a selective advantage.

Alginate has an additional role with clinical relevance; alginate is an antigenic determinant and its overproduction stimulates massive production of IgA and IgG antibodies against alginate (Høiby, 2011; Pedersen et al., 1992). A chronic infection in CF is generally defined as one with an alginate producing strain of *P. aeruginosa* which is present in sputum sample cultures for at least 6 months, together with rising levels of serum antibodies (Ratjen, 2001). In contrast, early transient infection is the term given if the infection is identified to be caused by non-mucoid *P. aeruginosa* in sputum cultures intermittently without the presence of detectable antibodies.

Mutations in the *mucA* gene are responsible for the development of mucoid *P. aeruginosa*, resulting in alginate overproduction (Lyczak et al., 2002). The biosynthesis and regulation of alginate is a highly regulated and complex multistep process (Ramsey and Wozniak, 2005). *MucA* belongs to an operon also containing *mucB*, *C* and *D* and *algT* (also termed *algU*), genes constitutively expressed in wild type non-mucoid strains. *MucA, B* and *D* gene products are negative regulators of AlgT; MucA is an inner membrane anti sigma factor that sequesters AlgT. (Govan and Deretic, 1996; Hogardt and Heesemann, 2010; Lyczak et al., 2002). Mutations in the *mucA* gene therefore leave AlgT uninhibited, enabling it to carry out its actions and bind to the promoter region of genes *algR* and *algD*, and enable transcription of AlgD. AlgD is a GDP-

mannose dehydrogenase, the first alginate-specific enzyme in the biosynthetic pathway, committing the cell into producing alginate.

AlgA and AlgC are other enzymes involved in the synthesis pathway, producing the alginate precursor GDP-mannose from glucose-6-phosphase. The subsequent production of GDP-mannuronate by AlgD is followed by polymerisation and further modifications, with the help of AlgG, AlgE, AlgI and AlgJ to produce alginate (Govan and Deretic, 1996; Lyczak et al., 2002). Other genes in the alginate biosynthesis operon (*alg8, alg44, algK, algX, algL,* and *algF*) are thought to be involved in polymerisation, transportation of alginate intracellularly, and exportation extracellularly (Govan and Deretic, 1996; Ramsey and Wozniak, 2005)

As mucoid *P. aeruginosa* is seldom found in natural environments, justifiably only 2% of *P. aeruginosa* infections in non-CF patients are mucoid. However, for patients with CF, up to 90% can be chronically infected with the mucoid type, indicating the specific and niche CF lung environment as a trigger for the non-mucoid to mucoid conversion (May et al., 1991). Mutations in *mucA* are known to be induced by oxygen radicals released activated from PMNs as part of the immune response to pulmonary infection, providing a plausible explanation for the high incidence of mucoid *P. aeruginosa* in CF (Høiby, 2002). Unfortunately, evidence of mucoid *P. aeruginosa* in the CF lung is linked to biofilm formation, and as such is also linked to chronic infection, an increase in exacerbations, and a poorer prognosis (Govan and Deretic, 1996).

1.3 *P. aeruginosa* biofilm growth in the CF lung

P. aeruginosa can form sessile populations known as biofilms, whereby bacteria form intricate communities and microcolonies encased in a bacteria-produced extracellular matrix (Costerton et al., 1995). The matrix has a crucial protective function and constituents of *P. aeruginosa* biofilms typically include exopolysaccharides (EPS) such as alginate, Psl and Pel; extracellular DNA; multimeric cell appendages such as pili and flagella; and various other surface proteins (Ghafoor et al., 2011; Mann and Wozniak, 2012). Biofilms are the predominant form of bacterial growth in nature, as opposed to planktonic growth which more commonly occurs in liquid media in microbiology laboratories (Li and Tian, 2012; Mann and Wozniak, 2012). As a whole, biofilms account for 65 to 80% of all bacterial infections in humans (Bispo, Haas, and Gilmore, 2015; Rasamiravaka et al., 2015). *P. aeruginosa* can form biofilms on a variety of surfaces (substratum), including water pipes, sinks, taps, contact lenses, urinary catheters, joint prosthesis, the endocardium, and in the CF lungs (Basak, Rajurkar, and Attal, 2013; Wu et al., 2014). In CF patients, chronic *P. aeruginosa* infection is linked to a switch to the biofilm mode of growth, and is associated with reduced responsiveness to treatment, a decline in clinical status and worsened prognosis.

Two contrasting views exist regarding *P. aeruginosa* biofilm development in the CF lung. Some have argued that biofilms are in contact with and directly attached to the lower airway epithelial cells, which is the basis of some theories in explaining the high prevalence of *P. aeruginosa* in CF. However, more evidence is suggesting that this is in fact not the case, and the formation of biofilms is associated only with the mucus layer (Moreau-Marquis, Stanton, and O'Toole, 2008). As described previously, a key research article to support the latter was one published by Baltimore et al. (1989), using a *P. aeruginosa* specific immunoperoxidase staining technique on post mortem lung tissues. Similarly, Worlitzsch et al. (2002), using immunolocalisation, transmission electron microscopy (TEM) and scanning electron microscopy (SEM), also found *P. aeruginosa* to exist as aggregates and 'macrocolonies' intralumenally. Collectively, data showed complete lack of bacteria attached to the airway epithelia, and for 94.5% of bacterial cells to be located 5 to 17 µm away from the epithelial surface and 6.5% to be located 2 to 5 µm from the epithelial surface. More compelling supportive evidence came from Niels Hoiby and colleagues in 2009 (Bjarnsholt et al., 2009), whereby in addition to conventional staining techniques, the team also utilised PNA-FISH (peptide nucleic acid probe based fluorescent *in situ* hybridisation) to show lack of *P. aeruginosa* adherence to the bronchial wall or epithelial cells in lung tissue. Rather mucoid *P. aeruginosa* biofilms were located in the sputum and surrounded by PMNs in the conductive zone (trachea, bronchi, bronchioles and terminal bronchioles) and next to epithelial cells of the alveoli and respiratory bronchioles in the respiratory zone (respiratory bronchioles, alveolar ducts and alveolar sacs) (Bjarnsholt et al., 2009; Høiby, 2011). Their experiments also showed non-mucoid planktonic bacteria to be phagocytosed by PMNs, not in common with bacteria in the biofilm.

1.3.1 Biofilm v planktonic mode of growth

The switch from planktonic to the biofilm mode of growth is recognised to be a survival strategy protecting bacteria from external stresses, host immune defences and medical interventions (Costerton, Stewart, and Greenberg, 1999; Donlan et al., 2002). Strikingly, bacteria in biofilms are up to 1000 fold more tolerant to antimicrobial chemotherapy than their planktonic counterparts (Høiby et al., 2010). Although the terms tolerance and resistance are sometimes used interchangeably in the literature, the two have fundamentally distinct meanings (Olsen, 2015). Resistance generally refers to an increase in the minimum inhibitory concentration (MIC) of antimicrobial agents due to permanent genetic mutations or the acquisition of resistance encoding genes via horizontal gene transfer. Tolerance refers to bacteria, which may or may not be resistant, that can withstand antimicrobial action due to reversible phenotypic changes in growth, the prime example being the switch from planktonic to biofilm mode. Bacteria originating from a biofilm grown in a planktonic state will often demonstrate susceptibility to antimicrobial agents which are ineffective against the biofilm.

In *P. aeruginosa* biofilms, microarray analysis identified that only around 1% of genes were differentially expressed, with half of these upregulated and half downregulated (Whiteley et al., 2001). In fact, only 73 genes in total showed at least a two-fold difference in expression with 34 genes activated and 39 repressed. Unsurprisingly, genes coding for products involved in motility were downregulated, including genes for the synthesis of pili and flagella. Genes associated with virulence factors were also down regulated; for example, the T3SS regulator showed a 2.5-fold downregulation. Multiple 50S ribosomal proteins and translation proteins however were upregulated between 2 and 7-fold. However, the most upregulated genes were those of a bacteriophage closely related to filamentous bacteriophage Pf1, with some showing a 8 to 83.5 fold increase in expression. The authors (Whiteley et al., 2001), therefore suggest phage induction to be important for gene transfer or perhaps the phage genome contains a toxin encoding gene. Small colony variants (SCV) of *P. aeruginosa* emerging during biofilm development were noted to have high densities of Pf4 phage filaments on the cell surface, in addition to enhanced attachment and microcolony development (Webb, Lau, and Kjelleberg, 2004). The filamentous Pf4 prophage was later shown to be essential for multiple stages of biofilm development, virulence and the emergence of SCVs (Rice et al., 2009). Proteomic analysis of planktonic *P. aeruginosa* cells and those in a mature biofilm revealed more than 800 proteins to have a six-fold or greater change in expression (Sauer et al., 2002).

1.3.2 Biofilm Life Cycle

The biofilm life cycle, shown in **Figure 4**, comprises of the following 5 stages: (i) reversible attachment of the bacterium following transport to the surface and each other, (ii) irreversible attachment of bacterium to surface and each other, (iii) early development of microcolonies and biofilm, (iv) formation of a mature biofilm, and finally (v) biofilm dispersal (Basak et al., 2013).

Several factors can trigger the formation of biofilms, these include nutritional cues or stress, alterations in levels of secondary messenger molecules, and even in response to sub-inhibitory concentrations of antibiotics (Basak et al., 2013). For example, sub inhibitory concentrations of tobramycin induce biofilm formation in both *P. aeruginosa* and *E. coli* (Hoffman et al., 2005). Other antibiotics have also shown a similar effect (Morita, Tomida, and Kawamura, 2014); sub inhibitory concentrations of carbapenems, namely imipenem, caused an increase in biofilm volume and more robust biofilms (Bagge et al., 2004).

1.3.2.1 Attachment

During the first stage of the biofilm life cycle, the pili and flagella are crucial, enabling transportation of the bacterium towards the substratum and towards each other. The flagella in particular allows motility of the bacteria such that they can overcome the repulsive electrostatic forces that may be present between individual bacteria and between bacteria and the substratum

(Laverty, Gorman, and Gilmore, 2014). Additional *P. aeruginosa* cell appendages known as Cup fimbriae (chaperone usher pathway), specifically CupA fimbriae, were shown to be required for *P. aeruginosa* attachment to a substratum (Ruer et al., 2007; Vallet et al., 2004). During the reversible stage of attachment, the bacteria are bound to the substratum by their poles and may still be susceptible to antimicrobial treatment. However, within a few hours, bacteria shift to attachment by their longidutional axis and molecular binding occurs between bacterial adhesins and the substratum (Basak et al., 2013). At this stage, attachment is irreversible and antibiotic treatment becomes less effective.

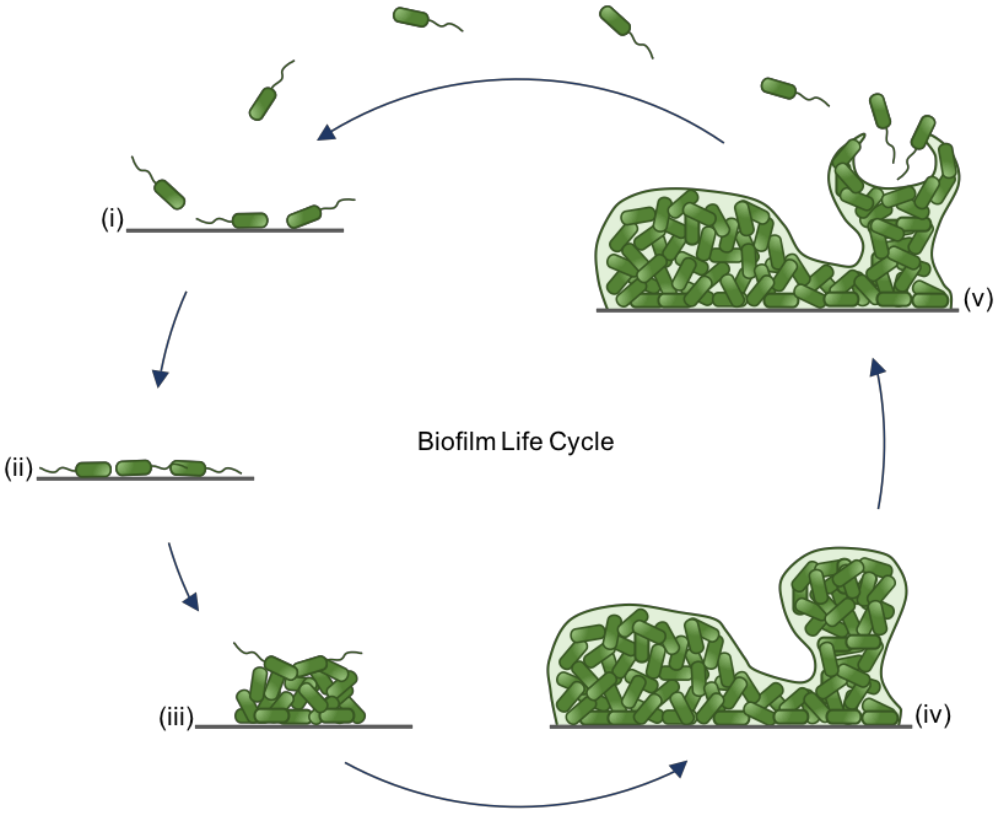


Figure 4. The biofilm life cycle. (i) the cycle begins with the reversible attachment of planktonic cells to a substratum, (ii) cells become irreversibly attached, (iii) as more cells attach to the surface, microcolonies start to develop and cells lose motility (iv) a mature biofilm is formed, with cells encased in self produced extra-cellular matrix, (v) cells from the biofilm disperse, re-establishing motility and able to form biofilms in other locations thereby re-starting the cycle.

1.3.2.2 Microcolony development and biofilm maturation

In the next stages of biofilm development, bacteria divide, begin synthesis of the matrix and polysaccharide components, and microcolonies develop (Kaplan, 2010). During these phases, loss of motility and decreased virulence is observed; for example, bacteria can exhibit loss of the flagellum. Components of the *P. aeruginosa* biofilm matrix include the exopolysaccharides

alginate, Psl and Pel. Ghafoor *et al.* (2011) found that although alginate was not crucial to biofilm development and can be partially compensated for by Psl and Pel production, it contributed to the preservation of bacterial cells and other studies demonstrate its involvement with biofilm tolerance mechanisms (Ghafoor et al., 2011; Ryder et al., 2007). Psl, a polysaccharide composed of mannose, galactose, rhamnose, glucose and small amounts of xylose, is involved in the structural stability of the biofilm, and, along with alginate, is required for the formation of the caps of mushroom shaped structures within mature *in vitro* biofilms (Colvin et al., 2011). Psl biosynthesis relies on the polysaccharide synthesis locus, an operon with 15 genes (Ryder et al., 2007). Glucose rich polymer Pel plays a role in biofilm cell density, compactness and in the stages of biofilm maturation (Ghafoor et al., 2011). Pel is crucial for pellicle formation and maintaining cell to cell interactions within the biofilm (Ghafoor et al., 2011). Its biosynthesis is dependent on an operon of 7 genes, *pelA* to *pelG*. Ghafoor et al. had found *P. aeruginosa* can still produce biofilms if any of the three single exopolysaccharides are not produced; biofilm formation is not possible only if both Psl and Pel are lacking. Another study had shown a PA14 strain unable to produce both Psl and Pel, was unable to progress beyond the stage of a monolayer of cells and produce a biofilm *in vitro*. (Colvin et al., 2011).

Extracellular DNA (eDNA) is another important component of the matrix. The origins of eDNA vary between different bacterial species; in *P. aeruginosa* biofilms the eDNA is produced as a result of autolysis of a bacterial subpopulation within the biofilm. Cell-cell communication system quorum sensing, controls the lysis of these cells (Montanaro et al., 2011). Concentrations of 3 to 14 mg/ml of eDNA have been found in CF sputum samples (Das et al., 2015). eDNA has important functionality in biofilm formation, stabilizing the biofilm structure by enhancing both the adhesion of cells to the substratum and cellular aggregation (Das et al., 2015; Montanaro et al., 2011). eDNA can also serve as a source of nutrients for bacteria in starvation mode and is involved in facilitating the twitching motility–mediated expansion of biofilms (Wei and Ma, 2013). Furthermore, eDNA can activate neutrophils, making *P. aeruginosa* biofilms pro-inflammatory (Wei and Ma, 2013). Experiments conducted by Whitchurch et al. (2002) showed that DNAse was successful in dissolving 12, 36 and 60 hour old, but not 84 hour old, *P. aeruginosa* biofilms *in vitro*. Recent investigations by Wang et al. (2012) have outlined interactions between eDNA and Psl, whereby the two components form eDNA-Psl fibres in a web like manner providing skeletal support within the biofilm. They also suggest that once interactions have taken place between matrix components such as eDNA and Psl, agents targeting an individual component in an attempt to breakdown the biofilm will not be successful, such as with DNAse I and more mature biofilms.

The protein component of *P. aeruginosa* biofilms includes cell appendages such as flagella, pili, adhesins, secreted extracellular proteins and outer membrane vesicle proteins (Fong and Yildiz, 2014; Wei and Ma, 2013). Proteins in the matrix are also key components and studies have shown that absence of these proteins results in reduced biofilm formation and affects the architectural

structure and stability of the biofilm. Proteomic analysis of the *P. aeruginosa* PAO1 matrix shows the majority of proteins to be outer membrane proteins (OMPs; 36%) and cytoplasmic proteins (29%), potentially due to cell lysis particularly considering the latter. Secreted proteins, periplasmic proteins and cytoplasmic membrane proteins and proteins of unknown cellular location form the remainder of the matrix proteome. Enzymes such as virulence factors alkaline protease and protease IV are also present in the matrix (Fong and Yildiz, 2014). Lectins LecA and LecB are also required for biofilm formation; LecA for the formation of biofilms on polystyrene and stainless-steel surfaces, and LecB for formation on glass surfaces. An important note as these materials can be the substratum for biofilms grown *in vitro* (Barnes et al., 2014; Chua et al., 2015; Mathee et al., 1999; Ramsey and Whiteley, 2004).

In the CF lung, additional substances contribute to *P. aeruginosa* biofilms. F-actin and DNA from necrotised neutrophils recruited as part of the immune response to infection, contribute to the composition of the biofilm matrix and enhance its formation abilities by providing an established framework (Walker et al., 2005). In fact, comparison of *P. aeruginosa* biofilms cultured *in vitro* with and without the presence of human neutrophils showed enhanced biofilm growth in cultures with neutrophils present. The density of the biofilm and number of bacterial cells present were both elevated (Parks et al., 2009).

1.3.2.3 Dispersal

In the final stage of biofilm development, cells are released from the biofilm, allowing them to colonise and begin biofilm growth on other surfaces; this is known as dispersal (Kaplan, 2010). The process by which the biofilm can engage in dispersal can be one of three. The first option, erosion, involves continuous release of cells singly or in small clusters from the biofilm during its formation. Second, may be the detachment of large sections of the biofilm in late stages of development known as sloughing. Both erosion and sloughing dispersal can be either active or passive processes. The third option, seeding, is only an active process and is also known as central hollowing; cells from within microcolonies of the biofilm have re-established motility and are released via small breaches in the microcolony structure.

Microcolonies of mature *P. aeruginosa* biofilms show mainly live cells on the outer parts (or walls) of microcolonies and high numbers of dead cells within the centre of biofilms (Webb et al., 2003). Further *in vitro* studies of *P. aeruginosa* biofilms grown in a flow cell system showed non motile cells forming the walls of microcolonies, and subpopulations of motile cells at the microcolony centre (Purevdorj-Gage, Costerton, and Stoodley, 2005). Webb et al. (2003) demonstrated the importance of prophage-mediated cell death in microcolony centres for the facilitation of dispersal, with the release of phage thought to be triggered by accumulation of ROS within the microcolony. Central hollowing of microcolonies has also been associated with rhamnolipids, extracellular surfactants which decrease the adhesiveness of cell to cell and to matrix interactions (Kaplan,

2010). This is evident as inhibition of rhamnolipid synthesis inhibits central hollowing (Boles, Thoendel, and Singh, 2005).

The triggering of biofilm dispersal has been an area of interest for many. Triggers include alterations in nutrient availability, oxygen depletion, changes in temperature or iron availability (McDougald et al., 2011). Fatty acid messenger *cis*-2-decenoic acid has been suggested to be responsible for inducing the dispersal of biofilms (Davies and Marques, 2009). Exogenous addition of *cis*-2-decenoic acid caused the dispersal of multiple species' biofilms including *P. aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, and *S. aureus.* *P. aeruginosa* endogenously produces *cis*-2-decenoic acid, and hence the authors put forward the idea that in small microcolonies levels of this fatty acid are low due to removal by diffusion and advective transport. However, in larger microcolonies, the rate of production is higher than the rate of removal and hence a concentration high enough to cause dispersal is reached. Although unlikely to be the sole cause for dispersal, this theory ties in with other suggestions that dispersal occurs once a critical size of the microcolonies has been reached (Harmsen et al., 2010). Molecules involved in quorum sensing, such as N-butanoyl homoserine lactone (C_4-HSL) and Pseudomonas Quinolone Signal (PQS), have also been implicated in initiating biofilm dispersal (Kaplan, 2010).

Many enzymes degrade the matrix of the biofilm and break bonds thereby allowing the release of cells for dispersal. These include glycosidases, proteases and deoxyribonucleases (Kaplan, 2010; McDougald et al., 2011). Perhaps the most well-known biofilm matrix degrading enzyme is dispersin B, a endogenous poly-N-acetylglucosaminidase produced by *Aggregatibacter actinomycetemcomitans* (McDougald et al., 2011). In *P. aeruginosa*, alginate lyase is a matrix degrading enzyme, and increased expression of this enzyme was shown to cause increased biofilm detachment (Boyd and Chakrabarty, 1994). Other effectors of biofilm dispersal include the previously mentioned phage, responsible for lysis of *P. aeruginosa* cells in microcolonies. Approximately 60 to 70% of bacterial genomes contain prophage sequences (McDougald et al., 2011).

Biofilm dispersed cells can go on to develop biofilms elsewhere by re-entering the first stage of the life cycle, ensuring sustenance of the bacterial population. Although presumed that cells dispersed from the biofilm would resemble planktonic cells, in fact, they are unique and physiologically different to both biofilm cells and planktonic cells. Single nucleotide resolution transcriptomic analysis by Chua et al. (2014) revealed 353 genes to have upregulated expression and 280 genes to have downregulated expression when comparing *P. aeruginosa* biofilm dispersed cells with planktonic cells. Upregulated genes included those associated with virulence, in particular the T2SS. This increased virulence was demonstrated in a *Caenorhabditis elegans* infection model, as organisms inoculated with biofilm dispersed cells resulted in higher mortality rates compared to those inoculated with planktonic cells. On the other hand, the transcriptomic analysis showed a downregulation of expression in genes involved in siderophores production in the dispersed cells,

and were shown to be more susceptible to iron stress. Chua et al.'s results showed that dispersed cells maintained their unique physiology whilst in the presence of a biofilm dispersing agent, whereas cells dispersed without induction maintained their phenotype for 2 hours or more before reverting to a planktonic phenotype.

1.3.3 Biofilm formation regulation

The switch from the planktonic to the biofilm mode of growth is not a random occurrence; rather an environmental adaptation and survival mechanism, requiring co-ordinated intercellular communication and complex intracellular signalling networks.

1.3.3.1 Bacterial communication: Quorum Sensing

Bacteria show high levels of co-ordination, and a communication system exists between individual bacterium. Many species, including *P. aeruginosa,* produce, detect and respond to small diffusible signal molecules which regulate many bacterial physiological processes and activities as part of a mechanism called quorum sensing (QS) (Li and Tian, 2012). QS comes into play once the bacterial cell density in a given environment had reached substantial levels (Sifri, 2008). In *P. aeruginosa*, the two main group derived benefits of the QS system are the regulation of virulence factor production and biofilm formation.

Small biochemical signalling molecules involved in QS, called autoinducers (AIs), are unique to each bacterial species (Li and Tian, 2012). The most well described AIs in Gram-negative bacteria are N-acyl-L-homoserine lactones (AHLs), part of the foremost lux-type QS identified from *Vibrio fischeri* (Høiby et al., 2010; Pesci et al., 1997; Sifri, 2008). In *P. aeruginosa*, two lux-type QS systems are present, the las and rhl systems. In the las system, autoinducer synthase LasI is responsible for the synthesis of N-(3-oxododecanoyl homoserine lactone) (OdDHL or 3O-C_{12}-HSL). AHLs can readily diffuse across bacterial cell membranes and enter the cytoplasm, following which the molecule can then bind to its cognate receptor; AI 3O-C_{12}-HSL binds to transcriptional activator protein LasR (Sifri, 2008; Wagner and Iglewski, 2008). The AI-protein complex then induces the expression of targeted genes, including *lasB, lasA, apr, toxA,* and also *lasI* thereby initiating a positive feedback loop or 'autoinducing circuit' hence the signalling molecules term (Pesci et al., 1997; Sifri, 2008). Comparatively, in the rhl system, RhlI is responsible for the synthesis of the AI N-butanoyl homoserine lactone (C_4-HSL), which binds to transcription activator protein RhlR. The AI-RhlR complex induces the expression of genes including rhlI (again causing positive feedback of signalling) and rhlAB, coding for a rhamosyltransferase needed for rhamnolipid production (Pesci et al., 1997; Sifri, 2008; Wagner and Iglewski, 2008). AHL's are only produced in high-cell density environments; accumulation of AHL's must occur and their concentration must reach a high enough level before a response (changes in gene expression) occurs (Annous, Fratamico, and Smith, 2009). Ultimately, QS results

in the repression or activation of target genes; around 10% of the *P. aeruginosa* genome are regulated by QS as identified by microarray analysis (Wagner et al., 2003).

Indeed, the las and rhl system are not independent to each other and are intertwined as the las system also exerts hierarchical regulatory control of the rhl system (Pesci et al., 1997). *P. aeruginosa* also produces a third signalling molecule, 2-heptyl-3-hydroxy-4-quinolone, also known as *Pseudomonas* quinolone signal (PQS), which links the las and rhl system (Wagner and Iglewski, 2008). PQS is synthesised by products of genes *pqsABCDE* and *pqsH*, and forms a complex with the PqsR receptor (Ciofu et al., 2014). Unlike AHL's, PQS is hydrophobic so cannot readily diffuse across membranes; hence release and trafficking of the signal is via the pinching off of small membrane vesicles from *P. aeruginosa*'s outer membrane.

QS in *P. aeruginosa* controls the expression, production and secretion of virulence factors, including elastase, proteases, rhamnolipid, pyocyanin and ETA (Prince, 2002). Bacterial motility is also controlled by QS as is many other factors contributing to biofilm architecture, integrity and timing of formation (Sifri, 2008; Wagner and Iglewski, 2008). The first study to link QS to biofilm formation found that mutants unable to produce 3O-C12-HSL had reduced biofilm thickness, lacked proper microcolony development and were more susceptible to sodium dodecyl sulphate (SDS) (Davies et al. 1998).

QS also regulates the synthesis of rhamnolipids and siderophores, key virulence factors implicated in biofilm formation (Rutherford and Bassler, 2012). Synthesis of rhamnolipids is particularly high during biofilm maturity where cell density is high (Davey, Caiazza, and Toole, 2003). As demonstrated by mutant studies (Davey et al., 2003), the production of rhamnolipids are important in maintaining void spaces and channels, known as open channels, which are part of the normal biofilm architecture and allow redistribution of nutrients, oxygen and removal of metabolic waste products . Additionally, rhamnolipids are known to be induced in the centre of the mushroom shaped biofilm structures and mediate the detachment of *P. aeruginosa* from biofilms and hence dispersal (Boles et al., 2005). The control of siderophore production is also crucial, as too high or too low iron concentrations have been shown to stimulate twitching motility and hence hinder biofilm formation (Singh et al., 2002). QS and the PSQ system also plays a role in autolysis and the release of eDNA (Wolska et al., 2015).

1.3.3.2 Intracellular signalling network: second messenger c-di-GMP

Bis-(3'-5')-cyclic-dimeric guanosine monophosphate (c-di-GMP) is a bacterial second messenger molecule which regulates many functions including the aggregative behaviour of bacteria, adhesiveness, virulence and biofilm formation. The intracellular levels of c-di-GMP are dependent on a balance between synthesis and degradation (**Figure 5**). High levels of c-di-GMP promote biofilm formation, increasing aggregation and adhesiveness, inducing matrix production, and reducing motility. Conversely, low levels of c-di-GMP promote motility and do not favour biofilm

formation. The c-di-GMP based signalling mechanism is considered to be the most complex second messenger signalling process to have been discovered in bacteria (Wolska et al., 2015).

C-di-GMP was first discovered to be involved in biofilm formation in 1987, where it was found to be a positive allosteric activator in *Gluconacetobacter xylinus*, enabling production of extracellular matrix component cellulose (Cotter and Stibitz, 2007; Ryan, 2013). The synthesis and degradation of c-di-GMP, is catalysed by regulatory proteins with GGDEF domains and EAL or HD-GYP domains, respectively (Ha and O'Toole, 2015). Diguanylate cyclases (DGC) with GGDEF domains promote the synthesis of c-di-GMP from two GTP molecules. C-di-GMP can then be degraded by phophodiesterases (PDE), first to an intermediate molecule pGpG by either EAL or HD-GYP domain proteins, and then to two GMP molecules by HD-GYP domain proteins, as shown in **Figure 5**. Most of these catalysing proteins also have additional signal input domains, and hence their activity is in response to intracellular cues or extracellular environmental stimuli (Ryan, 2013). Such stimuli can include oxygen, light, surface presence, chemical cues, and changes in cell density via QS (Srivastava and Waters, 2012).

Genomic analysis of *P. aeruginosa* reveals 41 proteins with GGDEF/EAL/HD-GYP domains, which regulate levels of c-di-GMP (Povolotsky and Hengge, 2012). Of these, 17 have GGDEF domains, 5 with EAL domains, 3 with HD-GYP domains, and 16 with both GGDEF and EAL domains (Wei and Ma, 2013). Those with both GGDEF and EAL domains can show both DGC and PDE activity, but often degeneration of one of the two motif occurs and hence the protein either has either DGC or PDE activity (Navarro et al., 2009).

In 2006, Amikam and Galperin, via sequence analysis, were the first to suggest c-di-GMP bound to a domain called PilZ ('pills'), a 118 amino acid protein encoded for by *P. aeruginosa* gene PA2960 with a previously unassigned function . A PilZ domain was identified to be present in multiple proteins including cellulose synthase, the enzyme activated by c-di-GMP enabling cellulose production in *G. xylinus*. In 2007, Merighi et al., identified 8 proteins of *P. aeruginosa* to contain a PilZ domain including the PilZ protein and Alg44, which is crucial for alginate biosynthesis. *In vitro* assays showed c-di-GMP bound to all the PilZ domains of all the proteins except for the PilZ protein, supporting the notion as PilZ as a c-di-GMP receptor. Hence, it is apparent c-di-GMP binds to the PilZ domain, and senses different intracellular levels of this second messenger molecule (Cotter and Stibitz, 2007). In addition to 'adapter protein' PilZ, c-di-GMP also binds to c-di-GMP riboswitches I and II and to transcription factors FleQ, VpsT, and Clp to name a few examples (Ryan, 2013). Hence, the c-di-GMP induced changes in gene expression are at the transcription, post transcription and post translational level (Cotter and Stibitz, 2007).

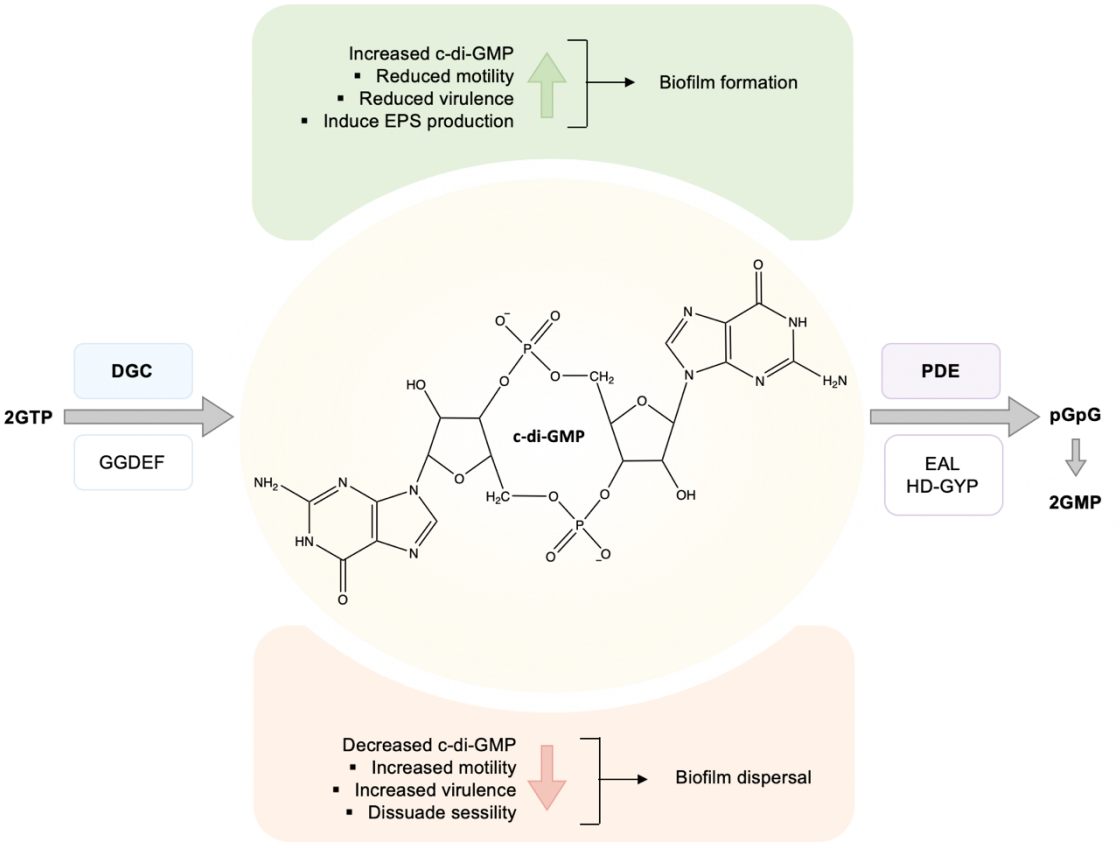


Figure 5. Synthesis, breakdown and effects of c-di-GMP. DGCs with GGDEF domains (red) synthesise c-di-GMP from two GTP molecule. PDEs with EAL or HD-GYP hydrolyse c-di-GMP into pGpG and then into two GMP molecules. The actions of c-di-GMP include decreasing cell motility and virulence, whilst promoting biofilm formation.

In addition to suppressing motility, increased c-di-GMP levels directly influence biofilm formation via EPS production (Ha and O'Toole, 2015). WspR is a GGDEF domain containing protein component of a chemotaxis-like system in *P. aeruginosa* which regulates EPS production and hence microcolony formation. Details behind this system are still unclear but it appears as though contact with a solid surface triggers the Wsp system and induces EPS production via increasing the expression of matrix component genes *pel* and *psl.*

1.3.4 Antimicrobial tolerance of *P. aeruginosa* biofilms

A combination of multiple mechanisms and factors account for the high tolerance of bacterial biofilms, distinct from the resistance mechanisms of planktonic bacteria, these are summarised in **Figure 6**. Initially the main mechanism thought to be responsible for high biofilm tolerance was the prevention of antibiotics penetrating through the biofilm matrix (Olsen, 2015; Stewart, 2002; Stewart and Costerton, 2001). Conversely, biofilms have a high water content, and many antibiotics can readily diffuse through the matrix but the penetration may be slow or incomplete

(Stewart, 2002; Stewart and Costerton, 2001) Hydrolysing enzymes can readily diffuse across the biofilm matrix and inactivate an antibiotic at a higher rate than the antibiotic can diffuse through (Stewart and Costerton, 2001). Slowed or incomplete penetration may also be a result of the antibiotic binding to matrix components, for example positively charged tobramycin binds to negatively charged alginate (Walters III et al., 2003). Secreted antibodies also struggle to penetrate through the biofilm due to binding in the matrix, as does toxic hydrogen peroxide due to the bacterial production of neutralising enzyme catalase (Fux et al., 2005). However, the lack of biofilm eradication via a gradual layer by layer elimination action indicates other biofilm tolerance mechanisms are present.

Biofilm physiology is linked to an adaptive stress response; for example, slowed penetration of an antibiotic through the biofilm matrix increases the contact time, enabling evolution of the response to the external stress (Fux et al., 2005). The expression of efflux pumps can also be triggered in response to an external stress (Chambless, Hunt, and Stewart, 2006).

Cells near the biofilm surface have ample oxygen and nutrient availability, whilst cells deeper within the biofilm closest to the substratum will be in a oxygen and nutrient limited microenvironment (Olsen, 2015). The resulting oxygen gradient has been shown to affect the activity of antimicrobials, such as aminoglycosides, and cause antibiotics to become ineffective (Borriello et al., 2004; Walters III et al., 2003) Hypoxia selects for bacterial resistance, thought to be via downregulation of energy metabolism genes and causing changes in gene expression, including genes involved in efflux pumps (Olsen, 2015; Schaible, Taylor, and Schaffer, 2012).

Gradients of metabolic substrate and products also develop, with the concentration of metabolic substrates decreasing from the top to the bottom of the biofilm, and metabolic products decreasing from the bottom upwards (Chambless et al., 2006). This triggers a starvation stress response causing subpopulations of bacteria deep within the biofilm to become more dormant and slow growing, resembling planktonic stationary phase bacteria (Nguyen et al., 2011; Olsen, 2015). Unfortunately the vast majority of antibiotics currently available work by targeting various aspects of cell growth, replication, and division, and hence are only effective against multiplying bacteria and ineffective against slow growing or non-multiplying stationary phase bacteria (Hu et al., 2010; Hu and Coates, 2006). The diffusion gradients, in particular gradients of metabolic products and waste, causes changes in pH and can interrupt the activity of certain antibiotics sensitive to pH (Stewart, 2002).

Biofilm cells are in close proximity to one another, enabling increased rates of horizontal gene transfer of resistance determinants, via transformation, transduction and/or conjugation (Olsen, 2015). These genes can be passed across not only bacteria of the same species, but across multiple species. Biofilms in the CF lung, although dominated by *P. aeruginosa*, are multispecies and the transfer of resistance encoding genes across different types of bacteria can result in entirely new

genetic combinations (Madsen et al., 2012). In particular, antibiotic-hydrolysing enzymes are highly transferred resistance determinants (Olsen, 2015).

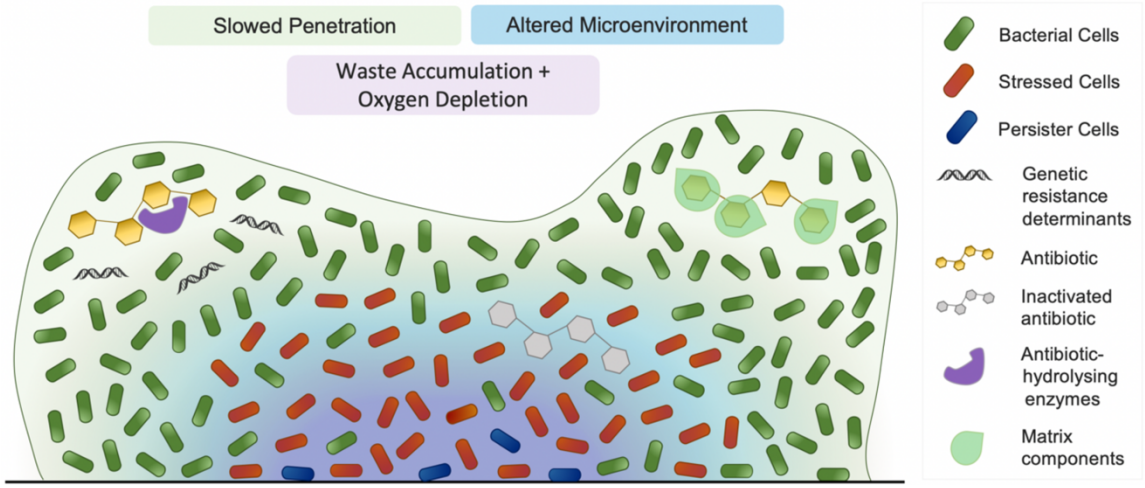


Figure 6. Biofilm tolerance mechanisms. Antimicrobials can be hydrolysed by bacterial enzymes, causing their depletion, or be bound by matrix components causing slowed penetration through the biofilm. The presence of an antimicrobial agent can also induce a stress response allowing bacteria to evolve and become non-responsive to the agent. The altered microenvironment and areas with waste accumulation and oxygen depletion causes changes in the bacteria, making them resemble planktonic stationary phase bacteria which cannot be targeted by conventional antibiotics. Dormant persister cells are also present which cannot be eradicated and can repopulate the biofilm even if the majority of other cells are killed.

Some recognise that bacteria in biofilms encounter higher rates of mutations, particularly the bacterium in microcolonies (Conibear, Collins, and Webb, 2009). Hyper-mutatability is seen with bacterial cells under stress, in conditions of starvation and treatment with antibiotics, such as cells within biofilms (Pace, Rupp, and Finch, 2005). Bacterium with elevated mutation rates evolve much faster and hence hypermutator bacterium are shown to have a higher incidence of multi-drug resistance compared with non-mutators. In a study by Ciofu et al. (2005), 54% of isolates from 79 CF patients with chronic infection showed the presence of hypermutatable *P. aeruginosa*. Also noted was that there was an increased chance of hypermutatable *P. aeruginosa* in older CF patients and those that had chronic infection for a longer period of time, which are certain to have biofilm growth in the lungs. The intensely pro-inflammatory environment of the CF lung is thought to be responsible for the high occurrence of hypermutatable *P. aeruginosa*. Experimental data supports the hypothesis that activated PMNs involved in the body's inflammatory response produce oxygen

radicals, causing chronic oxidative stress in the CF lungs, and oxidation of DNA contributes to, if not triggers, the development of hypermutatable *P. aeruginosa* (Ciofu et al., 2005).

Finally, persister cells represent a small subpopulation of cells which are dormant and highly protected, showing resistance to antibiotics, disinfectants and other antimicrobial agents (Lewis, 2005; Stewart, 2002). Persister cells are pre sent in planktonic bacteria, but higher numbers are present in biofilms, and make up between 0.1 and 1% of cells in the biofilm of *P. aeruginosa* (Dawson, Intapa, and Jabra-Rizk, 2011). This is the basis of the theory of how antimicrobial therapy can kill some or the majority of cells in the biofilm, however these persister cells remain and repopulate the biofilm. Furthermore, the presence of persister cells explains how even bacteria in very thin biofilms cannot be fully eradicated. It is thought that these persister cells are not genetically derived mutants but phenotypic variants which arise stochastically (Keren et al., 2004). 'Quiescence" is also a term used to describe these persister cells, as they display reduction in the expression of genes involved in cell metabolism and energy production and increase in those involved in cellular arrest.

1.3.5 Challenges of *P. aeruginosa* biofilms in CF

The formation of *P. aeruginosa* biofilms in the CF is a two-part problem. First, there is lack of a fast and efficient method of detection (Wu et al., 2014). Although *P. aeruginosa* infection can be confirmed through sputum sample cultures and the presence of a chronic infection identified via antibody levels, these tests take days to complete and there is no direct way of distinguishing the presence of *P. aeruginosa* biofilm. A biomarker, indicating *P. aeruginosa* biofilm growth, which could be quickly identified from easily acquired biological samples such as sputum, blood or saliva, would enable better orchestrated treatment regimens and clinical outcomes. Second, *P. aeruginosa* in the CF lung is difficult to clear despite aggressive antimicrobial chemotherapy due to biofilm tolerance mechanisms.

1.4 Involvement of NO and *P. aeruginosa* biofilms

NO is a highly diffusible simple gas and an ubiquitous physiological signalling molecule in multiple life forms including bacteria, plants, invertebrates and vertebrates (Barraud et al., 2006, 2015; Lamattina et al., 2003). This free radical diatomic gas is lipophilic and hydrophobic in normal atmospheric conditions. Endogenous NO is produced from L-arginine by enzymes known as nitric oxide synthases (NOS), of which there are multiple isoforms (Darling and Evans, 2003). Over the past few decades, research has uncovered the numerous important roles of NO. For example, the 1998 Nobel Prize in Physiology and Medicine was awarded for work that uncovered the importance of NO as a signalling molecule in the human cardiovascular system (Lamattina et al., 2003). Endogenously produced NO has vasodilatory effects on arteries and veins via relaxation

of vascular smooth muscle and was identified to actually be endothelium-derived relaxing factor (EDRF) (Ignarro, 2002; Ignarro et al., 1987). In addition to having vasodilator effects to maintain blood pressure in the cardiovascular system, NO is also an important signalling molecule involved in stimulating host defences in the immune system, regulating neural transmission in the brain, learning and memory, platelet aggregation, function and control of both the male and female reproductive systems, and cytotoxicity and cytoprotetion (Lamattina et al., 2003).

1.4.1 NO is a produced during anaerobic respiration in *P. aeruginosa* biofilms

Within the microcolonies of biofilms, it is recognised that cells in the centre of the microcolonies are in a nutrient starved state, subject to oxidative stress, and many undergo cell death (Webb et al., 2003). Oxidative stress is caused by the production of reactive oxygen intermediates, for example superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO^-). Accumulation of these molecules and lack of their removal causes damage to cellular lipids, proteins and DNA. Similarly, these cells also encounter nitrosative stress, caused by reactive nitrogen intermediates produced during anaerobic respiration.

P. aeruginosa is capable of both aerobic and anaerobic respiration in situations where oxygen is limited or completely lacking (Platt et al., 2008). Anaerobic respiration by *P. aeruginosa* requires nitrate (NO_3-), nitrite (NO_2-) or nitrous oxide (N_2O), to act as alternative electron acceptors (Schobert and Jahn, 2010). This is known as denitrification and involves 4 reductase enzymes: NarGHI, NirS, NorCB, and NosZ (detailed in **Figure 7**). NarGHI and NorCB are membrane bound, whilst NirS and NosZ are located periplasmically. These enzymes reduce nitrate (NO_3-) to dinitrogen (N_2), which is not the sole route but is the easiest for anaerobic energy generation, to enable ATP synthesis and cell growth (Hassett et al., 2009; Platt et al., 2008). Denitrification is also identified as dissimilation, which is in contrast to assimilation which reduces nitrate and nitrite to produce NH_3 and macromolecules.

The regulation of denitrification is complex and sophisticated, and multiple components are involved. Anr is perhaps the most important and central to activation of this pathway. Anr detects oxygen levels, acting as a sensing regulator, and can induce expression of the NarXL component system, Dnr and nitrate reductase NarGHI. NarL is a response regulator which is activated by nitrate sensor kinase NarX, and activates transcription of NirQ, NarGHI, and Dnr. Dnr is an essential component to the system; as well as sensing NO via ferrous heme, Dnr promotes transcription of the reductases and NirQ. NirQ is required to post-translationally activate NirS. Quorum sensing play a part in this pathway, and RhlR can repress transcription of the reductases to a certain degree. The PQS is also believed to be involved and have inhibitory and promotory effects on the reductases however specific details regarding this are yet to be established (Hassett et al., 2009; Schobert and Jahn, 2010).

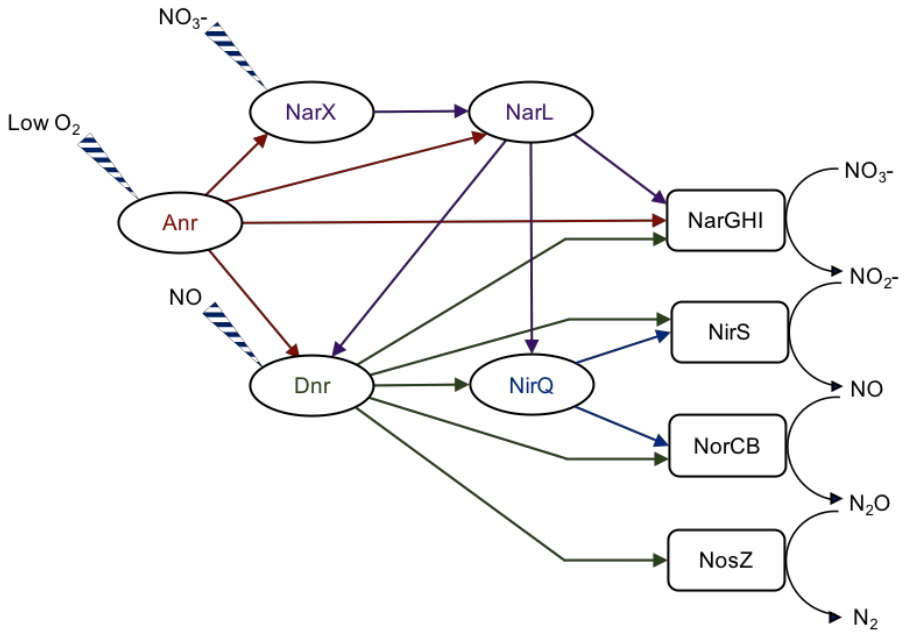


Figure 7. The denitrification pathway of *P. aeruginosa*. Four reductases NarGHI, NirS, NorCB, and NosZ (known as nitrite, nitrate, NO and nitrous oxide reductases respectively) are responsible for the reduction of nitrite (NO_3-) to dinitrogen (N_2). Nitrate sensor NarX activates response regulator NarL, which controls expression of NarGHI, NirQ and Dnr. Oxygen sensing regulator Anr promotes transcription of NarX, NarL, NarGHI and Dnr. NO sensor Dnr promotes expression the four reductases and NirQ.

As mentioned, NO is one of the intermediates in the denitrification pathway. Amongst its diverse functions, NO is also recognised to be a powerful antimicrobial molecule (Fang, 1997). Investigations into the effects of NO on *P. aeruginosa* confirm a toxic effect on biofilms as a result of NO accumulation (Yoon et al., 2002). Interestingly, accumulation of NO within mature biofilms and in the centre of microcolonies is known and this nitrosative stress linked to the death of cells in the microcolony core.

1.4.2 The CF lung is hypoxic with reduced NO levels

Interestingly multiple independent research groups have shown *P. aeruginosa* biofilms are subject to a microaerobic to anaerobic environment in the CF lung (Moreau-Marquis et al., 2008; Platt et al., 2008; Worlitzsch et al., 2002; Yoon et al., 2002). Estimations are that oxygen tension is severely reduced to below 2% if not completely hypoxic. This may seem paradoxical, yet plausible due to the destruction of lung tissue and presence of thick mucus and mucus plugs, that hypoxic intraluminal niches are present. This is supported by the isolation of obligate anaerobes from CF patients. In a 2009 study (Worlitzsch et al.,), 91% of 45 CF patients had isolation of one or more obligate anaerobic species from sputum samples including *Peptostreptococcus* spp, *Staphlyococcus*

saccharolyticus and *Actinomyces* spp. Further sputum analysis has also shown the increased expression of genes involved in the denitrification pathway in *P. aeruginosa* (Son et al., 2007).

Interestingly, it appears as though *P. aeruginosa* prefers anaerobic conditions. Worlitzsch *et al.* demonstrated that addition of motile *P. aeruginosa* on the CF airway surface penetrates into areas of the mucus which are hypoxic and then proceeds to excessively produce alginate. Yoon et al. (2002) corroborated this, and reported in their experiments that biofilms grown in anaerobic conditions showed 3 fold more growth and were more robust and compact compared to the biofilms grown in aerobic conditions.

In the CF lung however, levels of NO appears to be reduced in comparison with healthy lungs, and in contrast with other airway pathologies like asthma and bronchiectasis which show increased NO levels (Winter-de Groot and Ent, 2005). It is not clear why, however several reasons have been suggested. First, thick mucus in the lung inhibits diffusion of NO into exhaled air and so exhaled breath values do not accurately correlate with the levels of NO in the lung. This is supported by the increased concentration of NO_2^- and NO_3^- metabolites in sputum. Second, reduced NO could be due to a lack of substrate L-arginine. Third, the activity of NOS has been shown to be reduced in CF resulting in a reduction in NO production. Lastly, it has been suggested denitrification of NO by organisms such as *P. aeruginosa* may be responsible, resulting in increased NO consumption.

Michl et al. (2013), found that no correlation was evident between the level of exhaled NO, and with body mass index, FEV1, age, inflammatory markers or infection with either *P. aeruginosa* or *S. aureus*. NO was shown to be increased with patients on antibiotic suppressive therapy however. Conversely, other studies have demonstrated lower exhaled NO values with patients colonised with *P. aeruginosa*, compared to those without *P. aeruginosa* (Grasemann et al., 1997; Keen et al., 2007).

Irrespective of the cause, the reduced NO levels cause multiple negative effects including decreased bronchodilatation, and an increase in neutrophilic infiltration, viral replication, NO mediated oxidative injury and adherence of *P. aeruginosa* (Winter-de Groot and Ent, 2005). Hence, it is not surprising that reduced levels of exhaled NO are correlated with increasing lung disease severity (Keen et al., 2010).

1.4.3 NO causes the dispersal of *P. aeruginosa* biofilms

In 2006, Barraud et al. published work on the involvement of NO in the dispersal of *P. aeruginosa* biofilms. In contrast to previous research which demonstrates the toxic effects of NO on bacterial cells (Darling and Evans, 2003), it was demonstrated that sublethal concentrations in the nanomolar to micromolar range were able to induce the dispersal of *P. aeruginosa* in biofilms using multiple exogenous NO donors. At an optimal concentration of NO donor sodium nitroprusside (SNP), the

number of biofilms cells was reduced and the number of planktonic cells increased, giving a 10 fold decrease in ratio.

Comparison of biofilm development between wild type and mutant strains showed that a NO reductase deficient mutant (Δ*norCB*) showed higher numbers of dispersed cells and the biofilm contained more hollow voids and evidence of cell death. By comparison a NO_2- reductase deficient mutant (Δ*nirS*) which is unable to produce endogenous metabolic NO showed thicker and more confluent biofilms with no evidence of dispersal or cell death. This supports the notion that NO causes the dispersal of *P. aeruginosa* biofilms. Furthermore, NO in combination with conventional antibiotics, such as tobramycin, showed amplified treatment efficacy against *P. aeruginosa* biofilms compared with tobramycin alone, removing 80% of the biofilm cells. This data was indeed highly promising and represented a possible new therapeutic strategy to target *P. aeruginosa* biofilms.

1.4.4 Mechanism of NO induced dispersal

Much research has focused on investigating the mechanism of action behind the NO induced dispersal of biofilms. Further data from the 2006 publication by Barraud et al., detailed how low concentrations of NO enhanced the swimming and swarming motilities of *P. aeruginosa.* This increase in bacterial motility is consistent with dispersal and the reversion of bacteria from biofilm to planktonic state. It was shown that NO donor SNP caused a 25% increase in swimming motility and a 77% increase in swarming motility, and that this effect was abolished by a NO scavenger molecule 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO).

Following on from this, the same group linked the NO dispersal response to a reduction of intracellular c-di-GMP concentrations in biofilm and planktonic bacterial cells (Barraud, Schleheck, et al., 2009). Addition of NO stimulated PDE activity, causing degradation of c-di-GMP and hence a decrease in intracellular levels which has previously been established to be linked to biofilm dispersal. This is consistent with previous literature as PDE and DGC enzymes, which control the levels of c-di-GMP, respond and alter their activity based on environmental stimuli. The decrease in c-di-GMP effectively causes changes in gene expression that favour the planktonic mode of growth as opposed to growth in biofilms.

However, the precise mechanism behind NO induced dispersal in *P. aeruginosa* is not yet fully understood. Although, multiple other studies have shown NO to induce dispersal in other bacterial species (Barraud, Storey, et al., 2009). In *Shewanella woodyi*, H-NOx (heme-nitric oxide/oxygen binding) domains are conserved hemoproteins that are NO sensors and are often found adjacent to GGDEF and EAL domains (Cutruzzola and Frankenberg-Dinkel, 2015; Liu et al., 2012). This binding of NO to H-NOx proteins and the subsequent activation of PDE activity has been shown to

provide a molecular explain for alteration of c-di-GMP. However, H-NOX proteins are not present in *P. aeruginosa*.

The first protein identified to be involved in the c-di-GMP based theory behind NO induced dispersal was chemotaxis inducer BdlA (biofilm dispersion locus) in 2009 (Barraud, Schleheck, et al., 2009). BdlA is constitutively expressed but was shown to be upregulated further following exposure to NO (Barraud, Schleheck, et al., 2009; Petrova and Sauer, 2012); in a *bdlA* knock out strain of *P. aeruginosa* no significant differences in intracellular c-di-GMP concentrations in the presence and the absence of NO were noted, yet significant reductions were noted in the wild type. BdlA has a TarH or MCP domain (methyl accepting chemotaxis protein) and two conserved PAS (Per-Arnt-Sim) domains (Morgan et al., 2006). These encode for sensory receptors that can bind and respond to NO, and genomic *P. aeruginosa* analysis demonstrates an association between these and GGDEF and EAL domains (Cutruzzola and Frankenberg-Dinkel, 2015). Although the *bdlA* knock out strain was defective at biofilm dispersal, BdlA itself lacks GGDEF and EAL domains (Roy, Petrova, and Sauer, 2012). Therefore, the link between NO, BdlA and PDE activity came into question.

Along with *bdlA*, 6 other genes were initially proposed to be involved in NO induced dispersal: *fimX*, *morA*, *rocS1*, *PA0575*, *PA1181*, and *PA2072* (Barraud, Schleheck, et al., 2009). Identification of these genes was via a screening method and to be chosen had to satisfy at least three of the following 4 conditions: (i) was differentially expressed after NO exposure based on microarray data, (ii) the gene has EAL/GGDEF domain or known to alter c-di-GMP levels, (iii) the gene has a PAS domain, and (iv) the protein product of the gene has involvement with biofilm formation or motility (Barraud, Schleheck, et al., 2009). All 6 satisfied conditions (ii) and (iii). When each of these 6 genes were knocked out, dispersal still occurred in the presence of NO. In the same assays, the *bldA* knockout mutant did not show a significant dispersal effect with NO. This suggests that each of the 6 genes individually is non-essential to NO induced dispersal. However, as BldA is shown to be essential but does not have GGDEF or EAL domains, there could be an interaction or signal transduction pathway linking BdlA to FimX, MorA, RocS1, PA0575, PA1181, and PA2072.

In 2012, Roy et al., discovered 2 further proteins essential for NO induced dispersal, RbdA and DipA, which possess PDE activity and NO sensing PAS domains. *RbdA* and *dipA* knock out mutants did not disperse as the wild type did in response to NO and in response to other dispersal inducing agents such as glutmate, mercury chloride, and ammonium chloride.

The following year, in 2013, two further proteins were implicated in NO dispersal by Li et al., (2013), MucR and NbdA. Data from their experiments indicate both the proteins to exhibit PDE activity, whilst MucR also showed DGC activity. Again, mutant knock out studies were used to identify their impact on dispersal; the *mucR* knockout did not disperse when exposed to either NO or glutamate, however the nbdA knockout only showed lack of dispersal when exposed to NO.

Hence, so far, NbdA is the only PDE critical in the dispersal of *P. aeruginosa* biofilms induced specifically by NO. MucR and NbdA are the only two proteins produced by *P. aeruginosa* to have MHYT domains, a domain with seven predicated MSDs and thought to have a putative function to sense diatomic gases including oxygen, carbon monoxide and NO (Cutruzzola and Frankenberg-Dinkel, 2015; Galperin et al., 2001; Li et al., 2013). This sensory function is thought to occur through protein bound copper ions, co-ordinated by methionine and histidine residues on the proteins outer face. Once this has occurred the signal is transferred through GGDEF to EAL and PDE activity is stimulated hydrolysing c-di-GMP and resulting in biofilm dispersal, shown in **Figure 8.**

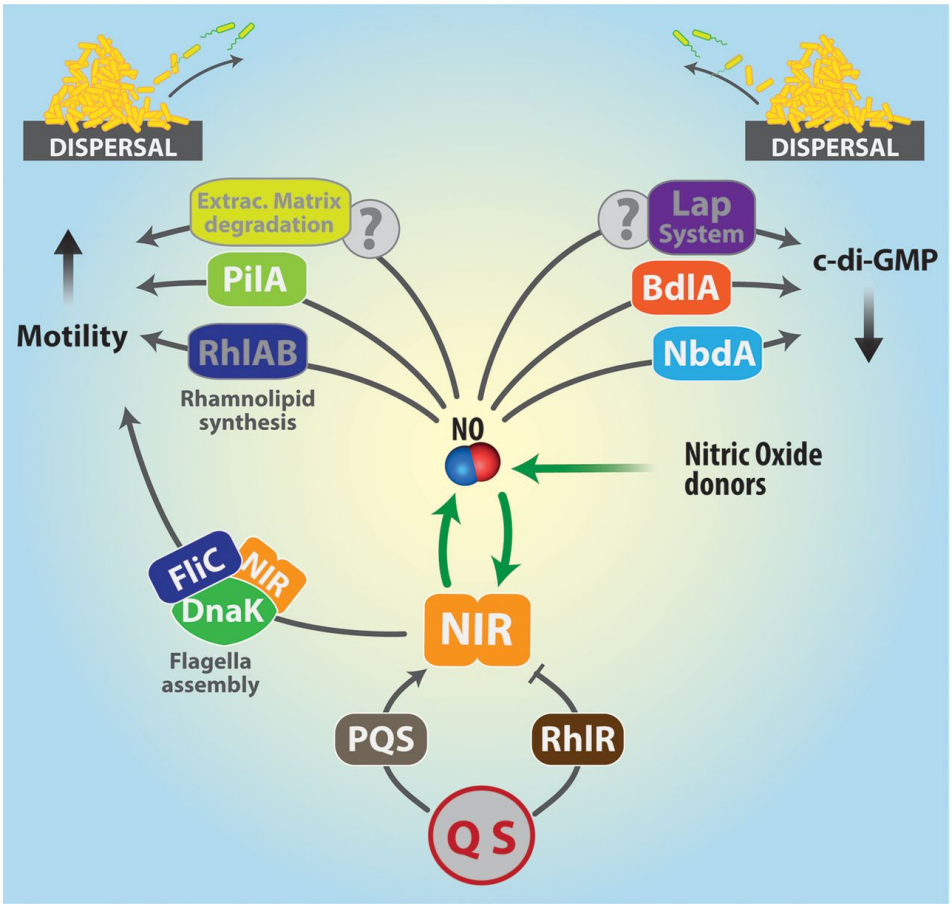


Figure 8. Factors involved in the NO induced dispersal response in *P. aeruginosa*. Endogenous NO can be generated via Nir, which is under the control of the rhl and PQS QS systems, whilst exogenous NO may be introduced using NO donors. NO is thought to decrease c-di-GMP levels via proteins including BdlA and NbdA. Another potential means of lowering c-di-GMP may be via the Lap system, though this still requires investigation in *P. aeruginosa*. An increase in motility is also thought to be initiated via an increase in pili (PilA) and rhamnolipid synthesis (RhlAB), degradation of the extracellular matrix, and via flagella assembly. Image reproduced with permission from Cutruzzola and Frakenberg-Dinkel (2015).

In addition to the c-di-GMP based pathway, membrane bound protease LapG has also been identified to be crucial to the NO dispersal response (Barraud et al., 2015). Knockout *lapG P.aeruginosa* mutants were found to be completely defective at dispersal in response to NO. In *Pseudomonas putida* and *Pseudomonas fluorescens* LapG cleaves LapA via receptor protein LapD. However, a LapA homolog does not exist in *P. aeruginosa* (Barraud et al., 2015).

Very recently, a novel family of bacterial haemoproteins called NO sensing proteins (NosP) have been discovered in organisms including *P. aeruginosa* following 11 years of research by the group, and are comparable to the H-NOX proteins found in bacteria not including *P. aeruginosa* (Bacon et al., 2018; Hossain and Boon, 2017). Even more interestingly, in P. aeruginosa, NosP is in the same operon as PA1976 (NahK), identified as a NosP associated histidine kinase, which has been previously associated with biofilm regulation (Hossain, Nisbett, and Boon, 2017). Based on their experiments, the authors proposed that NosP bound NO inhibits NahK activity, which includes phosphotransfer to histidine-containing phosphotransfer protein HptB. This is turn results in biofilm dispersal, however the specific players involved in the signalling pathway beyond this point have yet to have been identified.

1.4.5 Use of low dose inhaled NO in CF patients to disperse *P. aeruginosa* biofilms

After the NO induced dispersal effect was discovered, focus then turned to how it would be possible to develop this concept into a novel anti-biofilm therapy for patients. A small initial proof of concept trial was recently conducted in Southampton, UK, with inhaled NO gas administered to patients in combination with IV antibiotics tobramycin and ceftazidime (Howlin et al., 2017). Twelve hospitalised CF patients received NO gas via a nasal cannula overnight for 8 hours, for 7 consecutive days at a concentration of 10 ppm. Despite the short duration of this study, fluorescent *in situ* hybridisation (FISH) analyses indicated a significant reduction in *P. aeruginosa* biofilm volume and the number of aggregates with 20 or more cells.

1.4.6 Practicalities of using NO as a novel therapy for CF patients

Despite positive results from the proof of concept clinical trial, the method used to administer NO to the lungs had several disadvantages. Patients directly inhaled low doses of gaseous NO (gNO) via a nasal cannula. Although inhalation is a more specific route of administration compared to oral or intravenously administered NO-releasing drugs, gaseous NO is only currently available in the form of pressurised gas cylinders (Cairo, 2013).

Numerous safety precautions must be in place for the transport, storage and use of pressurized gas cylinders as they may cause or intensify fire and there is risk of explosion if the cylinders are heated (Air Products, 1998). These necessary precautions limit the use of the gas cylinders largely to hospitals and medical facilities with the capabilities of handling such equipment. Additionally,

the use of inhaled NO therapy involves a complex delivery device, calibration equipment, and monitoring by trained respiratory therapy staff (Yu et al., 2015). CF patients are particularly vulnerable to infection, so an increased number of hospital visits, or time in hospital, is not desirable. As such, barring intravenously administered antibiotics and surgery, all other current CF therapies have been adapted for home use.

In addition to practical issues, the cost of the current inhaled NO therapy is incredibly expensive, largely owing to the difficulty in handling the gas and its incompatibility with oxygen which results in the formation of toxic nitrogen dioxide (NO_2) (Yang et al., 2015). The sole FDA-approved clinical indication for the use of inhaled NO is persistent pulmonary hypertension of the newborn (PPHN); a 5 day course of this therapy in the US has a cost of approximately $12,000- $14,000 (Yang et al., 2015). Broken down, the cost of inhaled NO is approximately $6/L, with a minimum charge of $3000 for the opening of the tank of gas. Though expensive, the therapy for PPHN is a short term one, whereas this is unlikely to be the case with CF patients who currently require maintenance therapies for the duration of their life, thereby multiplying predicted costs drastically. Of final note, is that inhalation via nasal cannula may be suitable for newborns, however is less so for adults, and being physically connected to a large array of equipment renders the patient immobile.

Based on these impracticalities, it is clear an alternative approach to deliver NO to the lung is greatly desired. The ideal therapy would be one that is able to ensure selective and targeted delivery of NO to the lungs to avoid systemic effects, is practical for patients and easily administered, does not require hospitalisation and is cost effective.

1.5 Aims of this study

In this body of work, emphasis rests on the use of clinical CF isolates of *P. aeruginosa*; the overall aim is to investigate the relationship between CF isolates and NO, and investigate emerging potential novel NO-based therapies targeted at CF patients with *P. aeruginosa* infection. More specific objectives are as follows:

1) To investigate the effects of NO donor sodium nitroprusside (SNP) on clinical CF isolates of *P. aeruginosa*, and to investigate whether there is a correlation between the response to NO and isolate phenotype (Chapter 3)

2) To investigate if the c-di-GMP based mechanism for NO induced dispersal in PAO1 also stands true for clinical CF isolates (Chapter 3)

3) To explore the effects of the initial lead compound of a group of novel and targeted NO-releasing prodrugs, cephalosporin-3'-diazeniumdiolates (C3Ds), against CF isolates of *P. aeruginosa*, alone and in combination with antibiotics (Chapter 4)

4) To analyse the effects of a group of new generation C3Ds designed to have dual-action, and identify which compound from the group of analogues hold the most promise as a potential new CF therapy (Chapter 5)

5) To investigate the use of a novel NO delivery system that exploits electrochemical generation of NO gas from air, and whether this device could be used to treat *P. aeruginosa* infection in CF patients (Chapter 6)

Chapter 2 Materials and Methods

2.1 Bacterial strains and growth conditions

Laboratory strain PAO1 was obtained from the University of Washington, USA. Twenty clinical isolates of *P. aeruginosa* were obtained from the University of Southampton's culture collection, originally isolated from the sputum of twenty CF patients (mean age at informed consent: 28; range 19 to 65) based at University Hospital Southampton NHS Foundation Trust, UK. The mean age at informed consent was 28, with a range of age from 19 to 65 (NHS Research Ethics Committee 08/H0502/126). Details relating to the patients are shown in **Table 2**. CF physiotherapists assisted patients with the exportation of sputum into sterile containers. A research fellow then processed the samples in the laboratory: the sputum was digested with Mucolyse (Pro-lab Diagnostic) containing dithiotheritol (DTT) and phosphate buffer for 15 mis at 37˚C. *P. aeruginosa* was selected using cetrimde agar (Sigma Aldrich); a sterile loop was then drawn across the plate for a random selection of strains which were frozen down to form the culture collection. Multiplex polymerase chain reaction (PCR) was conducted to confirm *P. aeruginosa* in the samples.

For this study, samples from the culture collection were streaked onto selective cetrimide agar (Sigma Aldrich) to confirm isolation of *P. aeruginosa* and single colonies were selected to prepare fresh bacterial stocks in cryovials containing beads (Fisher Scientific), kept at -80˚C. Overnight cultures were prepared by inoculating a single bead of the frozen stock into 10 ml of Luria Bertani (LB) broth (Formedium) and incubated at 37˚C with shaking at 180 rpm. Biofilms were cultivated in M9 minimal media (pH 7; Formedium) containing 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, and supplemented with 2 mM $MgSO_4$ (Sigma Aldrich), 100 uM $CaCl_2$ (Sigma Aldrich) and 20 mM glucose (Sigma Aldrich). Non-selective tryptone soya agar (TSA) plates (Oxoid) and LB agar plates (Formedium) were used for general purposes such as colony forming unit (CFU) counting.

Table 2. Patient information for clinical isolates of *P. aeruginosa* used in this study

Isolate	Sex	Age*	Date sample acquired	Status*	Lung function (predicted values)*	History of PA infection/other notes	Current status of patient
PA05	F	16	02.11.10	NA	FEV1 = 47% FVC = 40%	First PA isolated in July 2000 (Age 7). Isolated again in Oct 2001, and in 2003. Chronic PA from late 2004 (Age 12). *M. abscesses* also detected in 2004. Mucoid PA isolated from sample.	Decd. (Age NA)
PA08	M	25	08.03.11	Exb.	Too breathless for lung function tests; Type II respiratory failure	First PA isolated in 1992 (Age 6). PA detected again Dec 1996. PA detected frequently as of Feb 1998, with SA also detected. Chronic PA from 1998 (Age 11). MRSA and *A. fumigatus* also detected in sample.	Decd. (Age 26)
PA10	F	20	01.03.11	Exb.	FEV1 = 41% FVC = 60.5%	Seen at SGH from 2008 onwards, notes sparse prior to this. PA detected in 2006 (Age 16). PA and SA detected in Mar 2009. Chronic PA from 2008/2009 (Age 18/19).	Alive
PA15	M	26	26.08.09	Well	NA	Mucoid PA first detected Aug 2009. Scanty growth of yeasts in sample.	NA
PA20	F	22	23.03.11	Exb.	FEV1 = 54% FVC = 68%	PA detected in 2000 (Age 12). PA detected again in 2005 (Age 17). Sample also grew: SA,HI, Streptococcus Group C, mucoid PA	Alive
PA21	F	18	12.05.11	Exb,	FEV1 = 26% FVC = 29%	PA, SA and HI detected in 1998 (Age 7). Chronic PA from 2000 (Age 9). Mucoid PA from sample.	Decd. (Age 21)

Isolate	Sex	Age*	Date sample acquired	Status*	Lung function (predicted values)*	History of PA infection/other notes	Current status of patient
PA26	F	29	25.05.10	Exb.	FEV1 = 19.6% FVC = 40.8%	First PA isolated in 1988 (Age 7). Colonised with PA and *B. cepacia* isolated April 1998. Scanty growth of yeasts April 2010. Heavy growth of *B. cepacia,* and type II respiratory failure at time of sample.	Decd. (Age 29)
PA30	F	19	17.05.10	Exb.	FEV1 = 43% FVC = NA	PA detected in Dec 2001, PA also detected in May 2010 MRSA detected in sample.	Alive
PA31	F	24	09.12.09	NA	NA	Left lung abscess at time of sample	NA
PA37	F	4	01.09.09	NA	NA	Negative PA antibody test in July 2009.	NA
PA39	F	18	04.04.11	Exb.	NA	Mucoid PA from at least Apr 2009. Heavy growth of PA and 3 other Pseudomonas species reported Feb 2011. Mucoid PA and yeasts in sample.	NA
PA44	F	34	27.08.09	Exb.	NA	Mucoid PA and SA growth in throughout 2009.	NA
PA47	M	22	25.05.10	Exb.	FEV1 = 65% FVC = 100%	First PA in 1991 (Age 3, clear following eradication therapy) PA detected again in 1998 (Age 10) Chronic PA from 2004 (Age 16). Homozygous F508del. SA also in sample.	Alive
PA49	F	38	24.05.11	Exb.	NA	Heavy PA growth detected at least from Mar 2010.	NA

Isolate	Sex	Age*	Date sample acquired	Status*	Lung function (predicted values)*	History of PA infection/other notes	Current status of patient**
PA55	F	20	03.09.09	Well	NA	Elective admission for IV antibiotics at time of sample, SA and *Streptococcus* Group A also detected.	NA
PA56	F	21	11.11.10	Exb.	Too breathless for lung function tests	Intermittent PA in 1997 and 1998 (Age 9 and 10) with 3 courses of IV antibiotics. Chronic PA in 2003 (Age 15). MRSA and yeasts also isolated from sample. Cause of death was malnutrition resulting from anorexia, independent of CF lung disease.	Decd. (Age 22)
PA57	M	21	14.04.11	Exb.	NA	*B. cepacia* and HI detected in Nov 2009. *B. cepacia*, HI, SA and yeasts also in sample.	NA
PA58	M	30	08.09.09	NA	NA	MRSA also detected in sample.	NA
PA66	M	17	01.10.09	NA	FEV1 = 70% FVC = 92%	Intermittent PA from 1996 (Age 4). PA detected again 1998. Chronic PA from April 2001 (Age 9). *B. cepacia* detected in 2005, eradicated in 2006. Homozygous F508del. Patient had sibling with CF who died despite having higher FEV1 which suddenly deteriorated after geographical relocation.	Alive
PA68	M	51	07.02.11	Exb.	FEV1 = 30% FVC = 50%	No correspondence prior to 2003. PA and *C. albicans* detected in 2003; Chronic PA from 2003 (Age 43)	Decd. (Age 53)

* = at time sputum sample was acquired. ** = status checked January 2019.

Exb. = exacerbation, NA = not available/unknown, Decd. = deceased,

PA = *P. aeruginosa*, SA = *S. aureus*, HI = *H. influenzae*.

2.2 NO donors, antibiotics, and cephalosporin-3'-diazeniumdiolates

Sodium nitroprusside (SNP; Sigma Aldrich) was prepared fresh in sterile Milli-Q water and filter sterilized before use. Care was taken to protect SNP from light during preparation, due to its light-sensitive nature. All antibiotics were acquired from Sigma Aldrich, aside from cefozopran hydrochloride which was acquired from Cambridge Bioscience, and cephaloram. All C3D compounds and cephaloram were synthesised by chemists at the University of Wollongong, Australia. All compounds were stored at -80˚C with desiccant and prepared fresh in DMSO or water (Sigma Aldrich) and then media before use. C3D compounds included were as follows: initial lead compound, DEA-CP (DiEthylAmine-NONOate Cephalosporin Prodrug) both potassium salt and carboxylic free acid forms (investigated in Chapter 4), and multiple new generation C3Ds (investigated in Chapter 5), detailed within **Table 3.** Additional details, including the molecular formula, molecular weight and chemical structure for all C3D compounds are shown in **Appendix A**.

Table 3. Eight control antibiotics (blue) and twelve new generation C3Ds used in Chapter 5

Agent	Abbreviation	Description	From
Cephaloram	LAM	1st generation cephalosporin antibiotic	UOW
PYRRO-cephaloram	P-LAM	Cephaloram with PYRRO/NO donor	UOW
AMINOPIP1-cephaloram	A1-LAM	Cephaloram with 1-Aminopiperidine NO donor	UOW
AMINOPIP2-cephaloram	A2-LAM	Cephaloram with 2-Aminopiperidine NO donor	UOW
Cefalexin	CEX	1st generation cephalosporin antibiotic	Sigma Aldrich
PYRRO-cefalexin	P-CEX	Cefalexin with PYRRO/NO donor	UOW
Cefuroxime	CXM	2nd generation cephalosporin antibiotic	Sigma Aldrich
Pyrro-cefuroxime	P-CXM	Cefuroxime with PYRRO/NO donor	UOW
Ceftazidime	CAZ	3rd generation cephalosporin antibiotic	Sigma Aldrich
PYRRO-ceftazidime	P-CAZ	Ceftazidime with PYRRO/NO donor	UOW
AMINOPIP1-ceftazidime	A1-CAZ	Ceftazidime with 1-Aminopiperidine NO donor	UOW
AMINOPIP2-ceftazidime	A2-CAZ	Ceftazidime with 2-Aminopiperidine NO donor	UOW
Cefotaxime	CTX	3rd generation cephalosporin antibiotic	Sigma Aldrich
Ceftriaxone	CRO	3rd generation cephalosporin antibiotic	Sigma Aldrich
Cefepime	FEP	4th generation cephalosporin antibiotic	Sigma Aldrich
PYRRO-cefepime	P-FEP	Cefepime with PYRRO/NO donor	UOW
AMINOPIP1-cefepime	A1-FEP	Cefepime with 1-Aminopiperidine NO donor	UOW
AMINOPIP2-cefepime	A2-FEP	Cefepime with 2-Aminopiperdine NO donor	UOW
Cefozopran	CZOP	4th generation cephalosporin antibiotic	Cambridge Bioscience
PYRRO-cefozopran	P-CZOP	Cefozopran with PYRRO/NO donor	UOW

UOW = Synthesised at University of Wollongong

2.3 Phenotypic characterisation of *P. aeruginosa* strains

PAO1 and the twenty clinical isolates of *P. aeruginosa* were phenotypically characterised for colony morphology, mucoidy, pigment production, planktonic and biofilm growth rates and antimicrobial susceptibility testing.

2.3.1 Colony morphology

P. aeruginosa strains were streaked onto nutrient-rich LB agar and incubated at 37˚C for 24 to 48 h. Strains were characterised as SCV if: a) the size of colonies measured between 1 and 3mm following 48 h incubation, and b) this phenotype was maintained for a further two subcultures, as previously defined for *P. aeruginosa* by Haussler et al (1999).

2.3.2 Mucoidy

To identify the isolates as mucoid or non-mucoid, the strains were grown on PIA and visually assessed after 48 h incubation. PIA was used as growth of CF isolates of *P. aeruginosa* on other agar medium has been shown to result in increased rates of reduced expression of the mucoid phenotype and reversion to a non-mucoid phenotype (Pugashetti et al., 1982).

2.3.3 Pigment production

The production of pigments was investigated by visual assessment of isolates grown on cetrimide agar, as well as LB and PIA. Cetrimide agar is a selective media which is known to enhance *P. aeruginosa* pigmentation; visual assessment was carried out following 24 to 48 h incubation at 37˚C.

2.3.4 Planktonic growth rates

Overnight cultures of *P. aeruginosa* strains were grown, then diluted into fresh broth and transferred to wells of a 96 well microtitre plate and placed into the FLUOstar Omega Microplate Reader (BMG Labtech) and incubated at 37˚C. Absorbance values (OD584 nm) were taken at 15-minute intervals for 62 h, with gentle shaking just before each reading.

2.3.5 Biofilm growth rates

The biofilm forming capabilities of *P. aeruginosa* strains were assessed via a crystal violet (CV) assay in a 96 well microtitre plate system, adapted from previous literature (Kwasny and Opperman, 2010; O'Toole, 2011). Overnight cultures were diluted 1 in 100 into freshly prepared M9 minimal media and added into a flat-bottom tissue culture treated 96 well microtitre plate

(Thermo Scientific) with a minimum of six replicate wells per strain; the location of wells altered such that for each group, 3 wells were located near the edge of the plate and 3 wells located nearer the centre of the plate to exclude any effect of well location on biofilm growth. Uninoculated M9 minimal media served as negative control. Four replicate 96 well microtitre plates were set up for each set of strains, for sacrificial analysis on days 1, 2, 3 and 5, and incubated at 37˚C. Daily media changes with fresh M9 minimal media were carried out for biofilms grown for more than 1 day. After incubation, the planktonic suspension was removed and discarded, and the biofilms washed twice with PBS to remove any non-adherent cells. Biofilms were then stained with 0.1% CV (Sigma Aldrich) for 15 mins. The CV was then removed, and biofilms rinsed with water 3 to 4 times, until clear, and left to air dry overnight. CV stained biofilms were resolubilised in 30% acetic acid (Sigma Aldrich) for 20 mins, and then transferred to a new 96 well microtitre plate. The absorbance was measured at 584 nm using a FLUOstar Omega Microplate Reader (BMG Labtech).

2.3.6 Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) of strains were calculated using the broth microdilution method in accordance with Clinical Laboratory Standards Institute guidelines (CLSI, 2012a). Two-fold serial dilutions of the antibiotic were prepared in a 96 well plate. Antibiotic dilutions were added to a new 96 well plate in triplicate, followed by the addition of inoculated cation-adjusted Mueller Hinton broth (CAMHB) broth, prepared by diluting an overnight culture 1 in 500 to achieve approximately 1 x 10^5 to 10^6 cells. A CFU count was carried out to confirm the exact cell number of the inoculum. Blank samples were prepared using antibiotic dilutions and uninoculated CAMHB broth. The 96 well plates were incubated overnight at 37˚C and the OD values read at 584 nm using the FLUOstar Omega Microplate Reader (BMG Labtech). The MIC was identified as the lowest concentration to inhibit bacterial growth according to turbidity.

2.4 Evaluation of NO release from NO donors

NO donors and novel NO releasing compounds used in this study were analysed for their NO release via two different methods, using a chemiluminescence detector and the colorimetric Griess Assay.

2.4.1 Chemiluminescence analysis

The release of NO from the novel compounds was assessed using a highly sensitive chemiluminescence detector (CLD 88Y, Eco Physics), as previously described (Bryan and Grisham, 2007; Feelisch et al., 2002). The CLD has a measurable NO range of 0 to 5000 ppb, a minimum detectable concentration of 0.05 ppb, a rise time (T_{90}) of <0.1 s. Briefly, the setup is as follows; a glass purge reaction vessel bubbled with air is connected to an iced scrubbing bottle

containing 1M sodium hydroxide, trapping traces of acid. The gas outlet from the scrubbing bottle is then in turn connected to the CLD. The CLD utilizes ozone (O_3) to react with NO; a product of this reaction is nitrogen dioxide in an excited state (NO_2*) which emits light upon returning to ground state, as shown in the following chemical equations:

$$(1)\ NO + O_3 \rightarrow NO_2^* + O_2$$

$$(2)\ NO_2^* \rightarrow NO_2 + h\nu\ (light)$$

This light is then quantified by a photomultiplier tube and directly correlates with NO concentration. PBS was added to the glass reaction vessel, heated to 37˚C and purged with air, to produce a baseline. SNP was prepared fresh in PBS just prior to the beginning of the assay and added to the vessel at a final concentration of 1mM. C3D compounds were prepared fresh in PBS and added to the reaction vessel at a final concentration of 10 μM, followed by 20 units of *Bacillus cereus* penicillinase (Sigma Aldrich). NO values were recorded using PowerChrom software. To quantify the amount of NO release, the area under the peaks were analysed using PowerChrom and multiple sodium nitrite solutions of increasing concentration were used as a standard.

2.4.2 Quantification of nitrite and nitrate using the Griess assay

The colorimetric Griess assay was used to indirectly measure the concentration of NO in solution, via quantification of NO breakdown products nitrite and nitrate. The protocol was adapted from Miranda et al., (2001). Griess reagents sulphanilamide and N-1-napthylethylenediamine dihydrochloride (NED; both Sigma Aldrich) were prepared in a 2% w/v solution in 5% hydrochloric acid (HCL), and a 0.1% w/v solution in water, respectively. In acidic conditions, nitrite reacts with the amino group of sulphanilamide to form the diazonium cation, which couples to NED to form the pink azo dye which can be measured spectrophotometrically (Tsikas, 2007). Vanadium (III) Chloride (VCl_3; Sigma Aldrich) was used as a reduction agent, to convert nitrate to nitrite to enable quantification. VCl_3 was chosen as it is less dangerous to handle than cadmium and more economical than the enzyme nitrate reductase. Five grams of VCl_3 was solubilised in 1M HCl to ensure saturation, and the solution filtered to remove the excess undissolved VCl_3.. Stock solutions of all three reagents were protected from light and stored in the fridge; if the colour of the solutions had changed, new stock solutions were prepared.

To calculate the nitrite and nitrate concentrations in a solution, two sets of triplicate aliquots were added to a 96 well plates, one for nitrite and one for nitrate measurements. To quantify nitrite concentrations, sulphanilamide was added to the first set of triplicate aliquots in the 96 well plate followed by addition of NED. To quantify nitrate concentrations, a premixed solution of VCl_3 and sulphanilamide (ratio 2:1) was added to the second set of triplicate aliquots in the 96 well plate, again followed by the addition of NED. NED was added sequentially following sulphanilamide and VCl_3 as this is reported to increase the assay sensitivity by 30%, compared to adding all agents

simultaneously. The plate was then incubated at room temperature for 30 minutes and protected from light, before absorbance values measured with the FLUOstar Omega Microplate Reader (BMG Labtech) at 560 nm. Serial dilutions of sodium nitrite and potassium nitrate prepared in water were used as standards. The limit of detection for this method is thought to be approximately 5 nM of nitrite (Tsikas, 2007).

For the analysis, standard curves were produced for nitrite and nitrate values respectively. As the values for the nitrate measurements represent both the nitrite already in solution and nitrite produced via the reduction of nitrate by VCl_3, the values from the first set of triplicates were deducted from the second set of triplicates to give the nitrate values, as denoted by the following, whereby 'A' represents absorbance:

$$A_{\text{nitrite}} = A_{\text{nitrite}} - A_{\text{nitrite blank}}$$

$$A_{\text{nitrate + nitrite}} = A_{\text{nitrate + nitrite}} - A_{\text{nitrate + nitrite blank}}$$

$$A_{\text{nitrate}} = A_{\text{nitrate + nitrite}} - A_{\text{nitrite}}$$

2.5 β-lactamase activity in *P. aeruginosa* strains

A nitrocefin assay was used to quantify β-lactamase activity. The use of nitrocefin is one of the methods recommended by the CLSI for assessing β-lactamase activity (CLSI, 2012b). Chromogenic cephalosporin nitrocefin undergoes a distinctive yellow to red colour change when hydrolysed by β-lactamase (Callaghan et al., 1972). *P. aeruginosa* isolates were grown overnight in LB broth, subcultured into fresh broth and grown to mid exponential phase. Nitrocefin (Cambridge Bioscience) was prepared in DMSO and PBS and added to the cultures to a final concentration of 50 μg/ml. Absorbance values at a wavelength of 490 nm were taken at selected intervals of incubation, with an increase in value at OD490 directly correlated to the level of β-lactamase activity.

2.6 Microtitre plate assays to investigate the effects of NO on *P. aeruginosa* biofilms

The effects of NO on clinical isolates of *P. aeruginosa* were analysed using a microtitre plate system, in a similar manner as previously described. An overnight culture was diluted 1 in 100 into freshly prepared M9 minimal media and added into a flat-bottom tissue culture treated 96 well microtitre plate (Thermo Scientific) and incubated at 37˚C for 24 h. Uninoculated M9 minimal media served as negative control. After incubation, the planktonic suspension was removed and discarded, and biofilm washed once with M9 minimal media to remove non-adherent cells. SNP or C3D compounds were prepared fresh in M9 minimal media, serially diluted and then added to the

wells with biofilm. A minimum of 6 replicate wells were used for each treatment group; the location of wells altered such that in each treatment group, 3 wells were located near the edge of the plate and 3 wells located nearer the centre of the plate to exclude any effect of well location on biofilm growth. Following treatment, the planktonic cell densities were recorded by measuring the absorbance. Biofilms were washed twice with PBS, stained with 0.1% CV for 15 mins, resolubilised in 30% acetic acid and then the absorbance was measured at 584 nm as previously described.

2.7 Construction of c-di-GMP reporter strains and measurement of c-di-GMP levels

The c-di-GMP reporter plasmid (p*CdrA*::gfp^{C}) was constructed using standard molecular cloning techniques by Rybtke et al., (2012). Briefly, the promoter of gene *cdrA* was fused with *gfp*; this was based on evidence showing the expression of *cdrA*, which encodes for a large adhesin, to be upregulated in response to increased levels of c-di-GMP. Rybtke et al., demonstrated the functionality of the reported strain, and confirmed increased fluorescence generated by the plasmid had correlated with increased c-di-GMP levels. The reporter plasmid was further modified to increase GFP stability by our colleagues at Imperial College London and kindly gifted to us, in *E. coli* strain TOP10.

2.7.1 Generation of c-di-GMP reporter strains.

Electroporation was used to introduce the c-di-GMP reporter plasmid into *P. aeruginosa* strains. Briefly, cultures of the donor *E. coli* TOP10 strain were grown in the presence of tetracycline and plasmid extraction carried out using the QIAprep Spin Miniprep Kit (Qiagen). Plasmid concentration of the extracted samples were quantified using the NanoDrop spectrophotometer (Thermo Fisher). *P. aeruginosa* strains were grown overnight in 12 ml of LB broth and prepared as electrocompetent as detailed by Choi et al., (2006); cells were washed with 300 mM sucrose three times and condensed into 200 µl. Electrocompetent *P. aeruginosa* cells and 500 ng of the extracted reporter plasmid were combined, added to a 2 mm electroporation cuvette, and a pulse applied for 5 ms using the MicroPulser Electroporator (Bio-Rad) on the Ec2 setting (2.5kV). A cuvette containing only *P. aeruginosa* cells as a positive control was also pulsed. Contents of the cuvette were added to fresh LB broth within one minute of the pulse being applied, before being streaked onto LB agar plates containing tetracycline and incubated at 37˚C overnight. MICs for tetracycline against *P. aeruginosa* strains were carried out and roughly 4x the MIC value was chosen as the concentration required for selection and isolation of transformed colonies. Transformed colonies were re-streaked onto fresh tetracycline LB agar plates at 4x and 8x MIC value and GFP fluorescence visualised microscopically to confirm successful construction of the reporter strain.

2.7.2 Development and optimisation of a biofilm-based c-di-GMP reporter assay

An assay to assess c-di-GMP levels in biofilm cultures was developed and optimised with PAO1. Overnight cultures of both the PAO1 wild type strain and PAO1 reporter strain were grown in LB. All media involving the plasmid incorporating strain included tetracycline at a concentration approximately 4-fold higher than the wild type strain MIC. Both cultures were normalised to the same OD in M9 minimal media, inoculated into clear flat bottom 96 well plate and incubated for 24 h. Wild type and reporter strain biofilms were then treated with SNP in M9 minimal media at concentrations of 0, 2, 4, 8 and 16 μg/ml for 20 h. Following treatment, the absorbance at 584 nm, and fluorescence at an excitation wavelength of 490 nm and emission wavelength of 530 nm of all wells was measured as an end point read. As part of data analysis, an equation from Overkamp et al., (2013) to correct for differences in the optical density of cultures, background fluorescence of the media, and auto-fluorescence of the wild type strain was used to calculate relative fluorescence (RF) values as follows:

$$\mathrm{RF} = \left(\frac{\mathrm{GFP_{reporter}} - \mathrm{GFP_{medium}}}{\mathrm{Od_{reporter}} - \mathrm{Od_{medium}}}\right) - \left(\frac{\mathrm{GFP_{wt}} - \mathrm{GFP_{medium}}}{\mathrm{Od_{wt}} - \mathrm{Od_{medium}}}\right)$$

This biofilm-based method was successful in measuring changes in c-di-GMP in biofilm cells and dispersed cells for PAO1. However, this method could not be reliably used with the *P. aeruginosa* clinical isolates due to various reasons including inadequate biofilm development or development of floating cells aggregates rather than biofilm development in the wells by the reporter strains.

2.7.3 Optimisation of a planktonic based c-di-GMP reporter assay

Following the unsuccessful utilisation of the *P. aeruginosa* clinical isolates with the biofilm based c-di-GMP reporter assay, a planktonic based assay was optimised based on methods from Rybtke et al., (2012)**.** Overnight cultures of the wild type and reporter strain were grown in 10 ml of LB, with the culture for the reporter strain containing tetracycline at 4x the MIC for the wild type. Each overnight culture was then sub-cultured into two universal tubes with 10 ml of M9 minimal media, one untreated control and one treated with NO**;** the OD of both the wild type and reporter strain was measured and the subculture in M9 standardised to ensure an equal inoculum. Universals containing M9 minimal media with and without NO and tetracycline served as controls. Following 20 h incubation, the samples were vortexed and aliquoted into a clear bottom black 96 well plate (Fisher), to allow absorbance and fluorescence measurement values. The equation from Overkamp et al., (2013) was used during data analysis as previously described.

2.8 Passaging of *P. aeruginosa* with low dose NO

In order to determine if *P. aeruginosa* could adapt and develop 'resistance' to the NO induced dispersal response, PAO1 was passaged with ultra-low doses of NO. Preliminary experiments revealed that a 1 nM concentration of SNP would not cause dispersal of biofilms and or significantly impact the growth rate of planktonic cells of PAO1, so this concentration was selected to be used during passaging. SNP was solubilised in water, filter sterilized at a stock concentration of 100 mM and diluted down to a concentration of 1 nM in LB. An overnight culture of PAO1 was prepared in 10 ml of LB broth; 5 µl of the culture was then transferred to 10 ml of LB broth and 5 µl transferred to 10 ml of LB broth containing 1 nM SNP. Both cultures were re-incubated at 37˚C, and the following day, 5 µl from the control sample was transferred to 10 ml of fresh LB broth, and 5 µl from the LB with 1 nM SNP culture transferred to 10 ml of fresh LB with 1 nM SNP. This was repeated 8 additional times. SNP solutions were prepared fresh daily, and CFU counts were conducted for every culture to monitor growth levels and ensure viability of cells. For the 10^{th} passage, cultures were diluted into M9 minimal media with and without 1 nM SNP and inoculated into 35 mm glass bottom well dishes (Mattek Cooperation). After 24 h hours growth, biofilms were either treated with M9 only or M9 supplemented with 1mM SNP for 20 h to trigger dispersal. Biofilms were stained with SYTO9 and propidium iodide (PI) and analysed using confocal laser scanning microscopy (CLSM) and COMSTAT, as described in Section 2.9.

2.9 Confocal laser scanning microscopy of biofilms

CLSM was used to analyse the effects of dispersal agents and antibiotics on *P. aeruginosa* biofilms. Overnight cultures were diluted 1 in 100 in M9 minimal media, and 2.5 ml volumes were used to inoculate 35 mm glass bottom microwell plates (MatTek Corporation). Microwell plates were incubated at 37˚C with gentle shaking at 50 rpm, with media changes at 24 h intervals if grown for more than 24 h. Biofilms were then treated with dispersal agents and/or antibiotics solubilised in M9 minimal media and re-incubated.

Following treatment, biofilms were washed twice with Hank's balanced salt solution (HBSS) to remove non-attached cells, and biofilm viability assessed using the LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies), containing dyes SYTO9 and Propidium Iodide (PI). SYTO9 stains bacterial cells with intact membranes fluorescent green, whereas PI stains cells with compromised membranes as fluorescent red (Molecular Probes Inc, 2001). Stock solutions of each dye were diluted to a concentration of 2µg/ml, added to the glass bottom microwell plates and incubated in the dark at room temperature for 20 minutes, as per manufacturer's instructions.

Biofilms were then examined with an inverted Leica SP8 confocal laser scanning microscope using the x63 oil immersion lens, with sequential scanning at 1µm intervals. Argon and DPSS laser lines

at 488 nm and 561 nm were used to excite the dyes, and the FITC filter set cube used to acquire images. A total of 5 replicate images were taken from each biofilm grown in the microwell plates. The locations of the well at which images were taken were kept constant for each plate to avoid bias and subjectivity. The first image was taken from the centre of the well, and four subsequent images were taken from the centre of each theoretical quadrant of the well. Images were obtained and analysed using the LAS AF software (Leica Microsystems GmbH). COMSTAT 2.0 software was used for quantitative analysis of obtained images (Heydorn et al., 2000).

2.10 Quantification of cells in *P. aeruginosa* biofilms

Overnight cultures were diluted 1 in 100 into M9 minimal media, and 3 ml was added to each well of a 6 well plate. Plates were incubated at 37°C with shaking at 50 rpm and the media changed daily if incubated for more than 24 hours. To quantify cells, the planktonic media was gently aspirated with a pipette and removed, and the biofilms washed twice with HBSS to remove non-adherent cells. One ml of HBBS was then added to each well and a cell scraper (Fisher) used to detach biofilm cells from the 6 well plate. The 1 ml of HBSS containing the detached biofilm cells, was then transferred to an Eppendorf tube and vortexed for 1 minute. CFU counts were carried by plating dilutions of the vortexed cells onto TSA plates. Agar plates were incubated overnight and colonies counted the next day.

2.11 eNO generator and accompanying apparatus

The eNO generator and NO_2 filters were kindly donated to us by Duncan Bathe, President of NitricGen Inc. Other apparatus that was required included an adjustable single-valve air pump (2.5w, flow rate 2 LPM; Hidom), plastic containers (Sistema) and polyvinyl chloride tubing, and 0.2 μm autoclavable air filters (Millipore). A CLD (CLD 88Y, Eco Physics) was used to measure low level NO gas concentrations (see Section 2.4.1 for CLD analyser details). A CrowCon Gasman handheld NO monitor (Cole Palmer), designed for industrial use, personal monitoring and portable safety applications, was used to measure higher NO concentration. The CrowCon monitor has a measurable NO range of 0 to 100 ppm, a minimum detection concentration of 0.1 ppm, and a typical response time (T_{90}) of 20 s. A ‘flow cap’ adaptor was connected to the Crowcon monitor to enable flow sampling and direct connection of the monitor to polyvinyl chloride tubing. A dock and accompanying software, Portables, were used to connect the CrowCon NO monitor to a PC, enabling settings to be changed and the data logs to be uploaded for analysis.

2.12 Growth of *P. aeruginosa* biofilms in varying oxygen concentrations *in vitro*

An overnight culture of a clinical isolate of *P. aeruginosa* was diluted 1 in 100 in M9 minimal media adjusted to pH 6.5 and used to inoculate 35 mm glass bottom dishes (CellVis). Half of the dishes were supplemented with 15mM of KNO_3 (Sigma Aldrish), required for *P. aeruginosa* survival in anaerobic conditions. Dishes both with and without KNO_3 supplementation were then placed into one of three conditions: aerobic, microaerophilic and anaerobic. Dishes with aerobically cultured biofilms were placed into a non-sealed container, the microaerphically cultured biofilms were placed into a sealed container with a CampyGen sachet (Fisher), and the anaerobically cultured biofilms were placed into a sealed container with an AnaeroGen sachet (Fisher). All three containers were then incubated for 24 h in 37˚C with 50 rpm shaking. CampyGen sachets reduce the oxygen concentration to approximately 6% within 30 mins, whilst the AnaeroGen reduces the oxygen concentration to below 1%. Following 24 h incubation, biofilms were stained with SYTO9 and PI and analysed using CLSM as previously described.

2.13 Statistical Analysis

The statistical test used for each data set is specified in the figure caption. Welch's T tests, or one way ANOVA with Dunnett's multiple comparisons test for data sets with multiple groups, were commonly used for statistical analysis. Ratio paired T tests were used for statistical analysis of c-di-GMP measurements (Chapter 3). For identification of potential correlations between two variables (Chapter 3), linear regression analysis was used, with the R^2 and p value detailed on the graph. Graphpad Prism 7.0 was used to run all statistical analysis. In all circumstances, levels of significance are indicated as follows: ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

Chapter 3 Phenotypic analysis of clinical CF isolates of *P. aeruginosa* and their response to NO

3.1 Introduction

Previous research involving NO and *P. aeruginosa* biofilms has almost exclusively involved PAO1. Though PAO1 is the most widely used *P. aeruginosa* strain and was the first strain of this species to have its full genome sequence identified, its historical isolation from a wound limits its relevance to CF. An Ovid SP search for "*Pseudomonas aeruginosa*", "nitric oxide", "biofilm", and "dispersal", and limiting resources to original research journal articles, produced 24 results (**Table 4**). In addition to PAO1, all but one of these studies had either used PA14, mutant/reporter strains, transposon library strains, non-CF isolates or isolates from a membrane bioreactor system. Again, these strains have limited relevance to CF.

The sole exception is the publication by our colleagues in Southampton primarily investigating the impact of inhaled NO gas on CF patients (Howlin et al. 2017; highlighted in bold in **Table 4**). Some *in vitro* investigations were also detailed; SNP treatment of expectorated sputum samples had visibly disrupted *P. aeruginosa* aggregates in five out of five patients. Twelve CF isolates of *P. aeruginosa* grown *in vitro* and treated with SNP had also indicated biofilm dispersal, though this was concluded based on increased planktonic cell turbidities and not direct biofilm biomass quantification. One of the twelve isolates had shown evidence of a reduction in biofilm biomass via changes in fluorescence values, however details for the other eleven were not shown.

Therefore, this study, in the interests of clinical relevance, focuses on the utilisation of clinical CF isolates of *P. aeruginosa*. As previously mentioned, CF isolates of *P. aeruginosa* are known to be genetically and phenotypically distinct from PAO1, and to each other, due to variations in adaptation mechanisms. Based on these differences it is not uncommon to note correlations or show varying efficacy of a drug or treatment dependent on phenotype or genotype.

In this chapter, the phenotypic characteristics of our panel of CF isolates (the same panel used by Howlin et al. (2017)) were examined, and our first objective was to identify potential correlations between isolate phenotype and the response of the CF isolate biofilms to NO treatment. In particular, we hypothesised that mucoidy and cell-pigmentation due to redox-active agents may influence NO-sensing and the dispersal response in *P. aeruginosa.* Our second objective was to conduct mechanistic investigations, by analysising c-di-GMP levels of the clinical isolates following NO treatment and to explore the potential adaptation of *P. aeruginosa* to NO as a dispersal trigger.

Table 4. Publications investigating NO and *P. aeruginosa* biofilms

No.	Author/Reference	Strains utilised
1	(Barraud et al., 2006)	PAO1 and mutants
2	(Van Alst et al., 2007)	PAO1, PA14 and mutants
3	(Barraud, Storey, et al., 2009)	*P. aeruginosa* strains not examined
4	(Barraud, Schleheck, et al., 2009)	PAO1 and mutants
5	(Kutty et al., 2013)	PAO1, and reporter strains NSGFP (P_{nirS}::gfp), and MH602 (P_{lasB}::gfp)
6	(De La Fuente-Núñez et al., 2013)	PA14 and mutants
7	(Li et al., 2013)	PAO1 and mutants
8	(Barnes et al., 2013)	Membrane bioreactor isolates
9	(Hui et al., 2014)	PAO1 and mutants
10	(Duong et al., 2014)	PAO1 and reporter strain NSGFP (P_{nirS}::gfp)
11	(Barraud et al., 2014)*	PAO1
12	(Kutty et al., 2015)	PAO1, PA14, and reporter strain MH602 (P_{lasB}::gfp)
13	(Alexander, Kyi, and Schiesser, 2015)	PAO1, and CMO (culture material; mixed biofilm culture)
14	(Reffuveille et al., 2015)	PA14 and *E. coli* 0157
15	(Nguyen et al., 2016)	PAO1
16	(Verderosa et al., 2016)	PA14
17	(Wood et al., 2016)	PAO1 and mutants
18	(Craven et al., 2016)	PAO1
19	(Hossain et al., 2017)	- (isolated proteins only)
20	(Howlin et al., 2017)	**Clinical CF isolates**
21	(Poh et al., 2017)	PAO1 (ATCC BAA-47)
22	(Bacon et al., 2018)	- (isolated proteins only)
23	(Zhu et al., 2018)	PAO1 and mutants
24	(Adnan et al., 2018)	PAO1

Search last updated 14.11.18. *Reference refers to a section in book entitled 'Pseudomonas Methods and Protocols'

3.2 Results

3.2.1 Phenotyping of CF isolates of *P. aeruginosa* shows high variability

PAO1 and the *P. aeruginosa* culture collection containing twenty clinical strains isolated from the sputum of adult and adolescent CF patients were subject to phenotypic analysis, firstly to investigate the variation amongst the panel, and secondly to allow correlations to be made with succeeding experiments. The cell morphology, mucoidy, pigment production, virulence, planktonic and biofilm growth rates, and AMS to three common CF antibiotics were analysed and are summarised in **Table 5.**

A high number of the clinical isolates were defined as SCV, a total of twelve out of the twenty clinical strains. Just three of the strains however, were identified as mucoid, PA05, PA10 and PA57, with the latter being identified as 'hypermucoid' and displayed excessive alginate production (documented in **Appendix B**). Pigment production varied greatly on a strain to strain basis, but the majority of isolates displayed evidence of producing the characteristic blue-green pyocyanin pigment. Six isolates produced pyoverdine, the florescent yellow and second most common *P. aeruginosa* pigment, whilst four produced the less common red-brown pyorubin pigment. Four of the twenty isolates produced no pigment at all.

Assays to investigate the planktonic and biofilm growth rates yielded interesting results; just over half of the clinical isolates had a slow rate of planktonic growth as expected with CF isolates, however not all of the isolates were good biofilm producers. It was important to assess the biofilm forming abilities of the clinical isolates to identify the strains that would be best utilised in future biofilm experiments. Surprisingly, eight of the twenty clinical isolates were shown to be 'poor' biofilm formers, including two of the three mucoid strains. Seven, including the third mucoid strain PA10, were classed as moderate, and another five classed as 'good' biofilm formers. five six 'good' biofilm formers and PA10, were prioritised as the strains to be carried forward into biofilm experiments.

Finally, the AMS of each strain to three common *P. aeruginosa* CF antibiotics was investigated. According to breakpoint values, just three isolates were identified as resistant to tobramycin, seven to ceftazidime and four resistant to colistin. No isolate was resistant to all three antibiotics; and ten of the twenty isolates were sensitive to all three.

Table 5. Phenotyping of *P. aeruginosa* isolates examined in this study

Strain	Isolate type[a]	Colonial morphology[b]	Muc.[c]	Pigment production[d]	Planktonic growth[e]	Biofilm growth[f]	AST[g]		
							TOB	CAZ	COL
PAO1	Laboratory	Smooth, round	-	PCN	Fast	Good	S	S	S
PA05	Clinical	Smooth, round	+	PCN, PRN	Slow	Poor	S	S	S
PA08	Clinical	SC	-	PRN	V. Slow	Poor	S	R	S
PA10	Clinical	Smooth, round	+	PCN	Slow	Moderate	S	S	S
PA15	Clinical	SC	-	-	Slow	Poor	S	R	S
PA20	Clinical	SC	-	PCN	Slow	Moderate	S	S	S
PA21	Clinical	Rough, flat, round	-	PCN	Fast	Good	S	R	S
PA26	Clinical	SC	-	PCN, PVD	Fast	Good	S	R	S
PA30	Clinical	SC	-	PVD, PRN	Fast	Good	S	S	S
PA31	Clinical	SC	-	PCN	Slow	Poor	R	S	R
PA37	Clinical	SC	-	PVD	Fast	Moderate	S	R	S
PA39	Clinical	Rough, wrinkled	-	-	Slow	Moderate	S	S	S
PA44	Clinical	SC	-	-	Slow	Good	S	S	R
PA47	Clinical	SC	-	PVD	Slow	Poor	S	S	R
PA49	Clinical	Rough, round	-	-	Slow	Moderate	S	S	S
PA55	Clinical	SC	-	PCN	Slow	Poor	R	S	R
PA56	Clinical	Smooth, round	-	PVD	Slow	Poor	S	S	S
PA57	Clinical	Smooth, round	++	PCN, PRN	Slow	Poor	S	S	S
PA58	Clinical	SC	-	PVD	Fast	Moderate	R	R	S
PA66	Clinical	SC	-	PCN	Slow	Moderate	S	R	S
PA68	Clinical	Smooth, round	-	PCN	Fast	Good	S	S	S

[a] all clinical isolates obtained from 16 – 65 year old patients with *P. aeruginosa* infection from SGH.
[b] strains grown on LB agar, assessed after 24 to 48 h incubation. SC = small colony
[c] mucoidy; strains grown on PIA, assessed after 48 h incubation; - , non-mucoid; + , mucoid; ++, hyper-mucoid
[d] strains grown on LB agar, cetrimide agar and PIA, assessed after 48 h incubation. PCN, pyocyanin; PVD, pyoverdine; PRN, pyorubin
[e] planktonic growth rates in LB broth, determined spectrophotometrically
[f] biofilms grown for 48 h in 96 well plates, stained with crystal violet and analysed spectrophotometrically for total biomass, Poor = OD value of less than 0.5; Moderate = OD value between 0.5 and 1; Good = OD value of above 1
[g] AST = Antimicrobial Susceptibility Testing. MIC determined by broth microdilution assay and compared with EUCAST breakpoint. S, sensitive; R, resistant; TOB, tobramycin (S ≤ 4mg/L, R > 4mg/L); CAZ, ceftazidime (S ≤ 8mg/L, R > 8mg/L); COL, colistin (S ≤ 2mg/L, R > 2mg/L)

3.2.2 The importance of agar selection and growth conditions in phenotyping assays with CF isolates of *P. aeruginosa*

In phenotyping the colony morphology, mucoidy, and pigment production of the isolates, growth on solid agar was required and different agar was utilised for each variable. LB agar was used to assess colony morphology, with this decision based on work by (Haussler et al., 1999). The authors recommend the use of LB agar with an incubation time of 48 h, and that SCV can be confidently identified if the phenotype is maintained for a further two subcultures. For determination of mucoidy, PIA was utilised for cultures. PIA was selected as previous studies had demonstrated the appearance varied with different media, and PIA was shown to have the lowest occurrence of false negative results (Ciofu et al., 2008; Pugashetti et al., 1982). Finally, cetrimide agar was utilised to assess the pigment production of the isolates, due to claims by the manufacturer that cetrimide agar enhances *P. aeruginosa* pigment production. To maintain consistency and accommodate for the slow growth of CF isolates, the incubation time for growth on all three agar types was set to 48 h, with cultures visually inspected once more following 7 days incubation.

As shown in **Figure 9**, the phenotype of *P. aeruginosa* isolates varies immensely dependent on the agar media the culture was grown on. With PAO1, shown in A, B and C, then colony size and morphology was relatively consistent, with just the enhancement of the blue-green pyocyanin pigment enhanced on cetrimide agar and PIA compared with LB agar. However, as an example, PA26 displayed distinct differences in colony size (shown in D, E and F); colonies grown on LB agar appeared to be SCV, however the colony size was larger on cetrimide agar and larger still on PIA. This was repeatedly seen with other clinical isolates. To confirm that the type of agar was the cause for the varying colony sizes, colonies grown on LB agar were subcultured onto LB agar, cetrimide agar and PIA. Colonies from the cetrimide agar and PIA plate were also subcultured onto all three types of agar again. The phenotype was maintained irrespective of the source of the colony. For example, a large colony grown on PIA showed a small colony phenotype when subcultured onto LB agar (PA26 is given as an example in **Appendix B**).

Varying alginate production was also shown and dependent on the agar medium. As shown by the PA57 example in **Figure 9**, mucoidy is clearly evident on the LB and PIA plate but not on the cetrimide agar plate. Agar cultures were kept incubated and visually examined on a daily basis for 7 days, to ensure no false negatives for mucoidy occurred. Following 5 days of incubation, the alginate production of PA57 was so high that alginate had dripped on the lid of the inverted plate (shown in **Appendix B**) leading to the classification of this isolate as hypermucoid.

Enhanced pyocyanin production was evident with cetrimide and PIA, as shown with PAO1 and PA26. Unlike PAO1 however, there was no evidence of green-yellow pigment with PA26 on the LB agar, again highlighting the importance of selecting agar medium. Again, a similar effect is noted with PA30, all three agar types have different pigment productions. Assessment for pigment

production took into consideration the growth of cultures on all three types of agar medium.

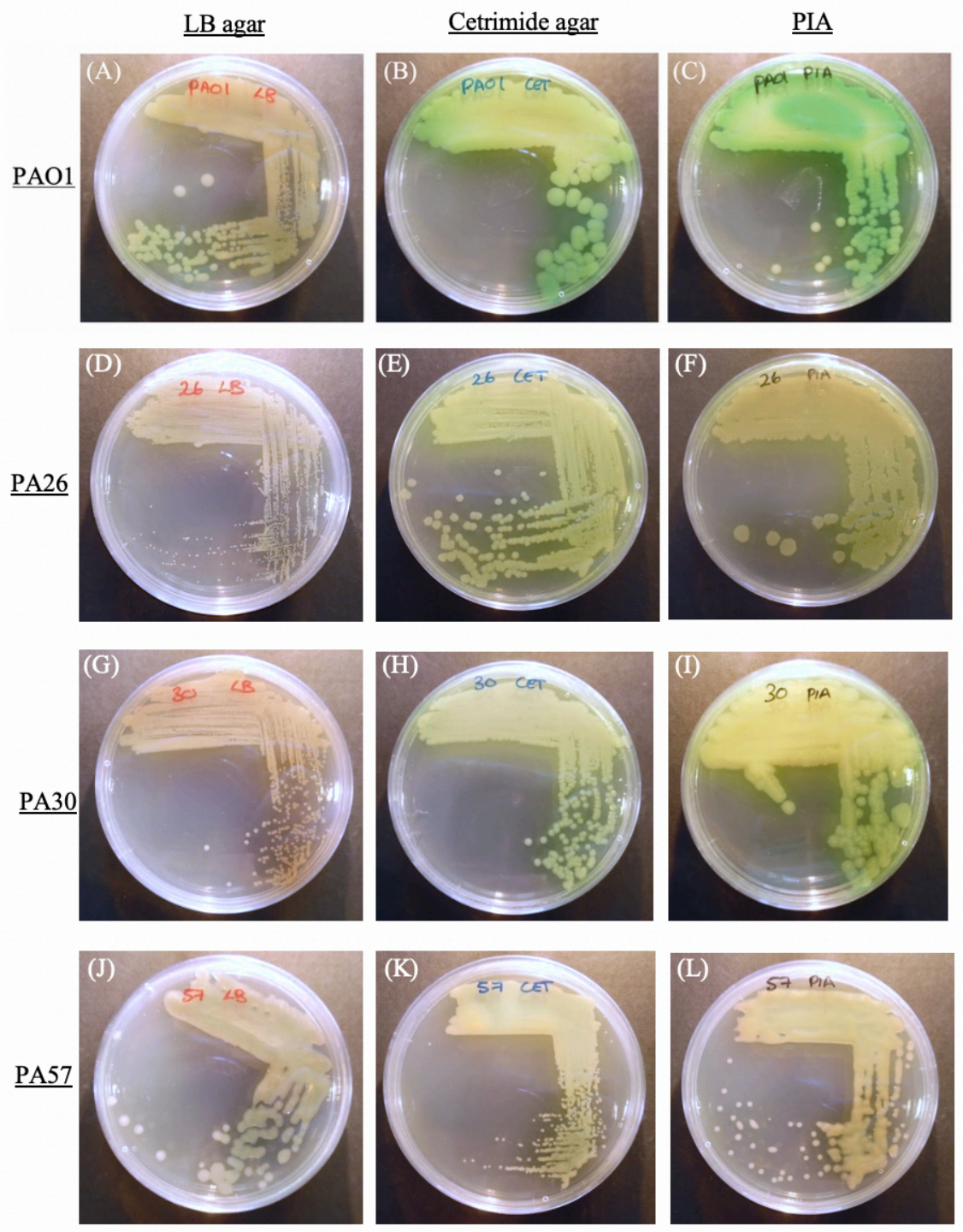


Figure 9. The importance of agar selection when phenotyping clinical isolates of *P. aeruginosa*. PAO1 and three CF isolates are shown as examples, PAO1 (A, B, C); PA26 (D, E, F); PA30 (G,H, I) and PA57 (J, K, L). Isolates in images A, D and G, are plated on non-selective LB agar; B, E, and H, on cetrimide agar, and C, F and I, on Pseudomonas Isolation agar (PIA).

3.2.3 Light exposure impacts the release of NO from SNP

Due to the ambiguity and lack of specificity in the methods of published literature concerning the NO release from SNP, a CLD was used to analysis the release of NO during different environmental conditions. SNP is known to be light sensitive; as shown in **Figure 10**, alternating periods of dark and light confirm NO release is increased when SNP is exposed to light. The NO concentration in periods of light were being recorded at just over 400 ppb compared to below 20 ppb during the periods of dark. Addition of carboxy-PTIO, a NO scavenger, provided additional confirmation of NO release, and caused the concentration of NO to drop almost instantaneously.

The release of NO from SNP during a 30 min period was compared in different conditions and in different media, as shown in **Figure 10B**. Interestingly, in the same light presence SNP released from solutions of PBS and M9 showed a comparable erratic release; release of NO is lower but more steady when SNP is in the dark or in presence of artificial light. To quantify this data, sodium nitrite standard solutions with known amounts of NO release were utilised in the CLD method (**Figure 10D**); 1 μM, 5 μM, 10 μM and 30 μM of sodium nitrite releases 50, 250, 500 and 1500 pmoles of NO respectively and was correlated to the area under the peaks from the CLD graph, shown in **Figure 10E**. This correlation resulted in an exact line of best fit, with the equation $y = 0.0031x$. Using this equation, the amount of SNP released from PBS and M9 in different light conditions was quantified (**Figure 10C**). Interestingly, there is no significant difference in NO release of SNP solubilised in PBS compared to M9 minimal media. However once again, it is confirmed that light exposure has a great impact; approximately 100 nmoles is released from SNP in natural light, compared to around 10 nmoles in the dark and just under 50 nmoles in artificial light dimmer than the natural light.

To quantify the NO release from SNP for a longer period of time, Griess reagents were utilised as opposed to the CLD, for practicality reasons. A 10 mM of SNP solution produced a positive reaction for both nitrite and nitrate (**Figure 11**). Lower concentrations of SNP were initially investigated; however, the nitrite and nitrate concentrations were close to or below the lower detection limit for the assay. Spectrophotometric analysis revealed that for a 50 μl sample of 10 mM SNP, 80.6 μM and 250 μM of nitrite and nitrate, respectively, were recorded, following a 20 h incubation period with light.

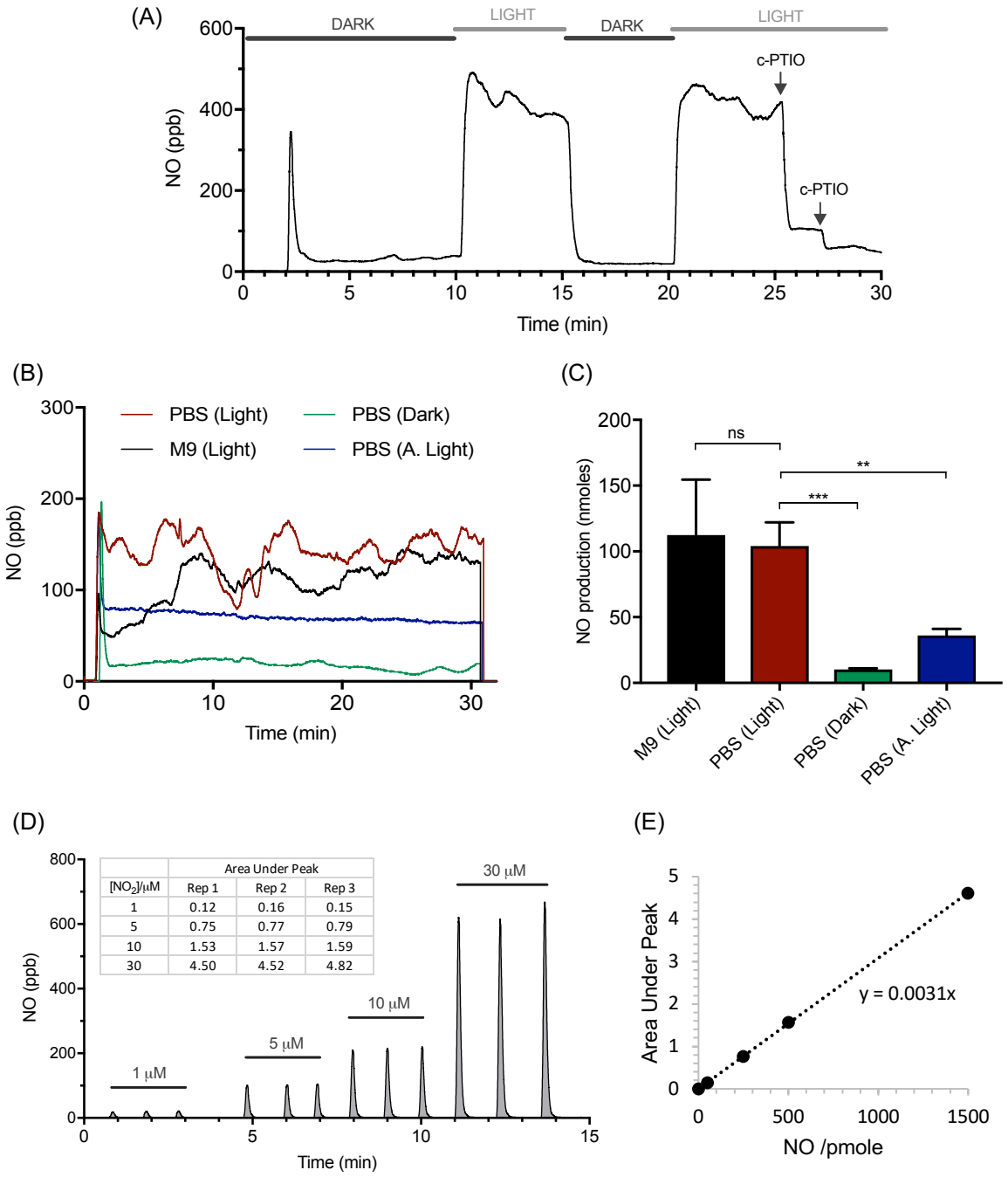


Figure 10. Measurements of NO release from SNP using a CLD. (A) The differences in NO release in alternating periods of natural light and dark; SNP (5 ml of a 1 mM solution) added to the system at 2 min, arrows signify addition of carboxy-PTIO. (B) NO release from 10 mM SNP in M9 media and PBS in (natural) light, dark or artificial light; runs shown representative of three repeats. (C) NO release from multiple sodium nitrite standard solutions, and values of the area under peak (AUP; table within graph). (D) Correlation between predicted NO release from sodium nitrite standard solutions and mean AUP values. (E) Quantification and comparison of the NO released in a 30 min period from SNP in M9 media and PBS in different light conditions. Bars represent mean values and SD from three runs. T-tests used for statistical analysis.

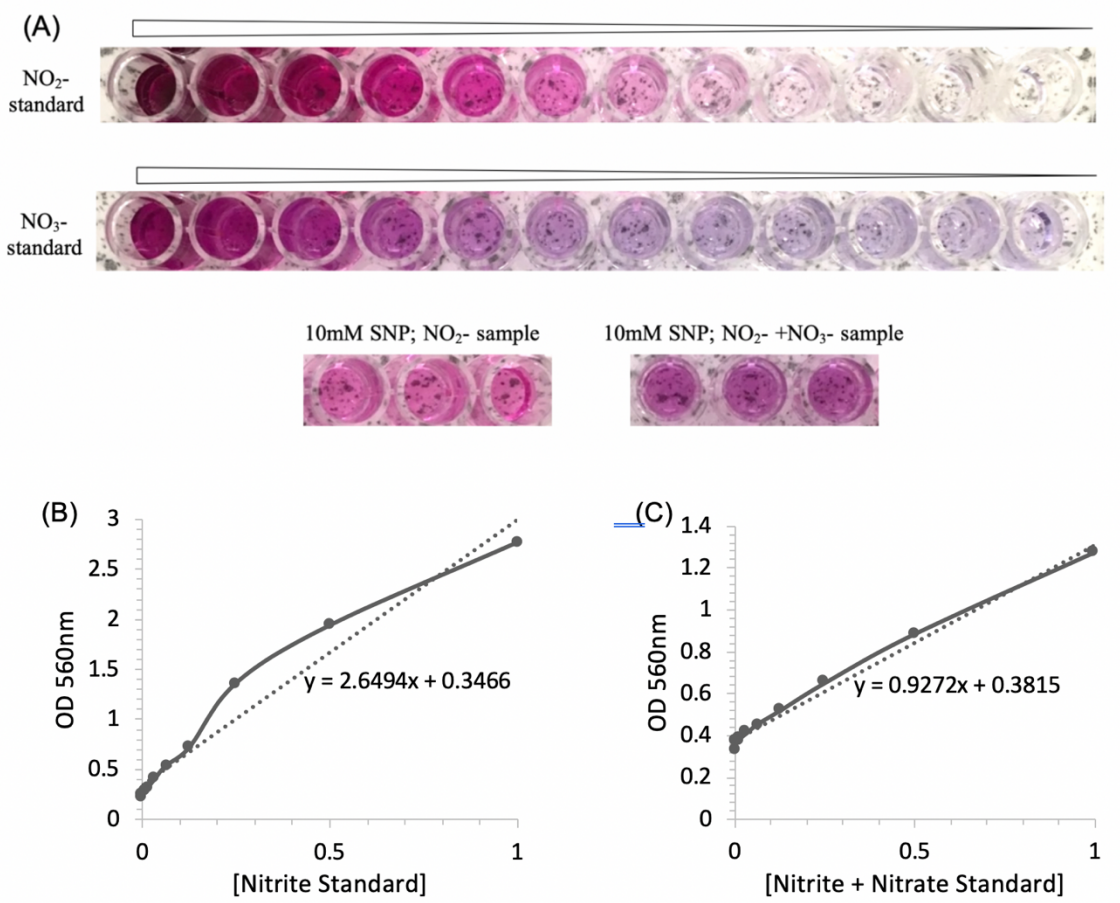


Figure 11. The release of NO from SNP measured using Griess reagents. (A) Example photographs showing the standard dilutions for nitrite (NO_2-) and nitrate (NO_3-), and samples of 10mM SNP, all following addition of Griess reagents. Nitrite and nitrate standard dilutions were made with sodium nitrite and potassium nitrate, respectively, with two-fold decreasing dilutions from left to right. (B) Correlation between OD value and nitrite standard concentration, plotted points are mean of three results. Equation of trendline shown on graph. (C) Correlation between OD value and nitrate standard concentration, plotted points are mean of three results. Equation of trendline shown on graph.

3.2.4 Response to NO is varied amongst clinical CF isolates of *P. aeruginosa*

To investigate the effects of SNP on biofilms formed by clinical CF isolates of *P. aeruginosa*, a CV staining method was utilised, and biofilms treated with SNP to produce a dose response curve. As shown in **Figure 12A**, PAO1 biofilms are dispersed as expected in response to SNP treatment, from concentrations above and including 500 nM in a dose dependent fashion. Planktonic growth values were increased indicating a dispersal effect as opposed to a bactericidal one (shown in **Appendix C**).

This assay, with identical conditions, was carried out with five clinical isolates selected from the panel, deemed to be good biofilm formers *in vitro*. A similar trend is seen with PA21, PA26, PA30 and PA68 (**Figure 12C-F**), whereby multiple SNP concentrations are able to disperse the biofilms and cause a statistically significant reduction in total biomass. However, for PA10, the opposite effect is seen (**Figure 12B**). Treatment with SNP resulted in an increase in biofilm biomass and no tested concentration resulted in biofilm dispersal. For all five isolates, the planktonic growth values were all increased, shown in **Appendix C.**

Following on from this unexpected discovery, four additional clinical isolates were assessed (**Figure 13**). For PA20, PA47, PA56 and PA66, all showed evidence of increased biofilm biomass following treatment with SNP, with varying degrees. Likewise to the previously mentioned isolates, planktonic growth values were also increased with these four isolates (**Appendix C**).

To eliminate the possibility that the concentration required to induce dispersal was above the highest tested concentration of 512 μM, PA10 biofilms were also treated with SNP concentrations up to 262 mM. As shown in **Figure 14**, biofilm dispersal was not observed; the biofilm biomass was reduced at concentrations above 65.5 μM, though as this coincided with a decrease in the planktonic growth value this indicates a bactericidal effect as opposed to a dispersal event.

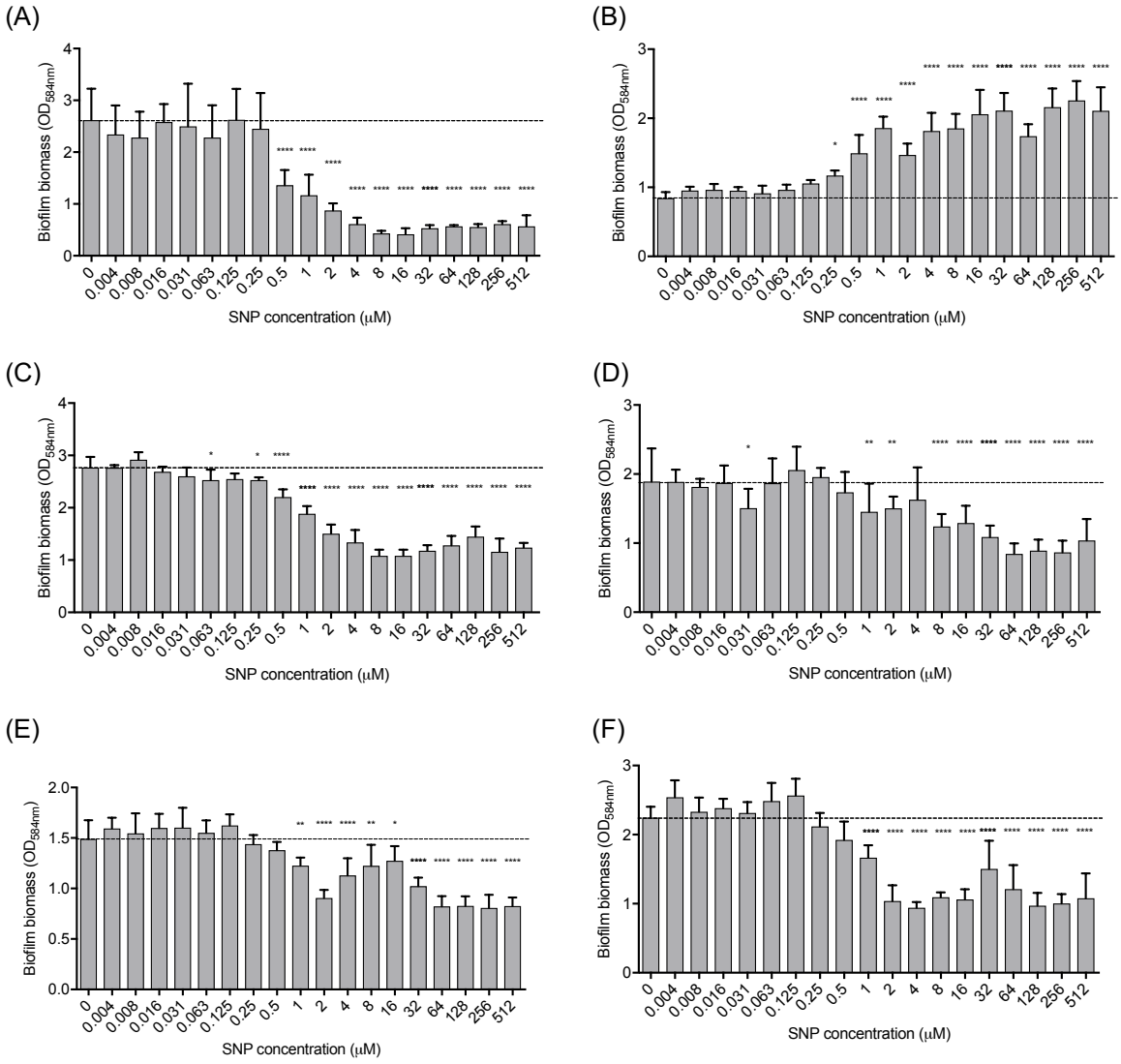


Figure 12. PAO1 and clinical *P. aeruginosa* isolate biofilms treated with SNP; (A) PAO1, (B) PA10, (C) PA21, (D) PA26, (E) PA30 and (F) PA68. Biofilms were grown for 24 h and treated for 20 h before staining with CV for biomass quantification. The mean of six technical replicates and SD is shown; dashed line is level with mean of untreated control. Experiments were repeated three times with similar results. One-way ANOVA statistical testing with Dunnett's multiple comparison test was used, all treatment groups compared to untreated control group. Correlating planktonic culture values in **Appendix C**.

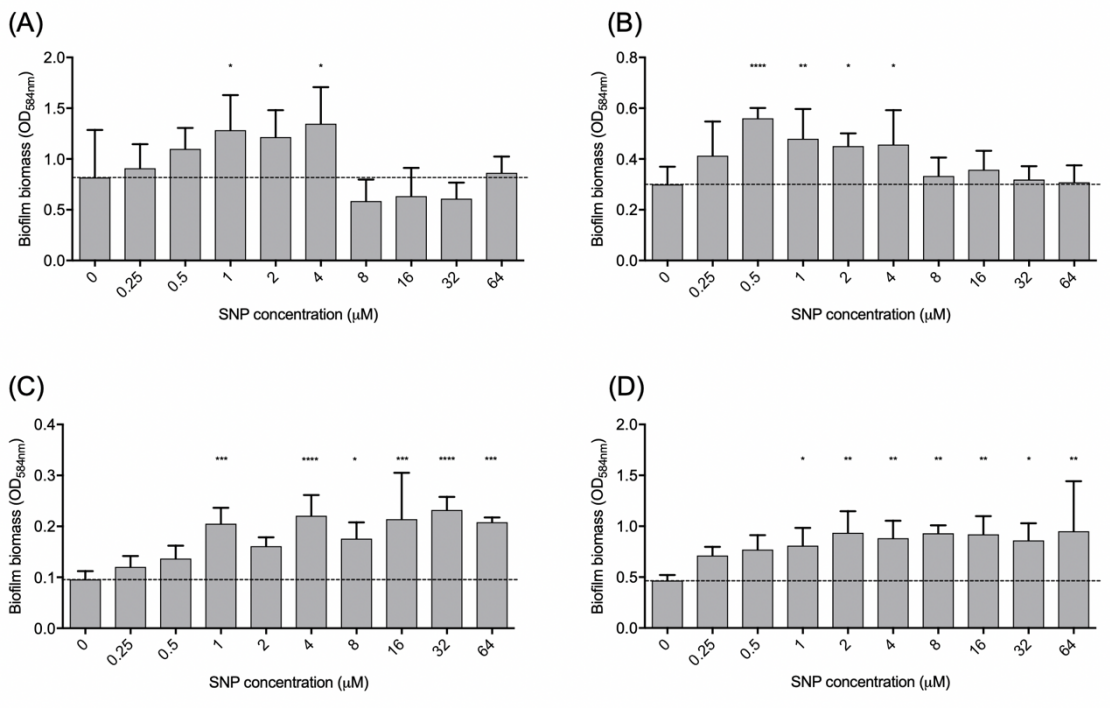


Figure 13. Additional clinical *P. aeruginosa* isolate biofilms treated with SNP; (A) PA20, (B) PA47, (C) PA56, and (D) PA66. Biofilms were grown for 24 h and treated for 20 h before staining with CV for biomass quantification. The mean of six technical replicates and SD is shown; dashed line is level with the mean of the untreated control. Experiments were repeated three times with similar results. One-way ANOVA statistical testing with Dunnett's multiple comparisons test was used with treatment groups compared to untreated control group. Correlating planktonic culture values in **Appendix C**.

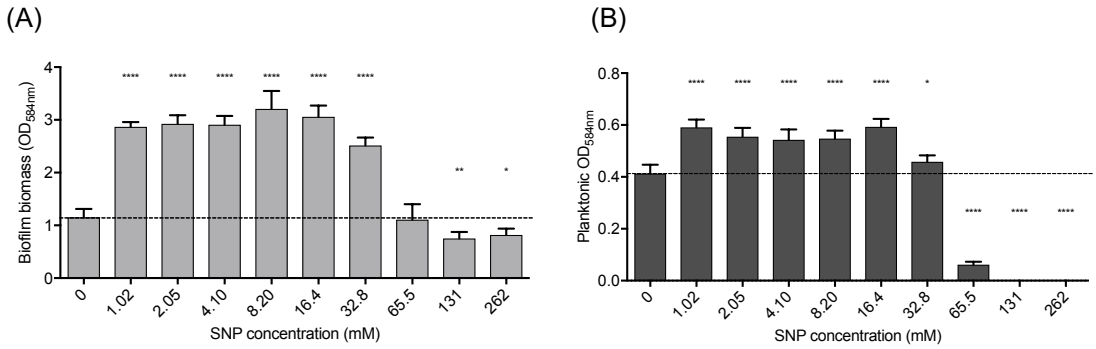


Figure 14. PA10 biofilms treated with increased concentrations of SNP. (A) biofilm biomass values, and (B) corresponding planktonic growth values following treatment with SNP concentrations ranging from 1.024mM to 262mM. The mean and SD of six technical replicates is shown; dashed line is level with the mean of the untreated control. Experiments were repeated three times with similar results. One-way ANOVA statistical testing with Dunnett's multiple comparison test was used with treatment groups compared to untreated control group.

3.2.5 Correlations between effect of NO and strain phenotype

Potential correlations between the biofilm response to NO and isolate phenotype were investigated and are shown in (**Figure 15**). In total, ten strains were included in the analysis: PAO1 and nine clinical isolates. No correlation was shown between biofilm response to NO and colony morphology, pyocyanin or pyoverdine production, or susceptibility to tobramycin, ceftazidime or colistin.

Linear regression analyses were carried out for phenotypic parameters that could be numerically quantified (**Figure 15D-H**). Interestingly, the comparison with the highest and significant R^2 value was the one comparing the isolate response to NO to the growth of the control untreated biofilm. Interestingly, all strains which had a value of 1 or less for the biomass of the control resulted in an increase in biofilm biomass following treatment with SNP, and all strains with a control biomass value of above 1 had resulted in significant decreases in biofilm biomass following the same SNP treatment. A similar trend was also seen with planktonic growth rate, although with lower and non-significant R^2 value; isolates with an OD value of below 0.4 at 12 h had shown increased biofilm formation with NO, whereas isolates with an OD value of above 0.4 at 12 h were dispersed.

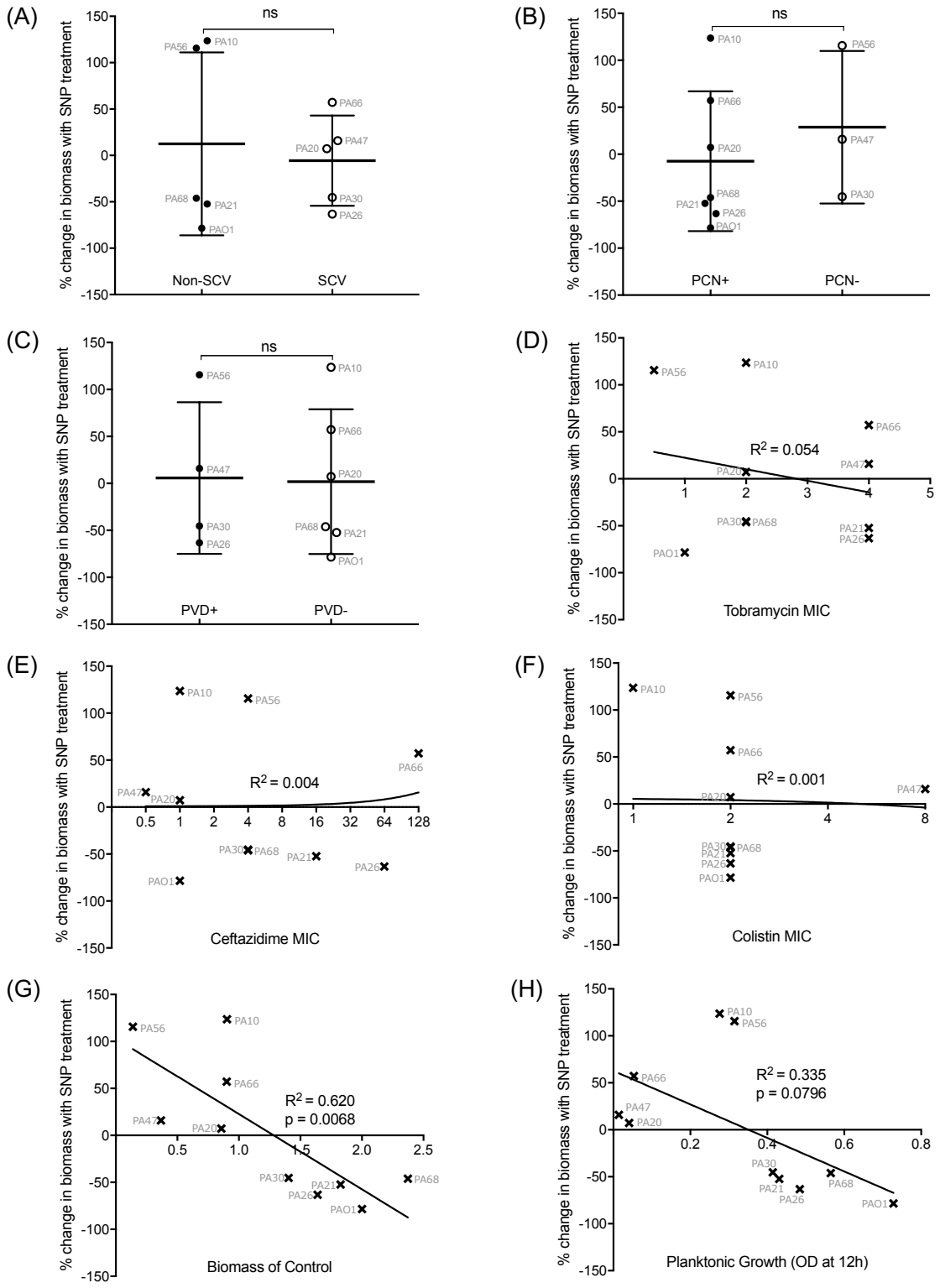


Figure 15. Correlations between the response to NO and isolate phenotype. Biofilm response to 64μM SNP treatment compared with: (A) SCV morphology, (B) pyocyanin production, (C) pyoverdine production, (D) tobramycin MIC, (E) ceftazidime MIC, (F), colistin MIC, (G) biomass of the control, and (H) planktonic growth. Mean of three biologicals replicates plotted. Welch's T test used for statistical analysis (A - C) and linear regression for (D - H).

3.2.6 c-di-GMP levels are decreased in clinical isolates of *P. aeruginosa* strains following exposure to NO from SNP

The dispersal action of NO on *P. aeruginosa* biofilms is thought to be associated with a decrease in bacterial second messenger c-di-GMP. To investigate how NO affected the c-di-GMP levels in PAO1 and CF clinical isolates, a c-di-GMP GFP-reporter plasmid was transformed into the *P. aeruginosa* strains. Initially, conjugation was selected as the transformation method, utilising the *E.coli* strain containing the plasmid provided to us. However, after multiple attempts, transformation was not successful. A second, more time-effective approach, involving plasmid extraction and electroporation of *P. aeruginosa* strains, was successful. PAO1 and five selected clinical stains were transformed and confirmed to contain the c-di-GMP reporter plasmid by way of antibiotic selection, microscopy and fluorescence values.

Previously, only planktonic based assays had been detailed in the literature to investigate changes in c-di-GMP levels with this reporter plasmid. In this study, a biofilm-based assay was successfully developed and optimised using PAO1. C-di-GMP levels were reduced in biofilms and dispersed cells with SNP treatment, and correlated to the biofilm dispersal action (**Figure 16**). Similar results were also obtained with PA21 showing a simultaneous reduction in c-di-GMP and biofilm biomass with SNP treatment. However, these results were not reproducible with other clinical *P. aeruginosa* isolates. Amongst other issues, the transformed strains were unable to form biofilms as well as the wild type strain or formed a large non-attached cell aggregates in the middle of the well rather than adhering to the well surface. Non-uniform suspensions result in inaccurate absorbance values, and reliable biofilm quantification values would be difficult to acquire in a 96 well based culture system. Due to these technical difficulties, a planktonic based assay was developed and produced more reliable results.

The c-di-GMP levels, for PAO1 and five clinical isolates of *P. aeruginosa*, with and without NO treatment using the planktonic based assay is shown in **Figure 17**. Although the raw values show variation, for each of the strains, the relative florescence and hence c-di-GMP level is decreased following NO treatment. For PAO1, PA21, PA26, PA30 and PA68, the decrease in c-di-GMP is expected following NO treatment as biofilms of strains were successfully dispersed. However, surprisingly, the c-di-GMP level is also decreased for PA10, one of the strains which had an increase in biofilm biomass following SNP treatment. For each strain, the reduction in c-di-GMP following NO treatment is statistically significant.

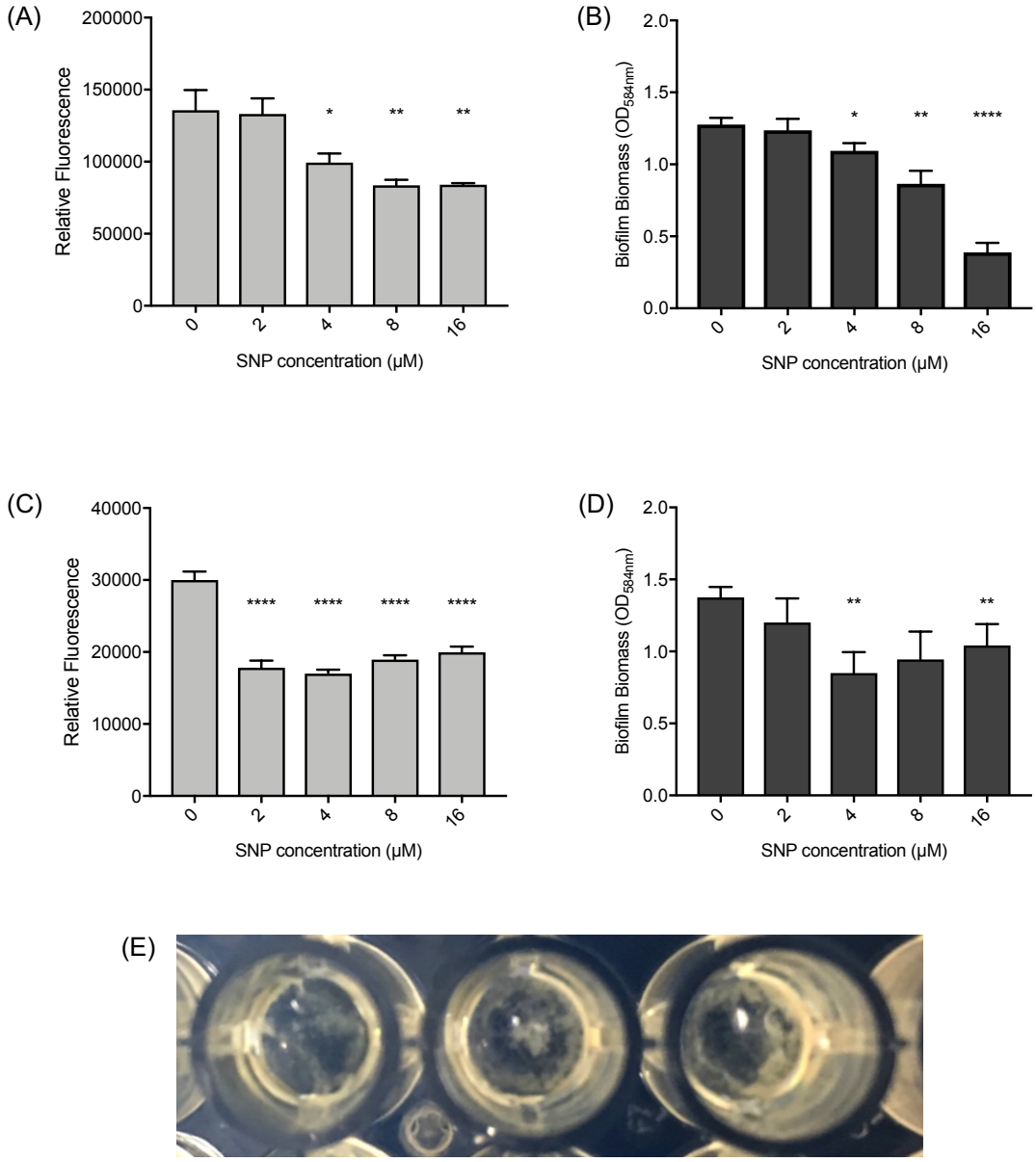


Figure 16. C-di-GMP measurements in *P. aeruginosa* biofilms and dispersed cells. (A) Reductions in relative fluorescence (and c-di-GMP expression) of PAO1 biofilms and dispersed cells with NO treatment. (B) Biofilm biomass values relating to c-di-GMP measurement in (A). (C) Reductions in relative fluorescence (and c-di-GMP expression) of PA21 biofilms and dispersed cells with NO treatment. (D) Biofilm biomass values relating to c-di-GMP measurements in (C). Graphs (A) to (D) are representative of two independent experiments; bars shown the mean and SEM of six technical replicates. T tests were used for statistical analysis. (E) Photograph of a transformed clinical isolate showing cell aggregation in suspension and inconsistent biofilm formation in the wells of a 96 well plate.

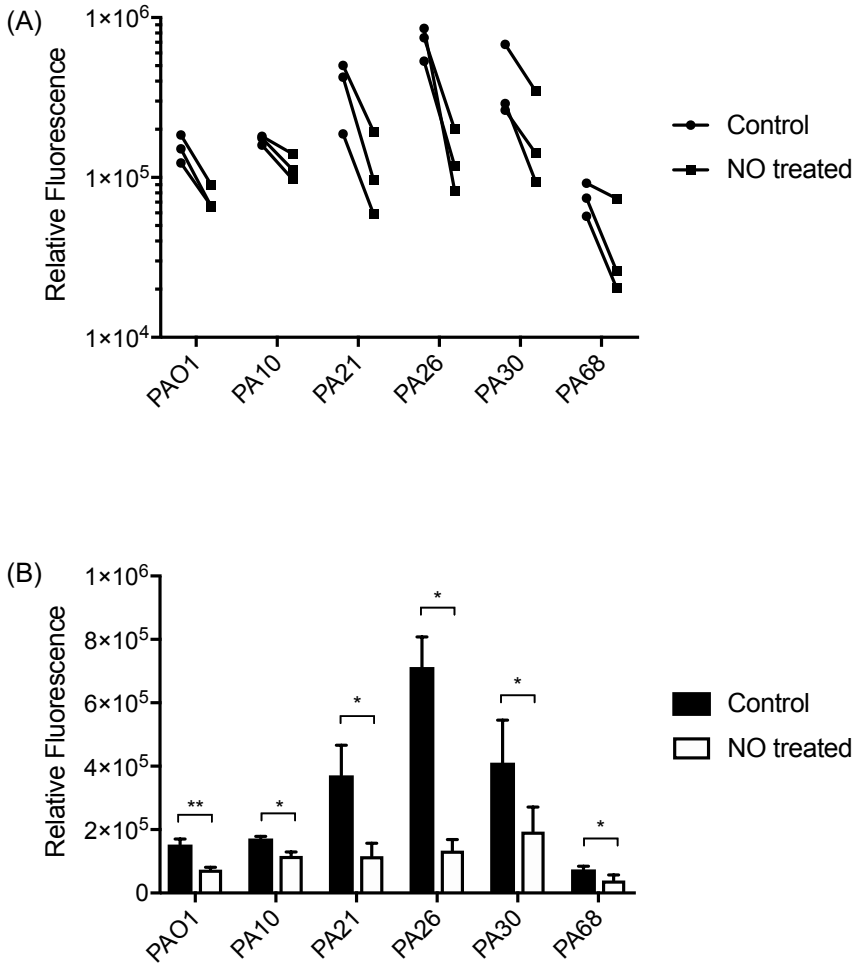


Figure 17. C-di-GMP measurements in *P. aeruginosa* planktonic cultures with and without NO. Planktonic cultures of PAO1 and five clinical isolates were sub-cultured into M9 minimal media and treated with 40 μM SNP for 18 h. (A) Reductions in relative fluorescence (correlating with c-di-GMP expression) of three biological replicates. (B) The mean and SD of three biological replicates, with ratio paired T-tests used for statistical analysis.

3.2.7 Passaging *P. aeruginosa* with low levels of NO shows evidence of some attenuation of the dispersal response

To investigate whether the dispersal response to NO is modulated if *P. aeruginosa* has been in the presence of low concentrations of NO for an extended period of time, a passaging experiment was carried out. PAO1 was the selected strain as it is historically a wound isolate and, unlike the CF isolates, would have had minimal exposure to NO. Planktonic cultures of PAO1 were passaged for 10 generations before biofilms were grown and treated with a concentration of SNP which should cause dispersal. Three sets of passaging experiments were carried out, shown in **Figure 18**. For the biofilms formed by PAO1 passaged naturally (i.e. without NO), in all three replicate experiments, the biofilm biomass is significantly reduced with the NO treatment group, confirming successful dispersal of the biofilms. As shown in **Figure 18D**, approximately 50-60% of the biofilm is dispersed with NO treatment in all three experiments.

For the biofilms formed by PAO1 passaged with low dose NO, some variation is seen in the dispersal response to NO treatment. In the 1st experiment, there is no statistically significant reduction in the biofilm biomass of the NO treated biofilm compared to the control biofilm indicating the biofilm was not dispersed. However, in the 2nd and 3rd experiments, there is a statistically significant reduction in biofilm biomass with the NO treatment, indicating the biofilm was dispersed. Comparison of the mean biofilm biomass reduction values (**Figure 18D**) show that biofilms disperse in response to higher dose NO treatment irrespective of whether PAO1 was passaged naturally or with lose dose NO. In experiment 1 however, the mean reduction in biomass drops from approximately 60% to approximately 30%.

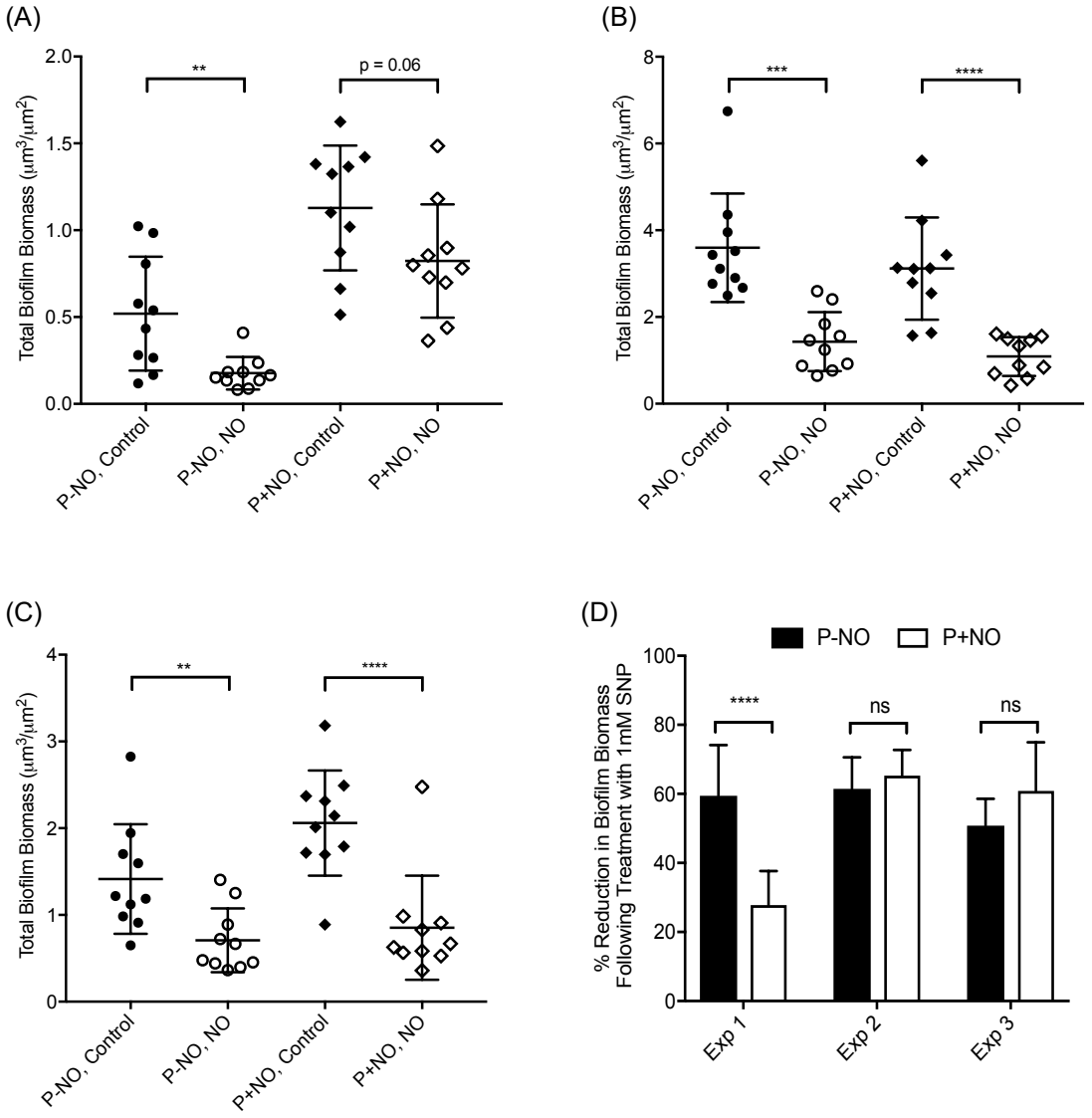


Figure 18. Total biomass values of biofilms formed by PAO1 passaged naturally, and PAO1 passaged with NO for 10 generations. Biofilms were then treated with higher dose NO to initiate dispersal, analysed with CLSM and biomass values quantified using COMSTAT. (A-C) Three biological replicates, passaged separately. Closed circles = passaged naturally (without NO), control biofilm; open circles = passaged naturally (without NO), treated with NO; closed diamonds = passaged with low dose NO, control biofilm; open diamonds = passaged with low dose NO, treated with NO. (D) The dispersal response following treatment with 1mM SNP of PAO1 biofilms passaged 10 times naturally (without NO) and passaged with low dose NO (1 µM). Exp 1, 2 and 3, relate to data shown in (A), (B) and (C) respectively. Black bars = passaged without low dose NO; white bars = passaged with low dose NO. Welch's T test was used for statistical analysis.

3.3 Discussion

3.3.1 Phenotypic analysis of CF isolates

Phenotypic analysis of the panel of clinical isolates of *P. aeruginosa* was a crucial starting point and enabled prioritisation of isolates to be utilised for future work. Amongst the phenotyping assays that were conducted, investigations into whether the strain was mucoid and their ability to form biofilms *in vitro* were particularly important.

Of the twenty clinical isolates, just three were identified as mucoid, following assessment of growth on PIA medium. Pugashetti et al., (1982) had shown PIA to be the only agar medium of 6 tested to display mucoidy in all 15 *P. aeruginosa* isolates they examined, whilst only 13 isolates were mucoid on LB agar and just 9 on nutrient agar. This is echoed results by Ciofu et al. (2008), noting that several CF isolates which were mucoid on PIA agar were non-mucoid on LB agar. Unfortunately, there appears to be inconsistency within the literature. Different research groups utilise different media for determinance of mucoidy, with varying incubation times, or simply state mucoidy was determined by "visual inspection on agar" with lack of additional details. This study supports these earlier findings that PIA is the media least likely to give false negative results, and a 48 h incubation period, as opposed to a 24 h period would also ensure a positive result is not missed.

The low proportion of mucoid isolates in this study is not entirely surprising. The rates of mucoid *P. aeruginosa* isolation from CF varies a great deal and is dependent on numerous factors. CF patients commonly acquire non-mucoid *P. aeruginosa* from the environment and following persistent infection and selective pressures in the lung, mucoid strains develop. A high proportion of non-mucoid isolates in our panel could indicate that *P. aeruginosa* was recently acquired, though this would be unusual based on the fact that the isolates were from adolescent and adult patients. Another long-known fact has been that mucoid *P. aeruginosa* can spontaneously revert back to non-mucoid when cultured *in vitro,* particularly if PIA agar is not utilised (Cullen et al., 2015; Pritt, O'Brien, and Winn, 2007). Rather than reversal of the original mutation, conversion back to the non-mucoid type is thought to be due to second-site suppressor mutations, in genes such as *algT* (Govan and Deretic, 1996). In the study by Ciofu et al. (2008), 69% of the non-mucoid isolates had secondary site mutations in *mucA* indicating these are 'revertants'. If further investigation was warranted, the non-mucoid isolates in this study could have been examined for mutations in *mucA* to identify if there are 'revertants'.

Interestingly, the biofilm forming capabilities of the clinical isolates were highly varied and not all of the isolates demonstrated good biofilm growth *in vitro.* Many isolates had shown biofilm formation inferior to PAO1. This could be attributed to the culture conditions; the assay for biofilm growth involved culturing air-liquid interface biofilms attached to a polystyrene tissue-culture

treated substratum. This is in stark contrast to their growth in the CF lung which are now associated more with self-aggregation as opposed to growth on an actual substratum. One isolate in particular, PA39, is reported as a poor biofilm former as biofilms did not develop on the polystyrene plate but rather as macroscopic non-attached aggregates present in the centre of each well. Many other studies have also looked at the biofilm forming capabilities of *P. aeruginosa* CF isolates. Perez et al. (2011) had also found, using a near identical methodology, that from a panel of CF isolates, many of them were classed as 'weak' biofilm formers *in vitro.* Lee et al. (2005) similarly saw that the majority of CF isolates had a reduced capacity to form biofilms in comparison to PAO1. Deligianni et al. (2010) showed that of 96 CF isolates, just over half were classed as weak or moderate biofilm formers and just under half as strong, though isolates were not compared to PAO1. In this study, the isolates that were identified as strong biofilm formers were prioritised for future biofilm assays.

Colony morphology, pigment production, planktonic growth rates and the antibiotic sensitivities to three common CF antibiotics for the panel of isolates were also examined. To assess colony morphology, isolates were growth on non-selective LB agar and the criteria set by (Haussler et al., 2003) used to identify SCV. Interestingly, in this study, two of the twelve clinical isolates defined as SCV demonstrated larger colony sizes on cetrimide agar, and even larger colonies still on PIA. From this, two conclusions are drawn; firstly, this study supports the recommendation by Haussler and colleagues that non-selective LB agar with a 48 h incubation period be used to identify SCV, as this phenotype would have been missed if selective agar was exclusively used for culturing. Secondly, the differing colony sizes could be attributed to variations in the agar media composition rather than the selectively/non-selectively of the media. We suggest that the glycerol content is responsible for the trend seen in colony size, as the glycerol content for LB agar, cetrimide agar and PIA is 0%, 1% and 2% respectively and shows a direct, positive correlation. The colony size of PAO1 and the majority of the CF isolates in the panel were unaffected by agar type and glycerol content, but noticeably influenced the colony size of two of the CF isolates in particular.

P. aeruginosa is known to enzymatically degrade lung surfactant lipid molecule phosphatidylcholine (PC) and utilise it as a nutrient source by metabolising its breakdown products which include fatty acids, phosphorylcholine and glycerol (Son et al., 2007). Glycerol uptake and metabolism is thought to be regulated by genes in the glp regulon under the control of negative regulator GlpR (Schweizer and Po, 1996). We postulate that the two isolates with colony sizes influenced by glycerol content in the agar may have mutations within this regulon and are able to more effectively use glycerol as a carbon nutrient source. Scoffield and Silo-Suh (2016) demonstrated that glycerol metabolism promoted biofilm formation, and, interestingly, both the CF isolates with hypothesised enhanced glycerol metabolism in this study were shown to be good biofilm formers. Further experiments are continuing in our laboratory to confirm the effects of glycerol of CF isolates and confirm the positive correlation seen here.

Somewhat unusually, a high proportion of the clinical isolates in this study (60%) were identified as SCV. Two main studies have previously taken place to investigate the frequency of *P. aeruginosa* SCV in CF patients. Thomassen et al. (1979) found SCVs in the sputum of 14% of 286 CF patients with *P. aeruginosa* infection sampled during a 3 month period. However, in a study by Haussler et al. (1999), SCVs were isolated from 38% of 86 CF patients with chronic *P. aeruginosa* infection, based on samples taken over a 2 year period. It is now recognised that SCVs of *P. aeruginosa* more commonly arise in those with chronic infection. This may explain the high proportion of SCVs found in this study, as the sputum samples were from adolescent and adult CF patients who are more likely to have chronic *P. aeruginosa* infection. The SCV phenotype is associated with an increased resistance to antibiotics, and a subgroup associated with hyperpilation, auto-aggregation, and increased biofilm attachment and formation, and a poorer clinical presentation (Malone, 2015). All of the isolates in this study that were resistant to tobramycin and colistin, as well as all aside from one resistant to ceftazidime were identified as SCV.

As expected, there was vast variation with regards to pigment production from the twenty CF isolates, and also variation as to the pigments produced with the different types of agar media used. Weiser et al. (2014) had found similar variations in pigment production when analysing 148 strains of *P. aeruginosa* from clinical, environmental and industrial sources, which complicated organism identification. Over half of the isolates in this study had demonstrated production of pyocyanin, the most common *P. aeruginosa* pigment and a key virulence factor, whilst pyoverdine and pyorubin production were also evident with isolates. Again, similarly to Weiser et al. (2014) distinction between the presence of pyocyanin and pyoverdine was sometimes difficult. Interestingly, phenotypic analysis carried out by Lozano et al. (2018) showed that mucoid isolates were more likely to produce pyocyanin and pyorubin compared to SCV. Although the isolate panel in this study is relatively small, all three mucoid isolates produced either pyocyanin or both pyocyanin and pyorubin, whilst two of the four isolates with no pigment production were SCV. A strong relationship occurs between the pyocyanin production and lung function decline, though the exact actions as to why this occurs is not entirely clear (Hunter et al., 2012; Rada and Leto, 2013). In addition to its actions as a virulence factor, pyocyanin is also associated with goblet cell hyperplasia, mucus hypersecretion, and causes pro-inflammatory changes in respiratory epithelial cells (Rada and Leto, 2013). Interestingly, *in vitro* studies have shown pyocyanin can also inactivate the vasodilatory actions of NO, discussed in greater detail in the next section.

Also, unsurprisingly, the majority of the CF isolates had slower planktonic growth rates when compared to PAO1. The slow growth of CF isolates is well recognised, though can be problematic for *in vitro* work. For example, for antimicrobial susceptibility testing the standardised 24 h incubation period is often too short to provide a result (Burns and Rolain, 2014). This was seen in this study with a number of isolates, and in these cases the incubation period had to be extended to 36 or 48 h before adequate turbidity was seen with the control, enabling identification of an MIC

value. The slower growth rate of CF isolates can be explained by their adaption to the environment in the CF lung, and are thought to be selected for *in vivo* by antibiotic pressure, as a reduced metabolism and division rate enables bacteria to resist the effects of antimicrobials. Slow growth *in vitro* is also an indication of auxotrophy. CF isolates are known to be auxotrophic mutants, having nutritional requirements not present in environmental strains, and are incapable of producing certain amino acids due to biosynthetic defects and the high amino acid content in CF sputum (Barth and Pitt, 1996; Taylor, Hodson, and Pitt, 1992). This sacrifice is thought to allow the energy conserved by utilising amino acids already present in the environment to be spent on other high energy consuming cellular processes to empower survival in the lung, such as alginate production.

Antimicrobial susceptibilities of the panel of isolates to tobramycin, ceftazidime, and colistin, three common CF antibiotics, demonstrated relatively low rates of resistance. Half of the twenty isolates were sensitive to all three agents, whilst none were resistant to all three; 15% were resistant to tobramycin, 35% resistant to ceftazidime and 20% resistant to colistin. These values are comparable to other studies observing resistance rates in CF isolates (Macdonald et al., 2010; Mustafa et al., 2016). For example, looking at 428 CF isolates, Pitt et al. (2003) found comparable values whereby 10% were resistant to tobramycin, 40% resistant to ceftazidime and 13% resistant to colistin. This data collectively shows that CF isolates in their planktonic form are still fairly susceptible to antibiotics, and antimicrobial tolerance in the lung can be accounted for by biofilm formation. This therefore also supports the notion of using a dispersal agent to release cells from the biofilm back into the planktonic mode of growth and enable killing by conventional antibiotics.

3.3.2 Release of NO from SNP

SNP is a NO donor molecule, used in clinical practice during hypertensive emergencies, to treat heart failure and to control hypotension during surgery, due to its vasodilatory actions. The release of NO from SNP is spontaneous, and results in the relaxation of vascular smooth muscle and inhibition of platelet aggregation and adhesion (Aldini et al., 2006).

SNP is also frequently used *in vitro* and was chosen as the NO donor molecule for use against *P. aeruginosa* biofilms in this study for a number of reasons. Firstly, SNP was the NO donor molecule used in the 2006 and 2009 publications by Barraud and colleagues when discovering the link between NO and biofilm dispersal. SNP is also a very frequently used NO donor molecule, and this would enable results from this study to be compared to others in the literature. Secondly, SNP is an inexpensive NO donor and readily available, this was imperative as its limited shelf life meant a new batch was required approximately every six months. Thirdly, and most importantly, SNP is stable in solution and has a very sustained release of NO in comparison to other NO donors with a short half-life (Bradley and Steinert, 2015).

SNP releases NO in response to heat and light; the energy absorption induces electron transfer from the Fe^{2+} centre to the NO+ ligand, weakening the bond and enabling release of NO (Wang, Cai, and Taniguchi, 2005). Unfortunately, many microbiological publications investigating the effects of SNP on biofilms have failed to specify the exact culture conditions, particularly regarding light exposure during incubation. In this study, we confirmed and clearly demonstrated the light sensitive nature of SNP using a highly sensitive CLD to show dramatically enhanced levels of NO release in the presence of light. Interestingly, we also discovered in natural light, though the release of NO was higher it was also more erratic, indicating SNP was incredibly sensitive to any change in light intensity. The release of NO from SNP in the dark and with artificial light was lower, however had a more consistent release over time. This data gave useful insight and aided in the development and design of assays utilising SNP against *P. aeruginosa* biofilms. Quantification of the NO release from SNP in PBS and M9 minimal media revealed no statistically significant difference between the two media, indicating the components of biofilm media has no impact on the NO release.

3.3.3 The varied response of CF isolate biofilms to NO treatment

In this chapter, we delved further into the impact of NO on CF isolates of *P. aeruginosa*. Using a biofilm assay near identical to the one utilised by Barraud et al in the 2006 publication, we were able to replicate their results and show evidence of biofilm dispersal with PAO1 following treatment with SNP. However, using the same assay, the results with clinical CF isolates were varied and unexpected. A total of nine clinical isolates were treated with NO; the biofilms of four isolates were dispersed with multiple concentrations of SNP in similar manner to PAO1, whilst the biofilms of five isolates were marginally or unquestionably increased following SNP treatment. Even amongst the isolates with biofilms susceptible to the NO dispersal signal, the dispersal rate varied.

A similar effect was noted by Kim et al. (2018); whereby SNP caused different rates of dispersal with clinical non-CF isolates of *P. aeruginosa* and could not disperse one of five isolates examined. The authors attributed this to differences in the biofilm structure, with NO being more effective with ‘porous’ biofilms. It is also to be noted however, that even in the 2006 publication by Barraud et al. it was made clear that an optimal concentration window of NO exists, and that SNP concentrations outside of this optimal window were shown to promote biofilm formation as opposed to initiating dispersal. Unfortunately, despite the large concentration range utilised in this study, we were unable to find a concentration window that could initiate dispersal in five clinical isolates. Twenty clinical isolates were available for analysis, although only nine were examined in this case due to the time consumption and labour intensity associated with these dispersal assays and the high concentration range.

3.3.3.1 The response to NO vs phenotype – correlation between response and growth

As phenotypic analyses of the clinical isolates were made, a possible connection between isolate phenotype and the biofilm response to NO was investigated. Of note, the literature has suggested a connection between pyocyanin and NO. Low concentrations of pyocyanin have been shown to inhibit the vasodilatory activity of NO released from pulmonary endothelial cells and multiple NO donors (Bozinovski et al., 1994; Hempenstall et al., 2015; Warren et al., 1990). Though the precise mechanism of this action is still unclear, its likely pyocyanin has a target downstream of NO production within the signalling pathway mediating vasorelaxation. More recently, NO released from SNP has been shown to reduce levels of pyocyanin in PAO1 and four non-CF isolates of *P. aeruginosa* (Gao et al., 2016); demonstrating a complex dynamic between pyocyanin and NO whereby both have inhibitory actions on one another. However, assessment of isolates in this study showed no correlation between pyocyanin production and the response to NO.

Colony morphology and mucoidy were also varied across all ten strains examined (PAO1 and nine CF isolates) with no obvious commonalities; though it must be noted just one of the 10 strains were mucoid, and this strain was not dispersed. Linear regression analyses showed no correlation between the isolate response to NO, and the AMS to three common CF antibiotics. Interestingly, there was a strong correlation between the response of the isolate to NO and the biofilm growth rate, and a weak/moderate correlation with the planktonic growth rate. PAO1 and the four clinical isolates successfully dispersed by NO were better biofilm formers and had faster planktonic growth rates than the five isolates not dispersed by NO, which were poor or moderate biofilm formers with slower planktonic growth rates.

3.3.3.2 Treatment of *P. aeruginosa* biofilms with a different NO donor supports the correlation between response to NO and biofilm growth rate

Other work in our laboratory has included investigation into the mechanism of the NO induced dispersal response, and isolates from the panel in this study were also utilised by (Cai, 2018) in her work. Of the seventeen isolates she examined, twelve were successfully dispersed by 250 μM of shorter-acting NO donor Spermine-NONOate following a 2 h treatment period. However, five isolates (PA08, PA15, PA20, PA49, and PA58) demonstrated an unchanged or marginal non-significant increase in biomass following treatment. Furthermore, a 2 h Spermine NONO-ate treatment and a 12 h SNP treatment were both unable to disperse planktonic cell aggregates formed by PA08, PA37, PA39 and PA66; in the case of PA08, the treatment caused a statistically significant increase in cell aggregate biomass.

The combined results from this study and the work by Cai demonstrate that the NO dispersal response is sensitive to different NO donors and/or treatment duration. This is demonstrated by the fact that three of the five isolates (PA10, PA56, and PA66) that showed biofilm promotion with 20

h SNP treatment in this study, were dispersed with 2 h Spermine NONOate treatment. One of the five isolates (PA20) was not dispersed by Spermine NONOate, whilst the fifth isolate (PA47) was not examined by Cai. Despite differences in the biofilm response to SNP and Spermine NONO-ate, a similar correlation is evident with Spermine NONO-ate treatment of biofilms whereby isolates that did not disperse were slow growing. PAO8, the isolate shown by Cai to have a statistically significant increase in cell aggregate biomass with both SNP and Spermine NONOate, had the slowest growth rate, by far, of all clinical isolates in the panel, further supporting the correlation found in this study.

3.3.3.3 Potential explanations for the isolate specific responses to NO

"Slow growth → Adaptation to anoxia → Denitrification → Utilisation of NO breakdown products as nutrient source":

The possible reasons for the varied response to NO are abundant and complex. An important factor to consider is the isolates not dispersed by NO were slower growing, a typical characteristic of CF isolates and indication of auxotrophy. As previously mentioned, slow growth and/or auxotrophy indicates adaptation to the environment of the CF lung. The lung is recognised as having hypoxic niche areas and studies have demonstrated anoxia in sputum (Cowley et al., 2015; Kolpen et al., 2014). An assumption could be made that isolates of *P. aeruginosa* that have a slower metabolism and growth rate, are therefore also likely to have adapted to the anoxic conditions in the lung. Line et al. (2014) demonstrated that physiological levels of nitrite in CF sputum were sufficient to support anaerobic respiration of *P. aeruginosa* using denitrification. This pathway is activated following detection of low O_2 by ANR, or of NO by DNR, and NO is an intermediary metabolite reduced to N_2O before N_2. Low doses of NO have been shown to modulate expression of genes associated with attachment, motility, and denitrification. Furthermore, the link between NO induced dispersal and denitrification has also been demonstrated by mutant studies; Nir deficient mutants do not disperse, whilst Nor deficient mutants display enhanced dispersal.

Therefore, one theory to explain the differing response to NO could pertain to the slower growing isolates are better adapted to anaerobic respiration, having the ability to utilise NO and/or NO breakdown products nitrite and nitrate as nutrient sources via the denitrification pathway. This also explains why rather than having no dispersal effect, NO treatment was able to promote biofilm formation with these isolates. Eichner et al. (2014) showed that *P. aeruginosa* had adapted to hypoxic conditions by increasing the expression of various genes beneficial during oxygen deprivation, and under the control of ANR. In addition to transcriptional changes, a genetic adaptation to anaerobiosis has been shown; CF isolates frequently have LasR inactivating mutations, selected for by the nutrient rich CF sputum. Argenio et al. (2007) found that *lasR* mutants of *P. aeruginosa* conferred a growth advantage with nitrogen sources, and had an enhanced usage of nitrite and nitrate. Hoffman et al. (2010) supported these findings, showing *lasR*

mutants to have decreased oxygen consumption and increased nitrate utilisation, which was conferred with increased growth and antimicrobial tolerance. Intriguingly, these mutants also showed higher levels of intracellular NO, and detoxification of the NO had also resulted in increased growth.

Additional information supporting this theory exists in the form of studies showing levels of exhaled NO are decreased in CF patients with *P. aeruginosa* compared to those without *P. aeruginosa* infection (Balfour-Lynn and Dinwiddie, 1996, Grasemann et al., 1997, and Keen et al. 2007). This is paradoxical as exhaled NO is usually increased during infection (Hopkins et al., 2006; Tripathi et al., 2007). The authors suggested that one explanation, amongst others, for this is the increased consumption of NO by *P. aeruginosa* as part of the denitrification pathway.

This theory also explains the opposing results seen with a 2 h and 20 h NO treatment period. Biofilms exposed to NO for 20 h will encounter accumulation of breakdown products nitrite and nitrate within the assay system, and this extended time is sufficient to allow for biofilm promotion. Comparatively, the concentration of breakdown products accumulated during a 2 h treatment period of NO will be lower, and the bacterial lag time will prevent any obvious increase in biomass. An important note to make is that this accumulation of breakdown products would not necessarily occur *in vivo.* Whilst nitrite and nitrate can accumulate within the wells of a 96 well plate *in vitro,* NO, nitrite and nitrate are metabolised in the human body and can be excreted via the bowel, liver, kidneys and saliva (Kelm, 1999). Therefore, if this theory holds ground, the increases in biofilm biomass with SNP treatment may be a strictly *in vitro* phenomenon.

"Immune cells release NO → PA resist NO killing → also resist NO dispersal response → Nor increase or NO scavengers":

An alternative theory relates to the previous exposure and adaptation of *P. aeruginosa* to exogenous NO. Epithelial cells, macrophages, and other immune cells release NO as an offensive strategy to attack invading pathogens and infectious agents (Tripathi et al., 2007). The persistence of *P. aeruginosa* in the lung, despite the excessive immune response to infection, suggests *P. aeruginosa* cells are in the presence of sub-lethal concentrations of NO and/or have developed mechanisms to resist NO-mediated killing. *P. aeruginosa* is already known to protect itself from nitrosative stress and toxic NO accumulation via Nor, converting NO to N_2O, which is then converted to non-toxic N_2 (Schobert and Jahn, 2010). The previous exposure to NO released from immune cells could have resulted in adaptation of *P. aeruginosa* to the dispersal response via the increased expression or upregulation of Nor to promote the breakdown of exogenous NO.

Interestingly, Yoon et al. (2007) investigated a *norCB* deficient mutant of PAO1 was still able to protect itself from toxic NO accumulation by controlling the endogenous synthesis of NO, and reassignment of metabolic enzymes to sequester NO. Loss of NorCB had resulted in reduced transcription of genes encoding for Nar and Nir, enzymes that synthesise endogenous NO from

NO_2- and NO_3-. Yoon and colleagues also showed increased NO levels resulted in derepression of genes such as *hmgA* and *hpd*, which bind to NO and function as NO scavengers to counteract high exogenous NO levels.

The ability of *P. aeruginosa* to scavenge NO was also investigated by Zhu et al. (2018) based on a previous discovery that multiple doses of NO did not enhance the dispersal of *P. aeruginosa* biofilms. PAO1 biofilms pre-treated with lower doses of Spermine NONO-ate did not disperse with a second higher dosage, and the pre-treatment caused induction of *Fhp* expression, another protein able to scavenge NO. This data supports the notion that previous exposure to NO can mediate the dispersal response to NO, and investigations into the regulation of flavohemoglobin Fhp in CF isolates would be of interest to support this theory.

3.3.4 Mechanistic investigations into the varied response to NO

Though much time and effort were invested into developing a biofilm-based assay to measure intracellular c-di-GMP levels and results were reproducible with PAO1 biofilms, the results with the clinical isolates were inconsistent and unreliable due a multitude of factors. Of note, the transformed clinical isolates were not stable enough to maintain the incorporated plasmid without antibiotic maintenance, formed biofilms inferior to the wild type, and formed large unattached cell aggregates within the wells which obscured absorbance readings. Rybtke et al., had encountered the same issue; they had utilised a Δ*wspF* PAO1 strain in an attempt to increase levels of c-di-GMP. However, this increase had caused planktonic cell aggregation due to the positive regulation of polysaccharides Pel and Psl by c-di-GMP. Therefore, without further genetic modification, we conclude that using a reporter plasmid is not particularly suited for measuring c-di-GMP in biofilms.

As a result, a planktonic based assay was utilised to observes changes in c-di-GMP with NO treatment, and the decrease seen in this study with PAO1 was comparable to the result by Rybkte et al. Five clinical isolates were also shown to have decreased c-di-GMP levels following NO treatment, including PA10, one of the non-dispersing strains. This therefore provides evidence against the theory of increased NO scavenging by *P. aeruginosa*, and indicates a modulation succeeding NO sensing and NO induced c-di-GMP level alteration. Comparatively, this result is supportive of theories involving adaption to anoxia and increased denitrification, and genetic changes downstream of, or unrelated to, the NO signalling pathway.

The association of low c-di-GMP levels with planktonic growth, and high c-di-GMP levels with biofilm growth has been described as a ‘general’ model. C-di-GMP regulates many factors and pathways that contribute to the biofilm formation, for example swarming, swimming, and twitching motility, and polysaccharide production. However, as reviewed by (Ha and O’Toole, 2015), some studies have generated results that are in contrast to this general model. For example, eDNA has

been demonstrated to promote the clumps of planktonic and biofilm cells, but has not been correlated with c-di-GMP(Allesen-Holm et al., 2006). The authors responsible for generation of the c-di-GMP reporter strain, in a more recent publication, had screened 5000 compounds against *P. aeruginosa* and tested their ability to reduce c-di-GMP levels. Anti-cancerous drug doxorubicin was found to substantially decrease c-di-GMP, but treatment with doxorubicin had caused biofilm formation with PAO1 (Groizeleau et al., 2016). The authors postulated that the promotion of biofilm could have resulted from the increased in eDNA released from a subpopulation of killed cells. Whether this concept could also be applied to the biofilm formation seen with clinical isolates in this study, in particular PA10, remains questionable; though examination into the variation in biofilm structure and relative biofilm compositions of each isolate may provide some clarity, and may be the basis of future work. To investigate additional clinical isolates would also be of interest, to verify the result seen with PA10; just five of the twenty clinical isolates were transformed and utilised in these assays due to the limiting factor of time and problem encountered with assay development and optimisation.

As the decrease in c-di-GMP with PA10 despite biofilm promotion with NO treatment was somewhat unexpected, further investigations into the differences in response to NO amongst the clinical isolates were carried out. PAO1, historically a wound isolate and therefore a strain not previously exposed to NO and low oxygen, was passaged 10 times with a low concentration of NO in an attempt to discover if any arising mutations would result in a change in the response to NO induced dispersal. Of three independently passaged experiments, two showed no statistically significant difference in the dispersal action of NO, whilst one showed an attenuation in response. Experiments similar to this one involving multiple generations and passaging with NO have not been reported in the literature; however an interesting publication to note is one by (Zhu et al., 2018). In their experiments, pre-treatment of the biofilms for 3 h with 10 μM spermine NONO-ate, followed by treatment with 100 μM spermine NONO-ate resulted in an attenuated dispersal response. They found expression levels of genes related to scavenging proteins were increased, leading to their conclusion of a NO tolerance mechanism via transcriptional changes and NO sequestering. Contrastingly, this study had cultured *P. aeruginosa* cells for 10 days with 1 nM SNP, in an attempt to increase clinical relevance and investigate whether adaptation to NO could be induced at the genomic as opposed to transcriptional level.

Had the results of the passaging experiment been more striking and conclusive, genomic analysis of PAO1 pre- and post- 10 days of incubation with 1nM SNP would have been worthwhile. Though it is plausible to have variation in the end result amongst three independently passaged replicates due to arising spontaneous mutations, multiple factors could be responsible for the inconclusiveness of this experiment. For example, based on previous biofilm assay data, 1 nM SNP was selected as it had no effect on biofilm or planktonic PAO1 cultures. However, it could be that this concentration was therefore too low to cause enough stress to induce genetic changes. It may

also be that the number of passages carried out in this study was insufficient. Whilst studies investigating the development of resistance to antibiotics often notice a change in response after just 10 or fewer passages (Carsenti-Etesse et al. 2001; Ołdak and Trafny 2005; Pollard et al. 2012), the potential adaptation mechanisms to low dose non-toxic NO will be different and may require a higher number of passages. Alternatively, it may be that transcriptomic, as opposed from genomic analysis of the isolates could reveal more information as to the mechanism and cause of the isolate specific response. For example, RNA-sequencing of isolates with and without NO treatment would reveal genes involved in the response to NO and show how this differs between various isolates.

Again, whilst these results were somewhat inconclusive, the lack of a definitive attenuation or abolishment of the NO-induced dispersal response is somewhat encouraging. Had this occurred following only 10 passages, this would have indicated a rapid genetic adaptation to NO and be cause for concern in relation to the development of NO-based anti-biofilm treatments. However, it must be stressed that this assay had a few limitations. Namely, the assay only consisted of 10 passages; whilst this is valid and an acceptable number for an initial passaging experiment, an experiment with 20 or 50 passages should be carried out to provide more conclusive results, and would be a priority for future work. Additionally, only one concentration of SNP was utilised during the sub-culturing process and for the dispersal treatment at the end of the experiment. Additional non-CF isolates aside from PAO1 would also be of interest to examine to verify the results seen are not limited to one strain of *P. aeruginosa.*

3.3.5 Conclusions

In this chapter, the first objective was to investigate the effects of NO on biofilms formed by clinical CF isolates of *P. aeruginosa* and to explore a potential correlation between response to NO and isolate phenotype. Crystal violet analysis of biofilms showed that response to NO varied substantially amongst nine clinical isolates of *P. aeruginosa*. Four of the nine had a response similar to PAO1, in which NO dispersed the biofilm in a dose dependent manner, whilst five of the nine isolates demonstrated lack of dispersal and evidence of biofilm growth promotion. Nine of the twenty clinical isolates available were treated with NO, as these assays were labour intensive and time was the major limiting factor. Interestingly, a correlation was found between the response of the isolate to NO induced dispersal and its biofilm forming capabilities *in vitro,* whereby isolates that were not dispersed had slower biofilm growth rates.

The second objective in this chapter was to investigate the c-di-GMP levels in the clinical *P. aeruginosa* isolates following treatment with NO and investigate the reason for the varied response to NO. Using a reporter plasmid system, the c-di-GMP levels were shown to be reduced following NO treatment in all isolates tested, including an isolate shown to have biofilm formation promoted with NO treatment. This suggests that the opposing response of the clinical isolate biofilms to NO is independent of the c-di-GMP pathway. A passaging experiment to investigate the possibility of

spontaneous mutations leading to a genetic based adaptation to the NO induced dispersal was conducted; some attenuation in the dispersal response was observed however results were not conclusive and further investigation is required. Two theories were proposed to explain the varied response of the clinical isolate biofilms to NO, one suggesting that an adaptation to denitrification and utilisation of NO breakdown products may be responsible for biofilm promotion, and the second suggesting an adaptation to NO in the lung, released from immune cells. Likely, is that there is no one explanation, but both of these theories contribute to the effect seen *in vitro.*

Chapter 4 Evaluation of DEA-CP against biofilms formed by clinical isolates of *P. aeruginosa* from cystic fibrosis patients

4.1 Introduction

Following the 2006 discovery of NO as a *P. aeruginosa* biofilm dispersal agent, various ways to utilise NO in clinical settings to target biofilms have been explored. In addition to the previously mentioned inhaled NO, these include NO donor compounds, stimulation of endogenous NO, NO releasing polymers and nanoparticle delivery systems, and NO hybrid compounds.

NO donors compounds have a long history of clinical use; the most common examples being glyceryl trinitrite (also known as nitroglycerine; GTN) and sodium nitroprusside (SNP) (Williams, 2003). These drugs exploit the vasodilatory actions of NO. GTN is often used to treat angina pectoris, and less commonly in other instances such as for uterine relaxation during obstetrical complications (Pace et al., 2007; Williams, 2003). Similarly, SNP can be used in cases of hypertensive crises, heart failure, and cardiac/vascular surgery (Hottinger et al., 2014). Although NO donors such as GTN and SNP are FDA approved and in clinical use already, their actions are systemic as they spontaneously release NO. Therefore, use of these agents in patients with CF who do not have hypertension or cardiac related pathologies may cause hypotension and other adverse effects.

The stimulation of endogenous NO may be achieved via the use of NIR enzymes, which, in *P. aeruginosa,* stimulate the production of NO from NO_2^-, or via the use of NOS enzymes which use L-arginine as a substrate for NO production (Kannan, Guiang, and Johnson, 1998). In 2006, Borriello et al., showed that arginine or nitrate supplementation to *P. aeruginosa* biofilms resulted in an increased susceptibility to ciprofloxacin and tobramycin under anaerobic (but not aerobic) conditions. Based on research since then we can hypothesise that these results may have been NO-mediated. An initial patient-blinded clinical trial using inhaled L-arginine in CF patients resulted in sustained improvements in lung function and increased NOS activity (Grasemann, Kurtz, and Ratjen, 2006), though a follow up randomised control trial (Grasemann, Tullis, and Ratjen, 2013) showed a minor FEV_1 improvement in the L-arginine treatment group, this was not statistically significant.

Nanoparticle drug delivery has been of interest in recent years, and NO releasing nanoparticles or polymers may be suitable as coatings for various medical devices prone to biofilm formation, such as urinary catheters, or in industrial settings (Duong et al., 2014). Again, these agents could be

formulated to be administered to humans systemically, or topically to treat skin infections, but are not appropriate for targeting lung infections.

Likewise with NO-based dual-action hybrid compounds; fimbrolides have been shown to have activity against QS in *P. aeruginosa* and development of fimbrolides-NO donor hybrid compounds were found to have the dual action of bacterial QS inhibition and biofilm inhibition (Kutty et al., 2013). Unfortunately, since this publication in 2013, no further work has been published on these compounds.

4.1.1 The development of cephalosporin-3'diazeniumdiolates

A promising alternative to inhaled NO and other NO releasing agents are cephalosporin-3'-diazeneiumdiolates. These compounds, developed by our colleagues at the University of Wollongong, Australia, have been designed to selectively release NO only at the site of infection, thereby concentrating NO where it is needed and reducing the risk of adverse effects seen with other agents, as shown in **Figure 19** (Barraud et al., 2012; Yepuri et al., 2013).

These prodrugs consist of a cephalosporin antibiotic that carries a chemically stable O2-linked diazeniumdiolate NO donor (NONOate) at the cephalosporin 3'-position. In the presence of bacterial β-lactamase, cleavage of the β-lactam ring leads to breakage the O-C bond and causes 'ejection' or a 'conjugate elimination reaction' of the unstable diazeniumdiolate anion. This in turn spontaneously releases NO. As cleavage can only occur in the presence of β-lactamase, this enables for a precisely localised and targeted anti-biofilm therapy and reduces systemic exposure to NO. Diazeniumdiolates were selected as the adjoining NO donor as these compounds only release NO after cleavage of the O-C bond.

In the first set of published data, these compounds were shown to be stable in solution and to selectively release NO in response to purified penicillinase, *P. aeruginosa* and *E.coli* cell extracts and culture supernatants using amperometric methodology (Barraud et al., 2012). The release of NO from the compounds in response to addition of *E.coli* suggested that cleavage could, in addition to β-lactamase, be triggered via the action of transpeptidase enzymes, the antibacterial target of all cephalosporins and other β-lactams. This concept is further explored in Chapter 5.

Compound 7, the potassium salt of Compound 3, was termed DEA-CP [DiEthylAmine NONOate-Cephalosporin Prodrug] and identified as a lead compound. DEA-CP has cephaloram (7-phenylacetamidocephalosporanic acid) as its antibiotic base, chosen as it can be synthesised with ease and, as a 1st generation cephalosporin, would be expected to show high reactivity towards β-lactamases. The diazeniumdiolate component of DEA-CP is DiEthylAmine-NONO-ate (DEA-NONOate); an agent with a half-life of 2 minutes at 37 °C at pH 7.4 and spontaneously dissociates

via a first order process to release NO. NO release is pH dependent, and 1.5 moles of NO is released per mole of parent compound (Keefer et al., 1996; Maragos et al., 1991).

Some preliminary microbiological assays were carried out in the original reports. In microtitre plate assays, 5 h old biofilms of PAO1 were allowed to develop, pre-treated with a sub-inhibitory dose of imipenem for 1 h (to trigger β-lactamase expression) and then treated with DEA-CP potassium salt for 15 mins, revealing a dose dependent reduction in biomass when stained with crystal violet. DEA-CP potassium salt (at 100 μM) was then used to treat PAO1 biofilms grown in a continuous-flow cell system; the effluent from the system showed an increase in OD_{600} readings indicating dispersal, however the biofilms themselves were not directly analysed. Finally, the use of microtitre plates was revisited and Compound 7 was combined with tobramycin and ciprofloxacin. CFU counts of the biofilms were reduced a further 1.8 and 1.5 log when DEA-CP potassium salt was combined with tobramycin and ciprofloxacin respectively. For these assays, biofilms were again pre-treated with imipenem for 1 h, and then treated with DEA-CP potassium salt and/or antibiotics for 1 h before analysis.

A follow up publication detailed the synthesis of various analogues of Compound 7 (DEA-CP potassium salt) and compared their different chemical stabilities, NO release and effects on PAO1 biofilms (Yepuri et al., 2013). The analogues have variations at the R1 (acyl-amino side chain) and R2 (O2-alkydianzeniumdiolate) groups. Of note is that for each of the compounds, the free carboxylic acid form could be converted to their water-soluble potassium salt forms by stirring with KOH at 0˚C and then freeze-drying; however, with some analogues this led to decomposition. Analysis of the NO release from all of the free acid forms of the compounds detailed showed variation, again with some displaying decomposition and lower inherent stability in aqueous solution. In addition to evidence showing dispersal action, lead compound DEA-CP was tested on L929 murine fibroblasts and showed no cytotoxicity (Barraud et al., 2015).

The C3D class of compounds thus represent an exciting new anti-biofilm approach that may greatly benefit CF patients. However, the effects of DEA-CP had not been investigated on any other *P. aeruginosa* isolate other than PAO1. In order to explore the clinical relevance of C3Ds, the objective in this chapter was to investigate the effect of DEA-CP on biofilms formed by clinical isolates of *P. aeruginosa* from adolescent and adult CF patients.

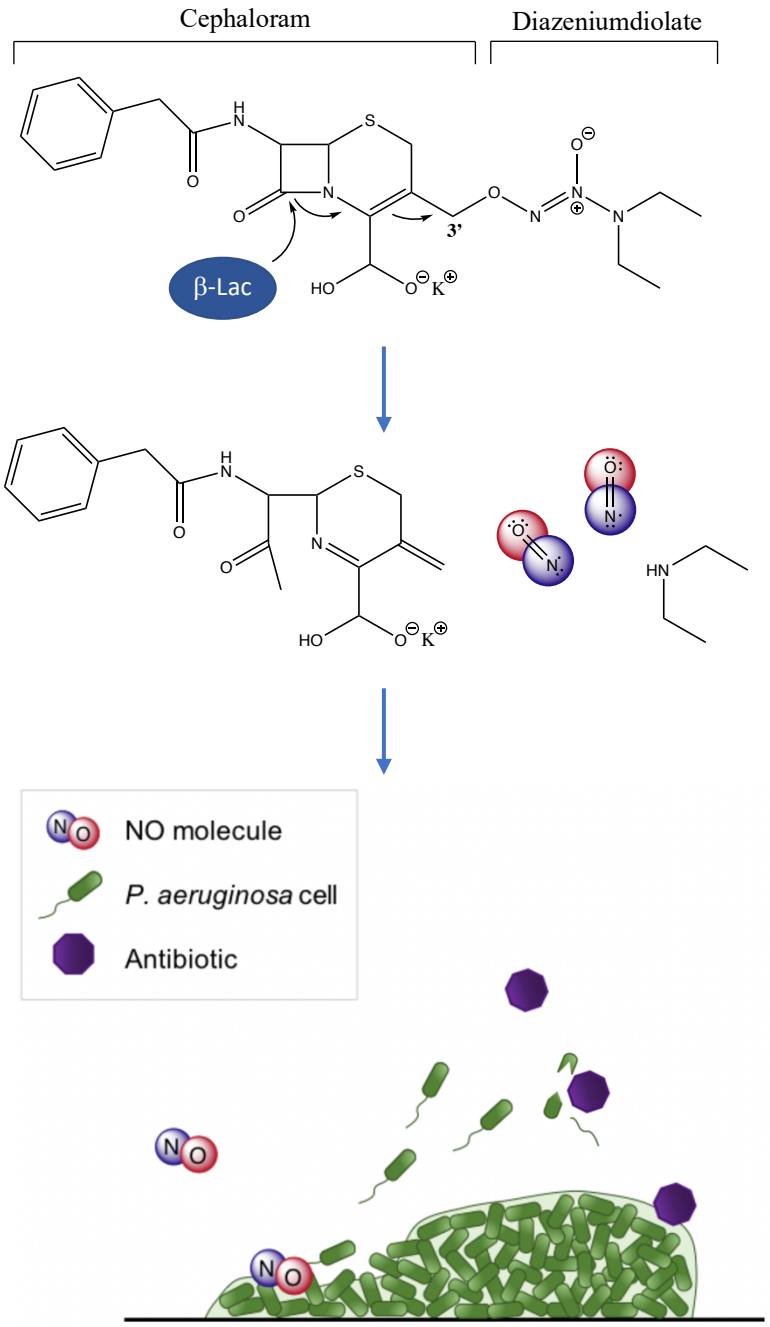


Figure 19. Chemical structure, mechanism of NO release, and proposed anti-biofilm action of DEA-CP (potassium salt). When in contact with bacterial specific enzyme β-lactamase (β-Lac), the β-lactam ring of the cephalosporin (cephaloram) is cleaved, liberating the diazeniumdiolate and releasing NO at the site of infection. The dispersal action of NO then enables the release of *P. aeruginosa* cells from the biofilm to facilitate their killing by co-administered conventional antibiotics.

4.2 Results

4.2.1 DEA-CP compounds selectively release NO in response to penicillinase

A highly sensitive CLD method was used to investigate and confirm the selectively of the novel compounds with regards to NO release. In addition to a quantitative output, this method generates a profile of NO release over time and the speed at which NO production begins. A shown in **Figure 20**, both DEA-CP potassium salt and free carboxylic free acid forms initiated the release of NO less than 1 minute following the addition of penicillinase. The release profile for both compounds shows a similar trend, with the peak NO release occurring within 6 minutes following penicillinase addition and a gradual decrease in production until the end of the experiment.

Noticeably, there are differences in the amount of NO production between the two compounds as the NO release from DEA-CP potassium salt peaks at over 100 ppb of NO whilst DEA-CP carboxylic acids NO production peaks at around 50 ppb. A small peak is shown following the addition of DEA-CP carboxylic acid to the reaction vessel before penicillinase is added. However, this peak cannot be conclusively attributed to the non-selective NO release from DEA-CP carboxylic acid. The peak could be contributed to traces of penicillinase left in the reaction vessel from the previous experimental run or by the sudden pressure change caused by the injection of the solution into the reaction vessel which was done manually.

The total amount of NO released from each compound (in nmoles as opposed to ppb) were calculated by comparison to peak areas of NO generated from multiple known concentrations of sodium nitrite injected into a reducing reaction solution. Per 10nmoles, DEA-CP carboxylic acid produced an average of 3.92 nmoles of NO, whilst DEA-CP potassium salt produced almost twice the amount, at 6.82 nmoles. Per mole of parent compound, 1.5 moles of NO production is to be expected from DEA-NONOate, leading to a yield of 26.1% and 45.5%, respectively, for DEA-CP carboxylic free acid and DEA-CP potassium salt during the first 30 minutes of NO release.

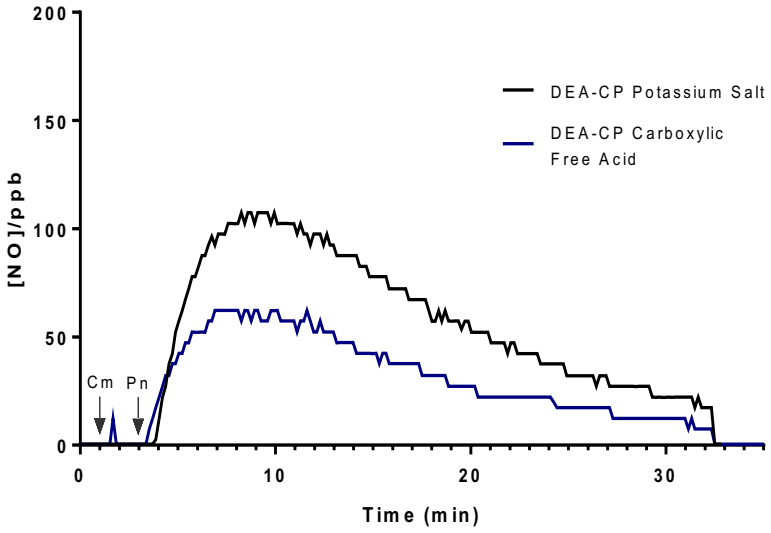


Figure 20. The selective release of NO from DEA-CP potassium salt and carboxylic free acid compounds. The release of NO was measurement using a CLD. Arrows signify the time at which the compound (Cm) and 20 units of penicillinase (Pn) were added. The readings were manually terminated 30 minutes post addition of the penicillinase.

4.2.2 β-lactamase production is highly varied between PAO1 and clinical CF isolates

Based on the requirement for DEA-CP to be cleaved by bacterial β-lactamase to release NO, the β-lactamase activity of PAO1 and the twenty clinical isolates in our panel were evaluated (**Figure 21**). Compared to PAO1, eight isolates showed a mean β-lactamase activity three or more fold higher (PA08, PA10, PA20, PA21, PA26, PA37, PA58, and PA66), with four of these being statistically significantly higher than PAO1. Ten isolates, including PA30 and PA68, had activity lower than PAO1, though for all of these isolates this difference was non-significant.

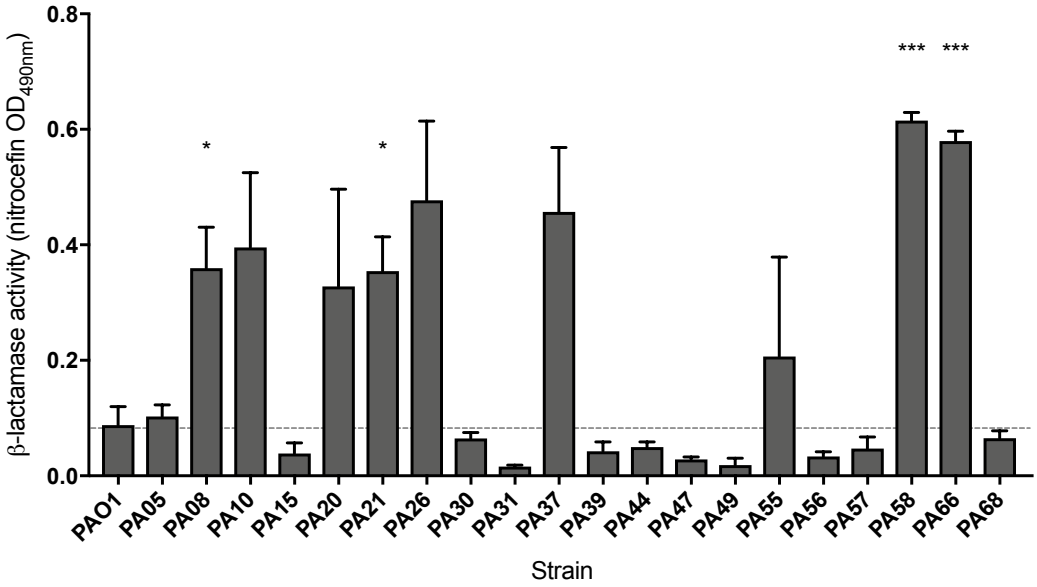


Figure 21. β-lactamase activity of PAO1 and clinical CF isolates of *P. aeruginosa*. OD values relate to the conversion of nitrocefin in 1 hour by bacterial β-lactamase and are in direct correlation with β-lactamase activity. The mean and SD of three biological replicates is shown. One way ANOVA testing with Dunnett's multiple comparisons test was used for statistical analysis.

4.2.3 Neither DEA-CP nor its antibiotic base cephaloram have bactericidal activity against *P. aeruginosa*

The core antibiotic of DEA-CP, cephaloram, is a first-generation cephalosporin and is not known to have activity against *P. aeruginosa*. However, previous evidence has shown that higher concentrations of NO are directly bactericidal to *P. aeruginosa*, so the bactericidal effects of both DEA-CP potassium salt and DEA-CP carboxylic free acid were investigated against PAO1 and three clinical isolates, as shown in **Table 6**. MIC values were above >128 μM for both DEA-CP potassium salt and DEA-CP carboxylic acid for all four *P. aeruginosa* strains tested. Higher concentrations were also tested but growth was present at concentrations as high as 4mM (data not shown), and hence there was no measurable MIC. For all four strains tested, neither DEA-CP potassium salt nor DEA-CP carboxylic free acid showed any bactericidal activity against the planktonic cultures.

Table 6. MIC values for DEA-CP potassium salt and DEA-CP carboxylic acid

	MIC (μM)	
Strain	**DEA-CP potassium salt**	**DEA-CP carboxylic acid**
PAO1	>128	>128
PA21	>128	>128
PA30	>128	>128
PA68	>128	>128

4.2.4 DEA-CP disperses biofilms formed by clinical CF isolates of *P. aeruginosa* as well as PAO1

Based on previous results, DEA-CP potassium salt was chosen for microbiologically evaluation. To explore the effect of DEA-CP potassium salt on multiple strains on *P. aeruginosa*, a crystal violet staining method was utilised to quantify differences in total biofilm biomass. **Figure 22A**, shows the response of PAO1 to DEA-CP concentrations ranging from 32 to 256 μM, with increasing dispersal action observed with increasing concentration. At the highest concentrations, DEA-CP was able to cause greater than 50% reduction in mean biofilm biomass, compared to the untreated control. This dispersal effect was replicated in the clinical CF isolates of *P. aeruginosa* (**Figure 22B-D**). Interestingly, there were differences with regards to the extent of dispersal on a strain to strain basis; PA21 and PA30 biofilms dispersed to a greater degree than the PA68 biofilms, with biofilm biomass being reduced by just under 50% at 256 μM of DEA-CP. For PA30 and PA68, 64 μM rather than 32 μM was required to initiate biofilm dispersal. In contrast, 4 μM was the lowest concentrations required to initiate biofilm dispersal for both PAO1 and PA21 (data not shown). Planktonic absorbance values were also acquired to confirm dispersal had occurred as opposed to bacterial cell killing. All planktonic measurements were either increased or remained the same with DEA-CP treatment compared to the control with all four strains, shown in **Appendix E**.

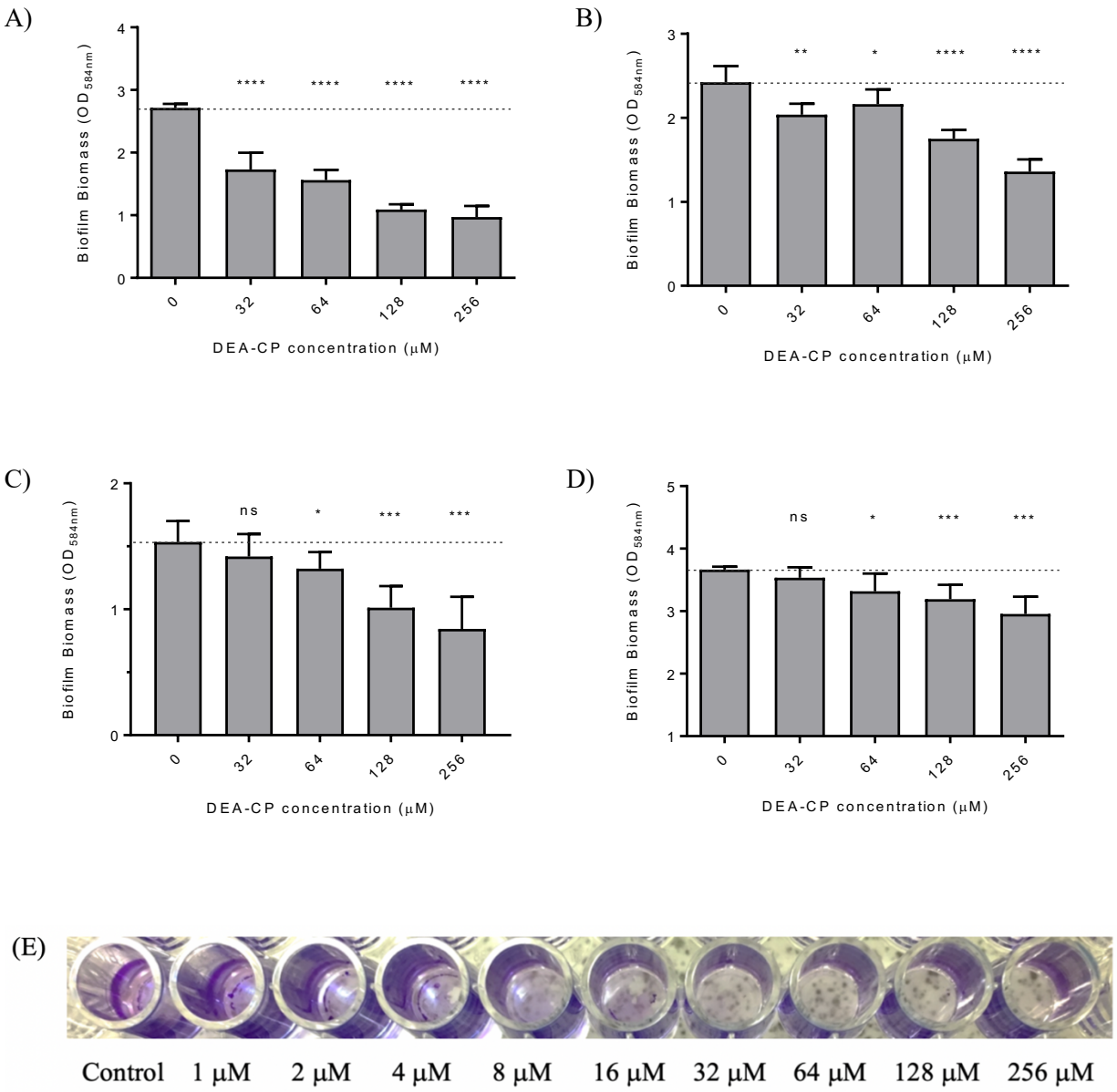


Figure 22. The effects of DEA-CP on *P. aeruginosa* biofilms formed by (A) PAO1, (B) PA21, (C) PA30, and (D) PA68, grown in 96 well plates. Biofilms were grown for 24 h, and then treated for 20 h with DEA-CP potassium salt before staining with crystal violet and resolubilised in acetic acid for quantification. Graphs show the mean and SD of six technical replicates. Ordinary one-way ANOVA was used for statistical analysis and each group was compared to the untreated control; ns = not significant. Experiments were carried out in duplicate for each strain with similar results (detailed in **Appendix E**). (E) Example photograph showing the effect of increasing DEA-CP concentrations on PAO1 biofilms, stained with CV.

4.2.5 DEA-CP is effective in combination with tobramycin against biofilms formed by a clinical *P. aeruginosa* isolate

CLSM with LIVE/DEAD staining was used to investigate the effects of DEA-CP in combination with common CF antimicrobial agent tobramycin. This method allowed for not only 3D visualisation of the biofilm and the localisation of live and dead cells, but analysis of parameters including total biofilm biomass, distribution and maximum biofilm thicknesses, and surface area coverage. Clinical isolate PA68 was chosen as this isolate was one of the best biofilm formers (as demonstrated in Chapter 3). As shown in **Figure 23**, the untreated control biofilm of *P. aeruginosa* clinical isolate PA68 displays substantial biomass comprised almost entirely of live cells. Biofilms treated with 4 μg/ml of tobramycin (4x MIC value) showed little change; reduction in biomass was negligible by visual analysis and the majority of cells were still viable. Treatment with DEA-CP potassium salt however produced a visually recognisable reduction in biofilm biomass. The combination treatment of DEA-CP and tobramycin appears to be the most effective treatment with very few cells left attached to the substratum, as seen in **Figure 23D** and **23H**.

COMSTAT analysis of the CLSM data corroborated the conclusions made by visual analysis. As shown in **Figure 24,** a statistically significant difference in the total biofilm biomass was seen with biofilms treated with DEA-CP compared to the untreated control. DEA-CP treatment alone was able to cause a 50% reduction in the mean biomass value, from 0.902 $\mu m^2/\mu m^3$ to 0.449 $\mu m^2/\mu m^3$. Conversely, treatment with tobramycin did not significantly reduce the total biomass of the biofilm compared to the untreated control and is thus ineffective. Comparison between tobramycin and the tobramycin-DEA-CP combination showed a statistically significant reduction in biomass with the combination treatment, with mean biomass values of 0.737 $\mu m^2/\mu m^3$ and 0.315 $\mu m^2/\mu m^3$. Although there is a difference between mean values, the total biofilm biomass was not statistically significant different between DEA-CP monotherapy and the tobramycin-DEA-CP combination.

In addition to total biofilm biomass, other biofilm parameters were also assessed, including thickness distribution, maximum thickness and total surface area coverage; mean values are shown in **Table 7**. DEA-CP was again found to be effective against the biofilm as a monotherapy, and the DEA-CP-tobramycin combination was more effective than tobramycin monotherapy. For all three parameters, there was a statistically significant difference between the control biofilms and DEA-CP treatment; tobramycin and the combination treatment; and DEA-CP and the combination treatment, indicative of a synergistic effect between the two agents. Biofilm thickness distribution, maximum thickness and surface area coverage were all approximately halved with the tobramycin-DEA-CP therapy compared to tobramycin monotherapy. Scatter plots of the data in **Table 7** are shown in **Appendix F**.

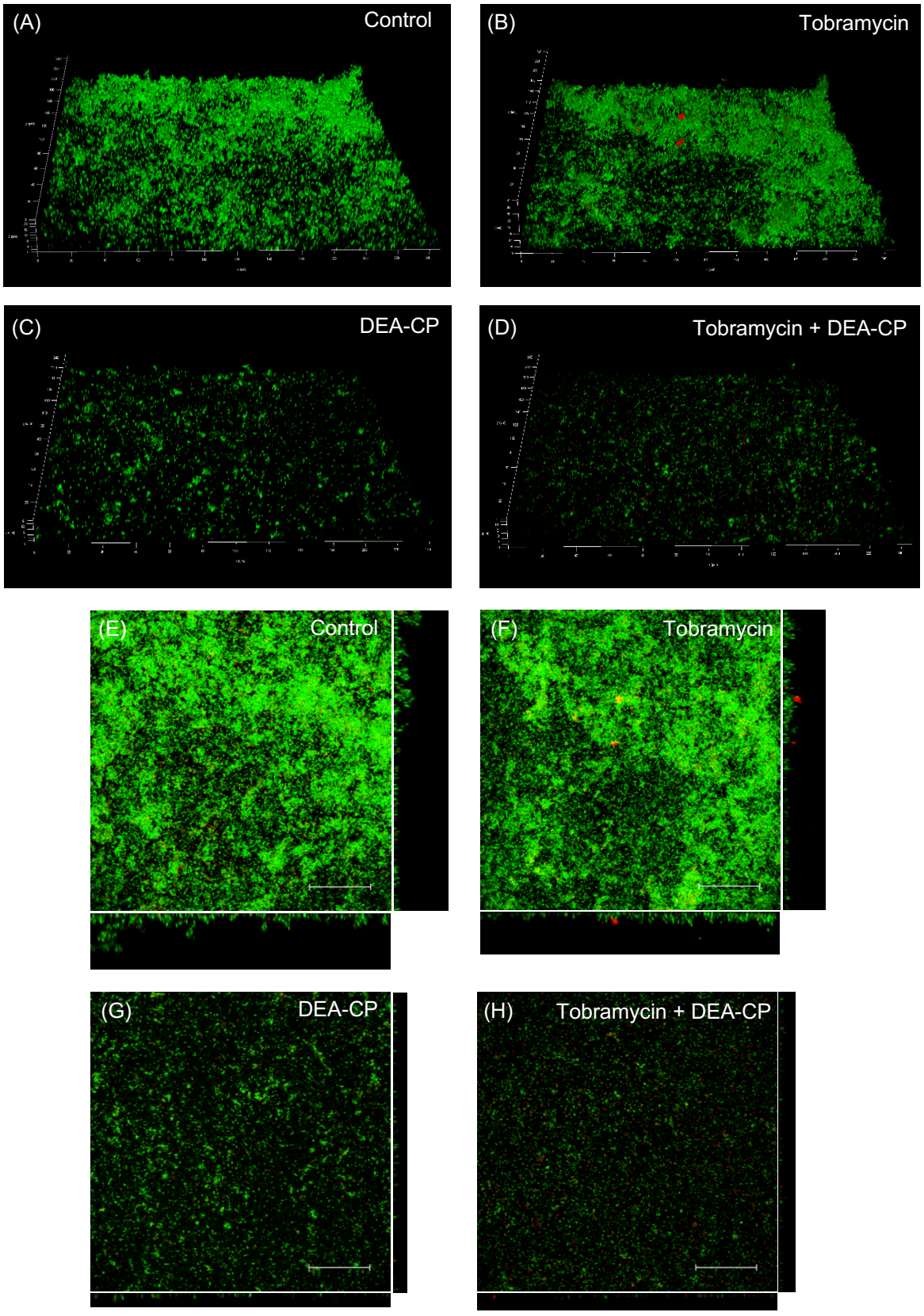


Figure 23. CLSM of 24 h old PA68 biofilms treated with 4 µg/ml tobramycin and 256 µM DEA-CP potassium salt for 20 h. Green and red staining represents 'live' and 'dead' cells respectively. (A) to (D) 3D images; x and y axes measure 246 µm by 246 µm, and (E) to (H) maximum projection images of biofilms with orthogonal X and Y sections; scale bar 50 µm. Representative images shown.

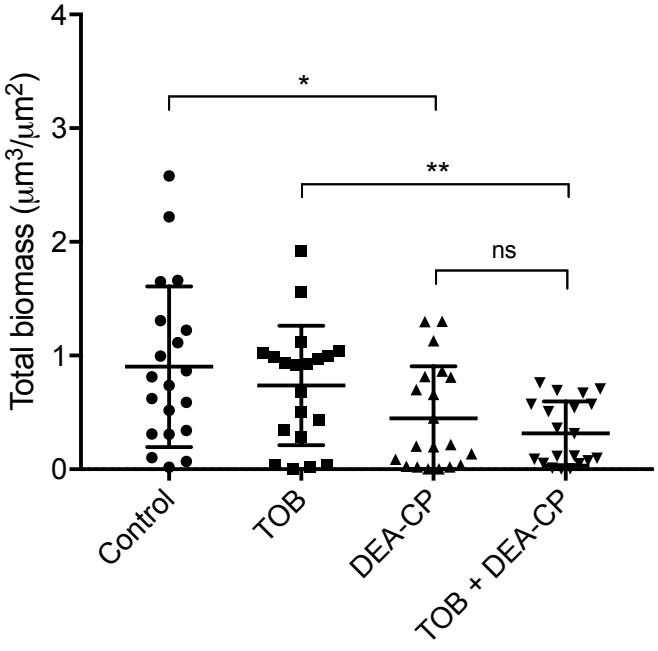


Figure 24. COMSTAT analysis of PA68 biofilms treated with tobramycin and DEA-CP, and visualised with CLSM. Values represent total biofilm biomass of biofilms, calculated by the addition of both live and dead biomass values. Scatter plot show all points, with the mean and SD. Welch's t-tests were used for statistical analysis.

Table 7. COMSTAT analysis of PA68 biofilms treated with DEA-CP and tobramycin assessing biofilm thickness and surface area

	Mean Values (±SEM)			
	Control	**TOB**	**DEA-CP**	**TOB + DEA-CP**
Thickness Distribution (μm)	2.6 (±0.4)	1.7 (±0.3)	1.4 (±0.2)	0.94 (±0.2)
Maximum Thickness (μm)	22.7 (±2.7)	19.7 (±2.3)	16.4 (±2.5)	11.2 (±1.2)
Surface Area Coverage (x1000, μm^2)	185 (±26)	160 (±22)	101 (±20)	83 (±14)

4.2.6 DEA-CP enhances the efficacy of colistin against biofilms formed by clinical *P. aeruginosa* isolates resulting in almost complete eradication of the biofilm

CLSM was used to investigate the effects of DEA-CP in combination with a second anti-pseudomonal antibiotic, colistin, against *P. aeruginosa* PA68 biofilms, as shown in **Figure 25**. Again, the control biofilms showed a large volume of biomass with a vast majority of live cells. Biofilms treated with 16 μg/ml of colistin displayed a substantial amount of dead or dying cells. However, large microcolonies comprised mostly of live cells were still present and are attached to the substratum such that a biofilm structure is still visible. A concentration of 16 μg/ml of colistin was selected as this figure was 8x the MIC value, and preliminary assays showed that this concentration was not effective in clearing the biofilm.

As previously shown, DEA-CP potassium salt has caused the dispersal of the biofilm, whilst not affecting cell viability. The combination of colistin and DEA-CP however showed a strong synergistic effect against the biofilm; visual analysis showed very few viable cells remaining and almost all of the biofilm has been removed from the substratum.

Quantification of total biofilm biomass (shown in **Figure 26**), and biofilm thickness distribution, maximum thickness and surface area coverage (shown in **Table 8**) supported the findings from the visual analysis. Again, DEA-CP treatment significantly decreased the total biofilm biomass, causing ~50% reduction in the mean value. Colistin monotherapy was effective in causing a significant reduction in biomass, however colistin-DEA-CP combination treatment was significantly better than both monotherapy treatments. The biomass for the colistin treatment group had a mean value of 0.186 $\mu m^2/\mu m^3$, but the combination treatment was able to reduce the mean biomass value to 0.039 $\mu m^2/\mu m^3$. The colistin-DEA-CP combination treatment also reduced the thickness distribution mean value to near zero. The mean maximum thickness of the control biofilm, 31.8 μm, was reduced to 13.1 μm by colistin monotherapy, and to just under 7 μm with the combination treatment. The colistin-DEA-CP combination was also superior to colistin with regards to reducing the biofilm surface area coverage; as compared to the control biofilm, the mean total surface area coverage was reduced by 87.6% by colistin and by 96.5% with the combination treatment. Scatter plots of the data in **Table 8** are shown in **Appendix F**.

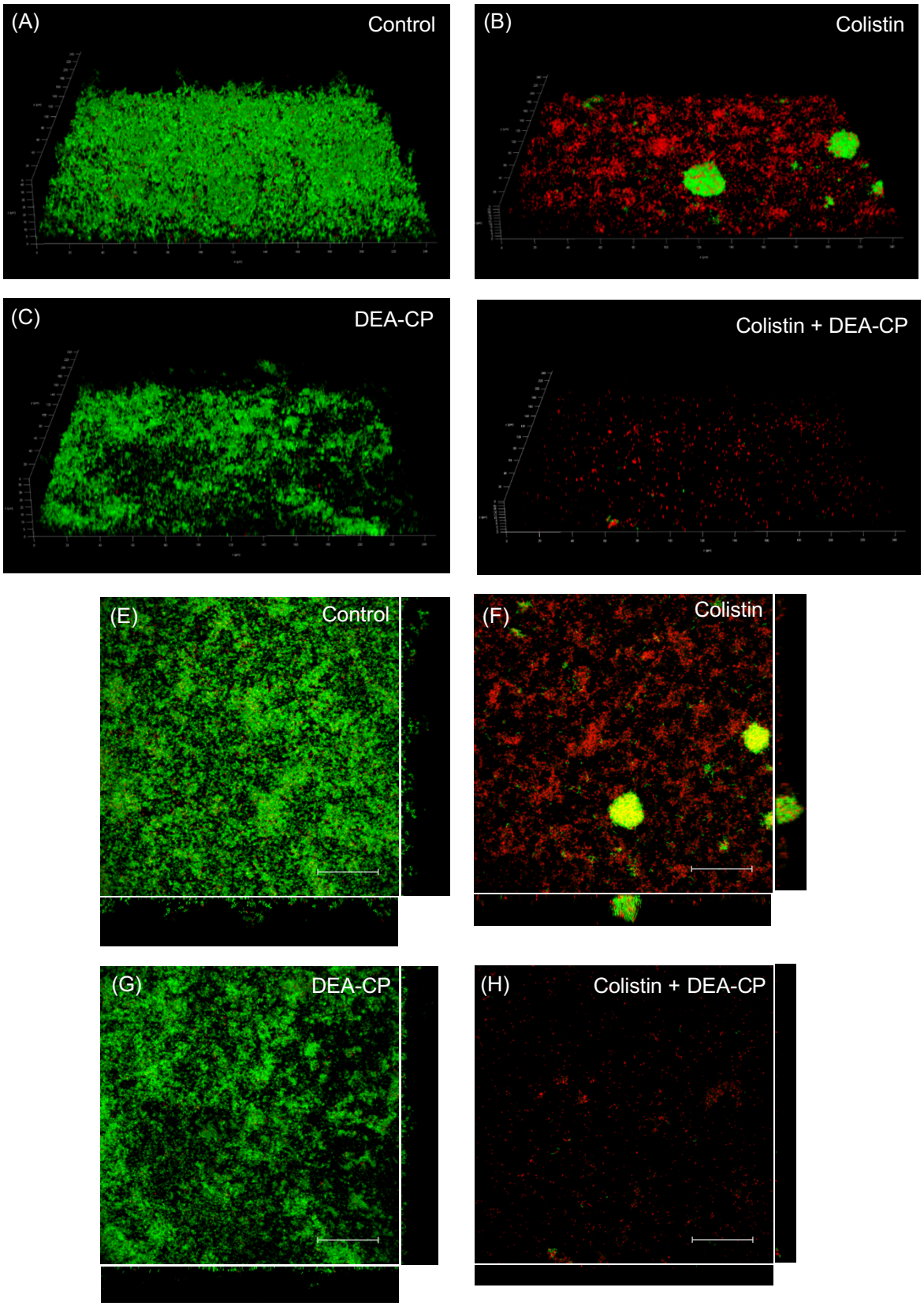


Figure 25. CLSM of 24 h old PA68 biofilms treated with 16 µg/ml colistin and 256 µM DEA-CP potassium salt for 20 h. Green and red staining represents 'live' and 'dead' cells respectively. (A) to (D) 3D images; x and y axes measure 246 µm by 246 µm, and (E) to (H) maximum projection images of biofilms with orthogonal X and Y sections; scale bar 50 µm. Representative images shown.

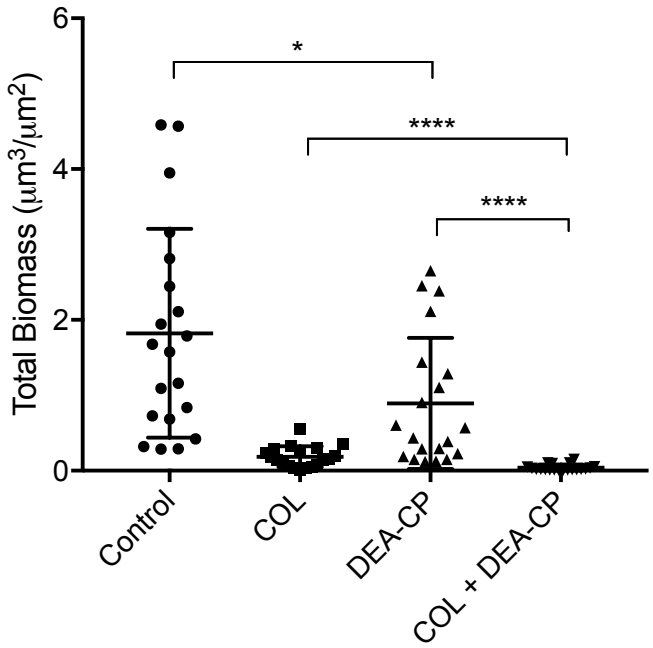


Figure 26. COMSTAT analysis of PA68 biofilms treated with 16 µg/ml colistin and 256 µM DEA-CP potassium salt, assessing total biofilm biomass, calculated by the addition of both live and dead biomass values. Scatter plot show all points, with the mean and SD. Welch's t-tests were used for statistical analysis.

Table 8. COMSTAT analysis of PA68 biofilms treated with DEA-CP and colistin assessing biofilm thickness and surface area

	Mean Values (±SEM)			
	Control	**COL**	**DEA-CP**	**COL + DEA-CP**
Thickness Distribution (µm)	3.1 (±0.6)	0.2 (±0.04)	1.1 (±0.3)	0.02 (±0.007)
Maximum Thickness (µm)	31.8 (±2.5)	13.1 (±1.2)	21.9 (±1.9)	6.7 (±0.7)
Surface Area Coverage (x1000, μm^2)	342 (±52)	42 (±6)	176 (±33)	12 (±3)

4.3 Discussion

4.3.1 The release of NO from DEA-CP

Ahead of microbiological analysis, it was necessary to confirm the release of NO from the DEA-CP sample provided to us by the University of Wollongong. A highly sensitive method using a CLD was utilised and verified the selective release of NO from DEA-CP following exposure of the compound to bacterial penicillinase. The results with the CLD corroborate and extend those published by (Barraud et al., 2012) and data provided to us by the chemists responsible for DEA-CP synthesis, in which an amperometric sensor was employed to detect the release of NO in solution. However, importantly, the amperometric based experiments were carried out on previous batches of DEA-CP. Therefore, experiments using the CLD were essential to verify the NO release of each batch of compound synthesised and sent to Southampton, and to ensure transportation process had not caused any alteration to the compounds.

Surprisingly, the CLD data also revealed different profiles of NO release from the potassium salt of DEA-CP and the carboxylic free acid. Following the addition of penicillinase, the potassium salt of DEA-CP released almost double the amount of NO compared to the free acid form of DEA-CP. The reason for this is still unclear, however may relate to an increased compound stability of the potassium salt. It is recognised that salt formulations of drug are used to increase aqueous stability, and address issues with chemical stability toxicity and absorption (Gupta et al., 2018). In particular, cephalosporins are more unstable in aqueous solution than other β-lactams and are commonly used in potassium or sodium salt forms (Deshpande, Baheti, and Chatterjee, 2004). In particular, cephalosporins are more unstable in aqueous solution than other β-lactams and are commonly used in potassium or sodium salt forms (Deshpande et al., 2004). Based on this data, DEA-CP potassium salt was chosen for use in subsequent assays.

4.3.1.1 Antibacterial activity

Previous research has demonstrated the bactericidal properties of NO (Mcmullin et al., 2005). The antibiotic base of DEA-CP, cephaloram, is a very old first-generation cephalosporin antibiotic that is now rarely used in clinic and known not to show antipseudomonal activity. However, the potential for an increased bactericidal effect with addition of a NO donor to cephaloram was worth investigating. The MIC for DEA-CP and its antibiotic base cephaloram against PAO1 and three clinical CF isolates were compared. For all four strains, DEA-CP was unable to reduce to MIC and in fact was higher than the highest tested concentration, as such no specific MIC value was able to be obatained.

4.3.1.2 DEA-CP against clinical *P. aeruginosa* biofilms

DEA-CP was shown to be successful in dispersing biofilms formed by *P. aeruginosa* strain PAO1 (Barraud et al., 2012; Yepuri et al., 2013). However, in those earlier studies the microbiological assays applied were essentially preliminary, in as much as no other *P. aeruginosa* strains were considered and PAO1 biofilms were grown for only 6 h. During the early stages of biofilm formation, planktonic cells attach to a substratum, first reversibly, then irreversibly. Cells then begin to produce the self-encased extracellular matrix, and, in the later stages of biofilm maturity, microcolony structures (Kaplan, 2010). Therefore, a 6 h incubation period is likely to represent the very early stages of biofilm formation. In our study, biofilms were cultured for a total of 44 h, representing more mature biofilms. Mature biofilms are more difficult to eradicate than nascent biofilms, and therefore may be a more accurate representation of *P. aeruginosa* infection in the CF lung (Hengzhuang et al., 2011). A flow cell experiment was also conducted in the previous studies, however dispersal with DEA-CP treatment was assessed by measuring the turbidity of the planktonic effluent as opposed to directly assessing the PAO1 biofilm.

Reassuringly, our experiments yielded similar results to those shown by Barraud et al. and DEA-CP was successful in dispersing PAO1 biofilms in a dose dependent manner and reducing the biofilm biomass by more than half. Excitingly, three CF isolates (PA21, PA30 and PA68) of *P. aeruginosa* were examined using the same experimental set up as PAO1, and were also successfully dispersed by DEA-CP. Again, this was in a dose dependent manner, but interestingly, the percentage reduction in biofilm biomass following DEA-CP treatment was varied and slightly lower with the three CF isolates compared to PAO1 biofilms treated with DEA-CP.

A potential explanation for differing rates of dispersal could have been due to different levels of β-lactamase activity amongst the isolates causing differences in NO release. Chromosomally encoded for β-lactamase AmpC is expressed at a low basal levels in environmental strains, and stable derepression leading to a higher expression of AmpC commonly occurs with CF isolates (Giwercman et al., 1990; Høiby et al., 2010). However, PA21 showed substantially higher β-lactamase activity than PAO1, and the activity for PA30 and PA68 was similar and only slightly reduced in comparison to PAO1. Another potential explanation invokes the possibility that NO dispersal responses may vary from strain to strain, or be linked to biofilm maturity, which was explored in Chapter 3.

Only three of the twenty available clinical isolates could be utilised in examining the dispersal effect of DEA-CP. A key limitation in this chapter was the finite amount of DEA-CP, as it is not commercially available and specially synthesised for this study. For this reason, experiments were carefully designed to maximise the number of isolates that could be examined, and two biological replicates instead of three were carried out for the dispersal assays for this reason.

4.3.1.3 DEA-CP in combination with CF antibiotics

Following evidence that DEA-CP disperses the biofilms of PAO1 and three CF isolates, the effect of DEA-CP in combination with anti-pseudomonal antibiotics were investigated on a CF isolate known to form good biofilms *in vitro*, using CLSM. The most commonly prescribed aminoglycoside antibiotic for CF patients is tobramycin, which targets the ribosomal subunits of Gram-negative pathogens, inhibiting protein synthesis (Young et al., 2013d). Unfortunately, like all other antibiotics, tobramycin is effective only against actively dividing cells, and is less effective against cells within a biofilm. Our study also showed that DEA-CP in combination with tobramycin, was a substantially more effective *in vitro* anti-biofilm treatment than tobramycin alone. Tobramycin itself showed no statistically significant effect on the *P. aeruginosa* biofilm, but the combination with DEA-CP reduced biofilm biomass by more than half. However, although the mean biofilm biomass with the combination treatment was lower than with DEA-CP alone, the difference was not statistically significant. This indicates that the combination lacked 'syngery' and that the addition of tobramycin to DEA-CP did not increase the biofilm clearance further.

DEA-CP was also examined in combination with colistin. Colistin, or polymyxin E, is commonly referred to as a 'last resort' antibiotic, but is frequently prescribed to CF patients with *P. aeruginosa* infection (Beringer, 2001) and administered by inhalation. Co-treatment with DEA-CP and colistin showed a striking anti-biofilm effect, leaving a barely detectable number of live bacterial cells. Clearance of the biofilm was drastically increased (by ~4.5-fold and ~22-fold respectively) with the colistin-DEA-CP combination treatment compared to either colistin or DEA-CP alone. These differences were statistically significant and indicative of a 'synergistic' effect.

The synergy observed with colistin and the lack of synergy observed with tobramycin indicates varying mechanisms of actions are in play. Colistin, a cationic polypeptide, targets the integrity of the Gram-negative membrane, first causing perturbation of the outer membrane and then resulting in leakage of cell contents by disrupting the cytoplasmic membrane (Beringer, 2001). Multiple studies have shown colistin and other membrane-perturbating peptides to synergise well with other antimicrobials against planktonic Gram-negative organisms, by facilitating their penetration through the membrane (Gunderson et al., 2003; Soren et al., 2015; Vidaillac, Benichou, and Duval, 2012). DEA-CP, which incorporates the core structure of first-generation cephalosporin antibiotic cephaloram, would not be expected to cross the Gram-negative outer membrane. In the case of the DEA-CP-tobramycin combination, tobramycin does not affect the cell membrane, so it can be assumed that the cleavage of DEA-CP occurs solely via the actions of extracellular β-lactamase. However, in the case of the DEA-CP-colistin combination, the membrane perturbation caused by colistin may facilitate DEA-CP in crossing the outer membrane, and therefore in addition to extracellular β-lactamase, DEA-CP could also be cleaved by periplasmic β-lactamase and transpeptidase enzymes. In addition to an increased rate of cleavage and NO release, cleavage of

DEA-CP periplasmically would release NO closer to the site of action within the cell, resulting in a more potent effect in inducing biofilm dispersal and accounting for the observed 'synergy'.

In addition to tobramycin and colistin, many other antibiotics used in CF would also have been interesting to have investigated in combination with DEA-CP. However, a number of limitations were present that prevented more than two antibiotic combinations from being examined. As previously mentioned, as DEA-CP is not commercially available, only a limited amount of compound was available, and experiments had to be carefully selected and designed. Therefore, tobramycin and colistin were prioritised and selected as the two agents most likely to show beneficial effects in combination with DEA-CP. For the CLSM assays, experiments consisted of two technical replicates and two biological replicates, again in an attempt to maximise the use of a limited amount of DEA-CP, and also due to the labour intensiveness and profound expense of CLSM use. Had these limitations not existed, the number of technical and biological replicates would have been increased to three, and antibiotics including ceftazidime and azithromycin would been examined in combination with DEA-CP.

4.3.2 Conclusions

In this chapter, the objective was to investigate the effects of DEA-CP on clinical CF isolates of *P. aeruginosa.* Following biochemical analysis to confirm the selective release of NO from DEA-CP, biofilm assays demonstrated that DEA-CP was successful in dispersing the biofilms formed by PAO1 and three clinical CF isolates of *P. aeruginosa*. Biofilm dispersal with DEA-CP was also demonstrated using CLSM, and anti-biofilm effects were noted when DEA-CP was combined with anti-pseudomonal antibiotics tobramycin and colistin. The DEA-CP-colistin combination treatment was shown to be particularly effective, resulting in almost complete eradication of the *in vitro* biofilms, which we hypothesise is due to the membrane-perturbating effect of colistin improving the rate and proximity of NO release in relation to bacterial cells.

Chapter 5 Comparison of twelve next-generation C3Ds against *P. aeruginosa* biofilms: Searching for an 'all-in-one' compound

5.1 Introduction

Following on from the success with prototype compound DEA-CP, next generation C3Ds were subsequently developed. The compound, PYRRO-C3D, consisting of a cephaloram base with a faster-acting diazeniumdiolate (PYRRO/NO, t ½ = 3 sec (Saavedra et al., 1997)) has previously been shown to have beneficial direct and adjunctive effects against biofilms formed by *Haemophilus influenzae* and *Streptococcus pneumoniae.* Against non-typeable *H. influenzae,* treatment of planktonic cells with PYRRO-C3D showed a direct antibacterial effect, and treatment of biofilms with PYRRO-C3D enhanced the efficacy of azithromycin (Collins et al., 2017). Interestingly, against *S. pneumoniae* biofilms, an organism that lacks β-lactamases, NO was still shown to be released from PYRRO-C3D, whilst simultaneously exerting anti-bacterial activity via its antibiotic, and not NO, component (Allan et al., 2017).

PYRRO/NO has a half-life of just 2 seconds, compared to DEA-NONOates 2 minutes (Saavedra et al., 1997). In theory, a faster release of NO would be more beneficial, preventing diffusion of the expelled diazeniumdiolate away from the biofilm before NO is released. Hence, a shorter diazeniumdiolate half-life suggests increased effectiveness due to a more concentrated NO release at the site of the biofilm and less systemic exposure.

In this chapter, twelve new C3Ds were investigated. The first six C3Ds generated contain PYRRO/NO, with a 1st, 2nd, 3rd or 4th generation cephalosporin base: PYRRO-cephaloram (1st gen; also known as PYRRO-C3D), PYRRO-cefalexin (1st gen), PYRRO-cefuroxime (2nd gen), PYRRO-ceftazidime (3rd gen), PYRRO-cefepime (4th gen), and PYRRO-cefozopran (4th gen). The intention of varying the cephalosporin base was to investigate the possibility of developing an "all-in-one" compound, able to disperse *P. aeruginosa* biofilms via the NO release and also have bactericidal action to kill planktonic cells.

Initially, it was thought that cleavage of the C3Ds was solely mediated by β-lactamase. However, it is now apparent that penicillin binding proteins (PBPs) can also trigger opening of the β-lactam ring and release NO. This is evident from the study by Allan et al. showing PYRRO-C3D to release NO when in contact with *S. pneumoniae*, an organism that does not produce β-lactamases. Therefore, as ceftazidime, cefepime and cefozopran have anti-pseudomonal activity, we can

theorise that their incorporation into C3D compounds could contribute to the desired dual antibacterial/anti-biofilm action against *P. aeruginosa.*

The Gram-negative cell membrane is a notorious bacterial defence mechanism, restricting the entry of numerous antimicrobials. However, research has shown that the chemical structure greatly influences molecule penetration and that positively charged, non-sterically hindered primary amino groups in a molecule can increase Gram-negative membrane penetration (Richter and Hergenrother, 2018). Therefore, in addition to the pyrrolidine-based donor PYRRO/NO, piperidine-based diazeniumdiolates (AMINOPIP/NO) were used to generate a further six C3Ds with primary amino groups, and in theory, would result in improved penetration of C3Ds through the *P. aeruginosa* membrane. Two variants with different alkyl chain lengths were chosen for study, to generate: AMINOPIP1-cephaloram, AMINOPIP2-cephaloram, AMINOPIP1-ceftazidime, AMINOPIP2-ceftazidime, AMINOPIP1-cefepime, and AMINOPIP2-cefepime.

In this chapter, the objective was to compare the twelve new C3D compounds for antibacterial and anti-biofilm effects and identify a lead compound for further development as a new antipseudomonal anti-biofilm agent.

5.2 Results

5.2.1 The release of NO from C3Ds shows high variation dependent on chemical structure and the antibiotic base

The release of NO from each of the C3D compounds was analysed using a CLD. All compounds were confirmed to release NO upon treatment with penicillinase, but each compound released a different relative amount of NO, as shown in **Figure 27**. For PYRRO-cefalexin, the peak NO concentration was 200 ppb, which tailed off gradually. Similar trends are noted with PYRRO-cephaloram and PYRRO-cefuroxime. The two additional compounds with cephaloram as the parent antibiotic, AMINOPIP1-cephaloram and AMINOPIP2-cephaloram, show an increased release of NO compared to PYRRO-cephaloram (**Figure 27B**). However, for the three compounds with ceftazidime as the parent antibiotic (**Figure 27D**), PYRRO-ceftazidime and AMINOPIP1-ceftazidime showed a similar NO release profile; whilst AMINOPIP2-ceftazidime had a higher peak of NO release. These three compounds show a more sustained and stable release of NO compared to the other compounds which have a higher initial peak and then tail off within minutes. PYRRO-cefozopran showed the highest initial peak of NO release of these compounds, at just over 600 ppb (**Figure 27E**). For the three compounds with cefepime as the parent antibiotic (**Figure 27F**), a similar trend was noted compared to the three compounds with cephaloram as the parent antibiotic. PYRRO-cefepime showed a relatively low amount of NO release peaking at below 100 ppb, whilst AMINOPIP1-cefepime and AMINOPIP2-cefepime reached peaks of approximately 300 ppb and 500 ppb respectively.

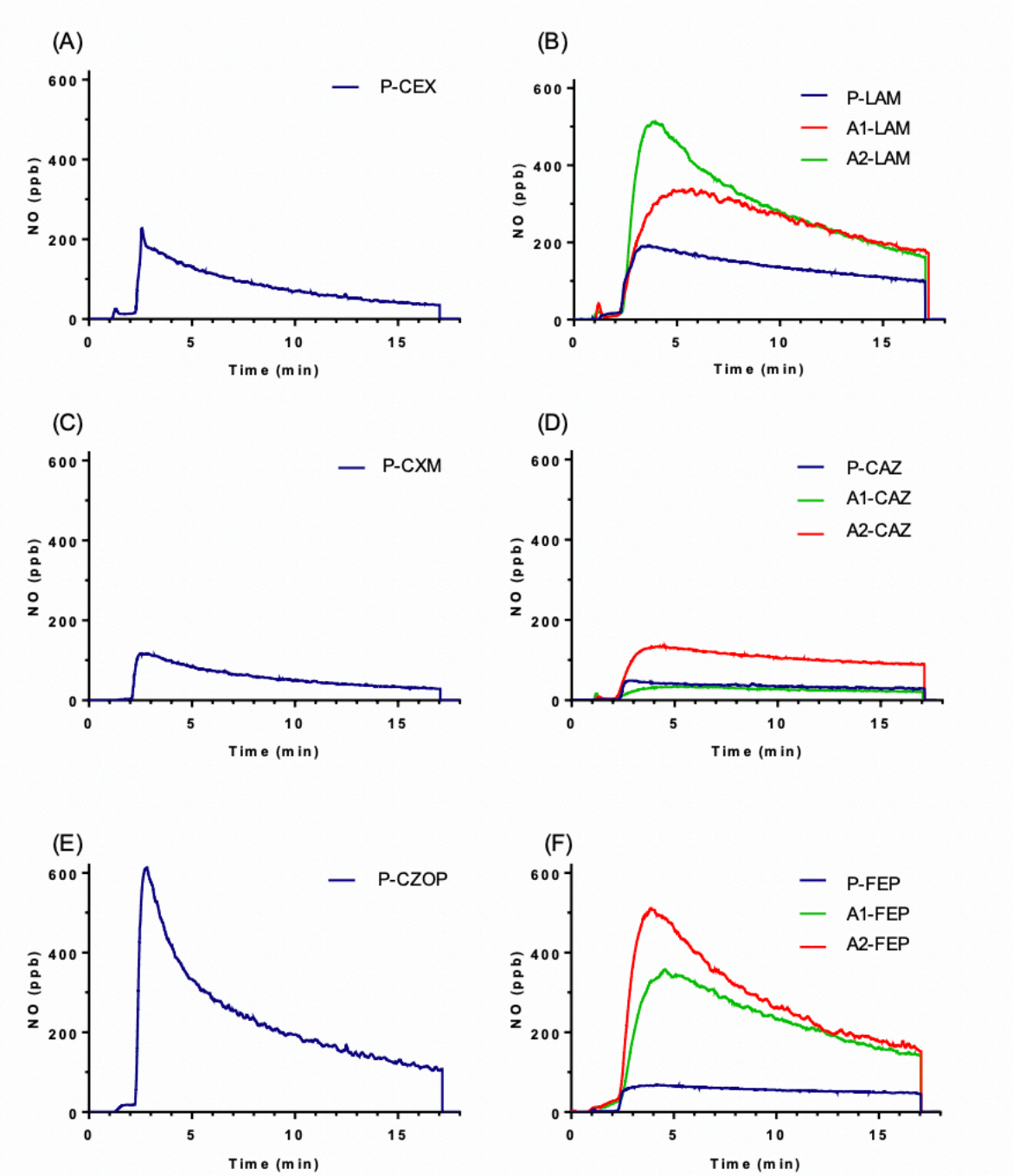


Figure 27. Release of NO from twelve next-generation C3D compounds upon treatment with penicillinase. Compounds are grouped into graphs according to their antibiotic core; (A) PYRRO-cefalexin, (B) Cephaloram based compounds, (C) PYRRO-cefuroxime, (D) Ceftazidime based compounds, (E) PYRRO-cefozopran, and (F) Cefepime based compounds. For all compounds, a final concentration of 10 μM in PBS was added at the 1 minute mark, followed by addition of 20 units of *B. cereus* penicillinase at the 2 minute mark. The readings were manually stopped at the 17 minute mark (15 minutes following the addition of the penicillinase). Data shown are representative examples from three replicates.

The total amount of NO released, in nmoles rather than ppb, from each compound during the 15 minute period was quantified (using the same method utilised in Chapter 3) and is shown in **Figure 28.** PYRRO-cefozopran, AMINOPIP2-cephaloram and AMINOPIP2-cefepime were the three compounds shown to release the most amount of NO during the same period, at approximately 80 nmoles. Comparisons were also made between the three compounds with the same parent antibiotic. For all three agents (cephaloram, ceftazidime and cefepime), the compound with the AMINOPIP2-group released a statistically significantly higher amount of NO than the compound with only the PYRRO-group. Interestingly, AMINOPIP1-cefepime has a statistically significant higher amount of NO release compared to PYRRO-cefepime. However, this trend was not replicated with AMINOPIP1-cephaloram or AMINOPIP1-ceftazidime; these two compounds have a non-significant difference or a statistically significant lower amount of NO release compared to PYRRO-cephaloram and PYRRO-ceftazidime respectively.

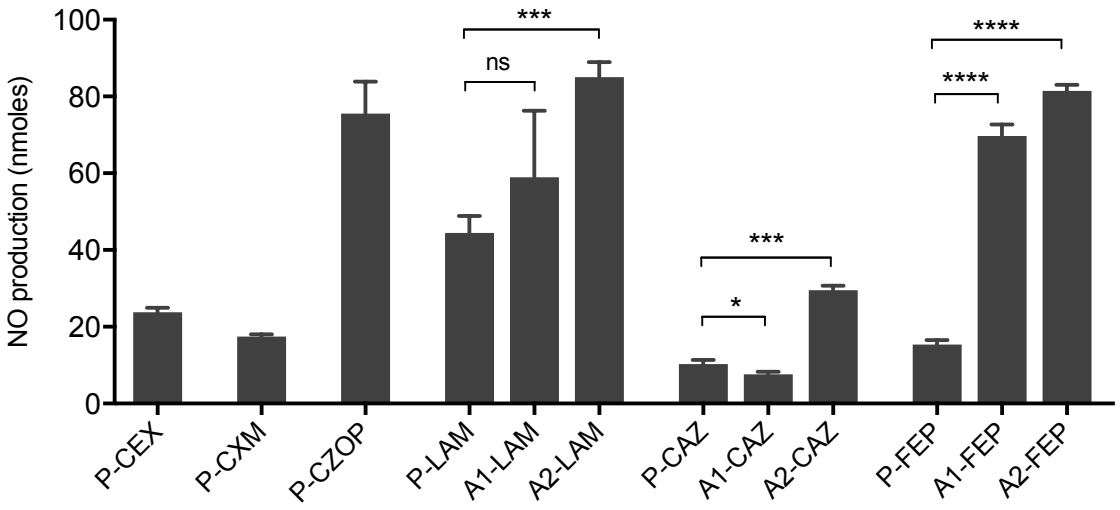


Figure 28. Total amount of NO released from twelve C3Ds during the first 15 minutes following exposure to penicillinase. Bars show the mean and standard deviation, from three replicate experiments. Welch's T tests were used for statistical analysis.

5.2.2 Ceftazidime-based NO donor compounds have enhanced bactericidal activity compared to the original ceftazidime antibiotic

In order to investigate the potential of an 'all-in-one' compound which, in addition to acting as a dispersal agent, also shows bactericidal effect, the MIC of the 12 new C3Ds were measured alongside their parent antibiotics. As the MIC for one particular agent can vary greatly dependent on the strain tested, PAO1 and ten clinical isolates were included in the MIC assessment. **Table 9** shows the MIC values for the first and second generation cephalosporin antibiotics and their C3D derivatives. The MIC value for cefalexin and PYRRO-cefalexin was above the highest tested concentration of 128 μg/ml. A similar result was seen with cefuroxime and PYRRO-cefuroxime; where all tested strains aside from two showed an MIC of >128 μg/ml. Cephaloram, PYRRO-cephaloram, AMINOPIP1-cephaloram, and AMINOPIP2-cefphaloram all showed an MIC of >128 μg/ml against all strains.

Table 10 shows the MIC values for the third and fourth generation cephalosporin antibiotics and their C3D derivatives. For ceftazidime, the MIC for PAO1 was 1 μ/ml, whilst the MIC for the clinical isolates ranged from 1 to 128 μg/ml. Addition of the pyrrolidine NO-donor group to ceftazidime caused an increase in MIC across all strains. Excitingly, the AMINOPIP1-ceftazidime and AMINOPIP2-ceftazidime compounds on the other hand, demonstrated MIC values equal to or less than that of the ceftazidime antibiotic, for 10 out of the 11 strains tested. For example, the PA26 MIC of 64 μg/ml for ceftazidime, increased to >128 μg/ml for PYRRO-ceftazidime, but was reduced to just 8 and 16 μg/ml for AMINOPIP1- and AMINOPIP2-ceftazidime, respectively. For cefepime, the MIC for PAO1 was 0.5 μg/ml and the range for the clinical isolates was 2 to 16 μg/ml. Again, with PYRRO-cefepime, there was an increase in MIC value across all strains relative to cefepime. However, for AMINOPIP1- and AMINOPIP2-cefepime, although the MIC values were lower than that of PYRRO-cefepime, they were all much higher than the cefepime values. The MIC values for ceftriaxone and cefotaxime were also tested as while they contain different substituents at the 3' position they all contain the same aminoacyl side chain as cefepime. The MIC values for AMINOPIP1-cefepime and AMINOPIP2-cefepime were similar to those of ceftriaxone and cefotaxime. For cefozopran, the MIC value for PAO1 was 1 μg/ml, whilst for the clinical isolates the MIC ranged from 4 to >128 μg/ml. Interestingly, aside from PA05, the MIC value for PYRRO-cefozopran was increased relative to cefozopran for 10 of the 11 *P. aeruginosa* strains.

Table 9. MIC values for 1st and 2nd generation cephalosporin antibiotics used in this study, and their respective C3D derivatives

Strain	MIC(µg/ml)							
	CEX	P-CEX	CXM*	P-CXM*	LAM	P-LAM	A1-LAM	A2-LAM
PAO1	>128	>128	>128	>128	>128	>128	>128	>128
PA05	>128	>128	64	>128	>128	>128	>128	>128
PA10	>128	>128	128	>128	>128	>128	>128	>128
PA21	>128	>128	>128	>128	>128	>128	>128	>128
PA26	>128	>128	>128	>128	>128	>128	>128	>128
PA30	>128	>128	>128	>128	>128	>128	>128	>128
PA37	>128	>128	>128	>128	>128	>128	>128	>128
PA44	>128	>128	>128	>128	>128	>128	>128	>128
PA56	>128	>128	>128	>128	>128	>128	>128	>128
PA58	>128	>128	>128	>128	>128	>128	>128	>128
PA68	>128	>128	>128	>128	>128	>128	>128	>128

* indicates final value is based on results from three independent experiments

Table 10. MIC values for 3rd and 4th generation cephalosporin antibiotics used in this study, and their respective C3D derivatives

Strain	MIC (µg/ml)											
	CAZ*	P-CAZ*	A1-CAZ*	A2-CAZ*	FEP*	P-FEP*	A1-FEP*	A2-FEP*	CRO	CTX	CZOP*	P-CZOP*
PAO1	1	32	1	1	0.5	32	4	4	4	4	1	32
PA05	1	2	1	1	4	4	8	8	2	2	8	4
PA10	1	4	0.5	0.5	2	8	8	8	1	1	4	8
PA21	16	64	16	8	4	>128	64	128	32	128	8	128
PA26	64	>128	8	16	4	>128	32	128	64	128	16	>128
PA30	4	128	4	4	4	128	32	32	16	16	8	>128
PA37	128	>128	16	16	4	32	16	32	32	32	16	16
PA44	4	128	16	16	16	128	128	128	64	64	>128	>128
PA56	4	64	2	4	4	128	16	32	16	16	8	128
PA58	16	128	4	8	8	>128	64	128	32	128	4	128
PA68	4	64	4	4	4	128	32	32	16	16	8	>128

* indicates final value is based on results from three independent experiments

5.2.3 C3D compounds have varying effects on *P. aeruginosa* biofilms

The effects of the parent antibiotics and their C3D derivates on *P. aeruginosa* biofilms were examined using CLSM and COMSTAT analysis. The effects of cefalexin, PYRRO-cefalexin, cefuroxime, PYRRO-cefuroxime, cefozopran, and PYRRO-cefozopran on PA68 are shown in **Figure 29**. Unfortunately, statistical analysis revealed there is no significant reduction in either total biofilm biomass or biofilm thickness distribution for any of these six agents. Comparison between each of the three antibiotics and their respective C3D, again, showed no statistically significant difference. For biofilms treated with cefozopran, the biofilm biomass and thickness was actually increased compared to the untreated control, whilst the biofilms treated with PYRRO-cefozopran showed reduced biomass and thickness compared to both the untreated control and the cefozopran treatment group. However, these differences were narrowly classed as a non-significant. The difference between cefozopran and PYRRO-cefozopran for total biofilm biomass and thickness distribution resulted in p values of 0.0997 and 0.0567 respectively following the t-test analysis.

The effects of cephaloram and cephaloram-based C3Ds on PA68 biofilms are shown in **Figure 30**. Cephaloram and PYRRO-cephaloram had no significant impact on biofilm biomass or thickness. In contrast, AMINOPIP1-cephaloram and AMINOPIP2-cephaloram had caused a notable and statistically significant reduction in both parameters. The mean biofilm thickness distribution was reduced by a third with AMINOPIP2-cephaloram treatment from 2.51 μm to 1.66 μm. AMINOPIP2-cephaloram appeared to be marginally more effective than AMINOPIP1-cephaloram, but there was no significant difference on comparison between these two compounds.

For biofilms treated with ceftazidime, as shown in **Figure 31**, no significant change was seen in biomass or thickness relative to the untreated control despite using a concentration of 128 μg/ml – 32 fold higher than its MIC value against this strain. Treatment with PYRRO-ceftazidime showed a lower biomass value and lower thickness distribution value, but only the latter was statistically significant. Similarly, there was no impact on either biomass or thickness following with treatment with AMINOPIP1-ceftazidime, but excitingly, treatment with 128 ug/ml of AMINOPIP2-ceftazidime was effective and significantly reduced the total biofilm biomass and mean thickness distribution value compared to the untreated control. Comparison between ceftazidime and AMINOPIP2-ceftazidime also revealed a statistically significant reduction in total biofilm biomass, with mean values of 1.65 and 1.22 $\mu m^3/\mu m^2$ respectively.

Lastly, biofilms treated with cefepime and cefepime-based NO donor compounds are shown in **Figure 32**. Despite a high concentration of cefepime, equivalent to 32x the MIC (128 μg/ml) the compound had no effect on the PA68 biofilm. PYRRO -cefepime also had no notable effect. Treatment with AMINOPIP1-cefepime caused a statistically significant reduction in both mean total biomass and thickness distribution compared to the untreated control, and to the cefepime

treatment group. The mean thickness distribution of the control biofilm reduced from 2.14 $\mu m^3/\mu m^2$ to 1.29 $\mu m^3/\mu m^2$ with AMINOPIP1-cefepime treatment. Treatment with AMINOPIP2-cefepime caused a non-significant reduction in mean total biomass biofilm but a significant reduction in mean thickness distribution.

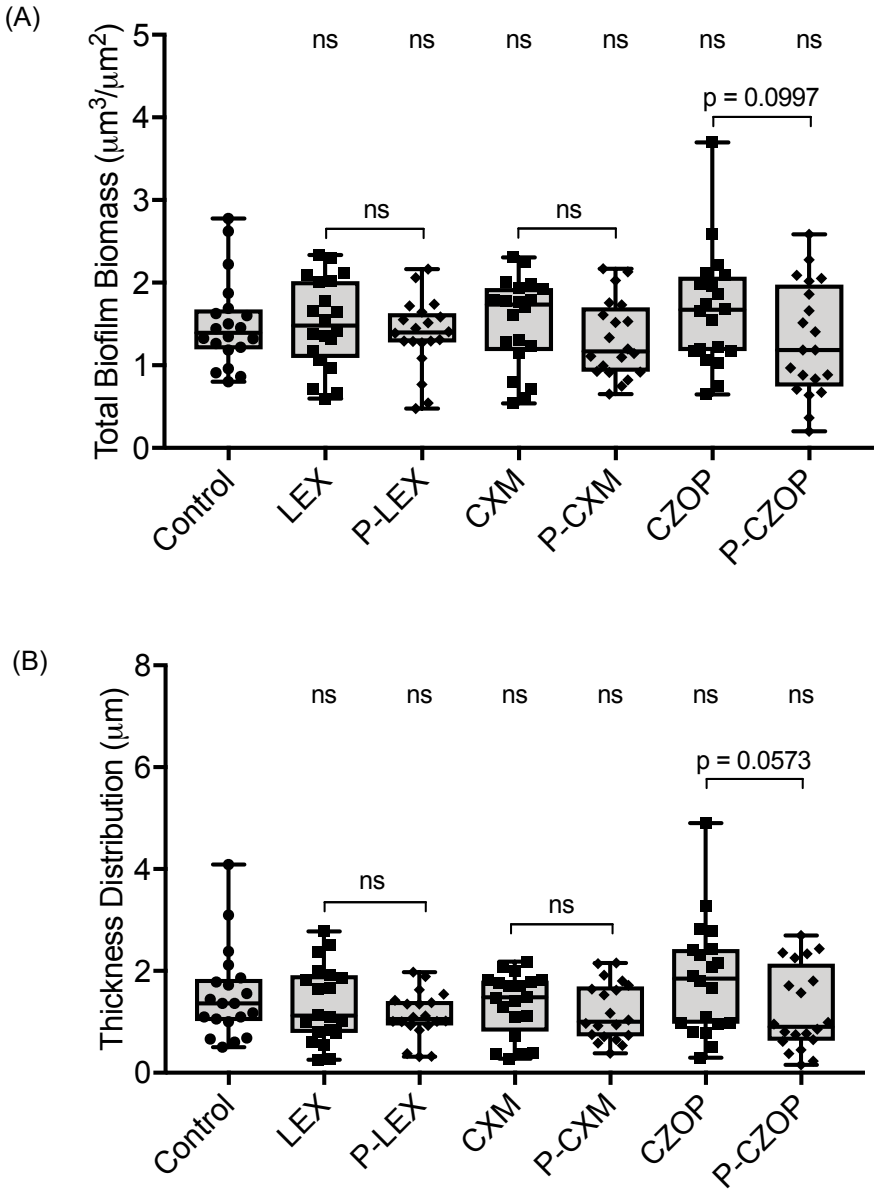


Figure 29. The effects of cefalexin, cefuroxime, and cefozopran, and their respective C3D compounds on PA68 biofilms. Biofilms were grown for 24 h and treated for 3 h at 128 ug/ml for all agents, before analysis with CLSM and quantification of images using COMSTAT 2.0. (A) the total biofilm biomass, and (B) the thickness distributions of biofilms. Bars shown the mean and SEM of 20 values, (n=4 from two biological replicates). Welch's T-test was used for statistical analysis, with each treatment group compared to the control, and each antibiotic treatment group compared to the treatment group with its corresponding NO donor compound.

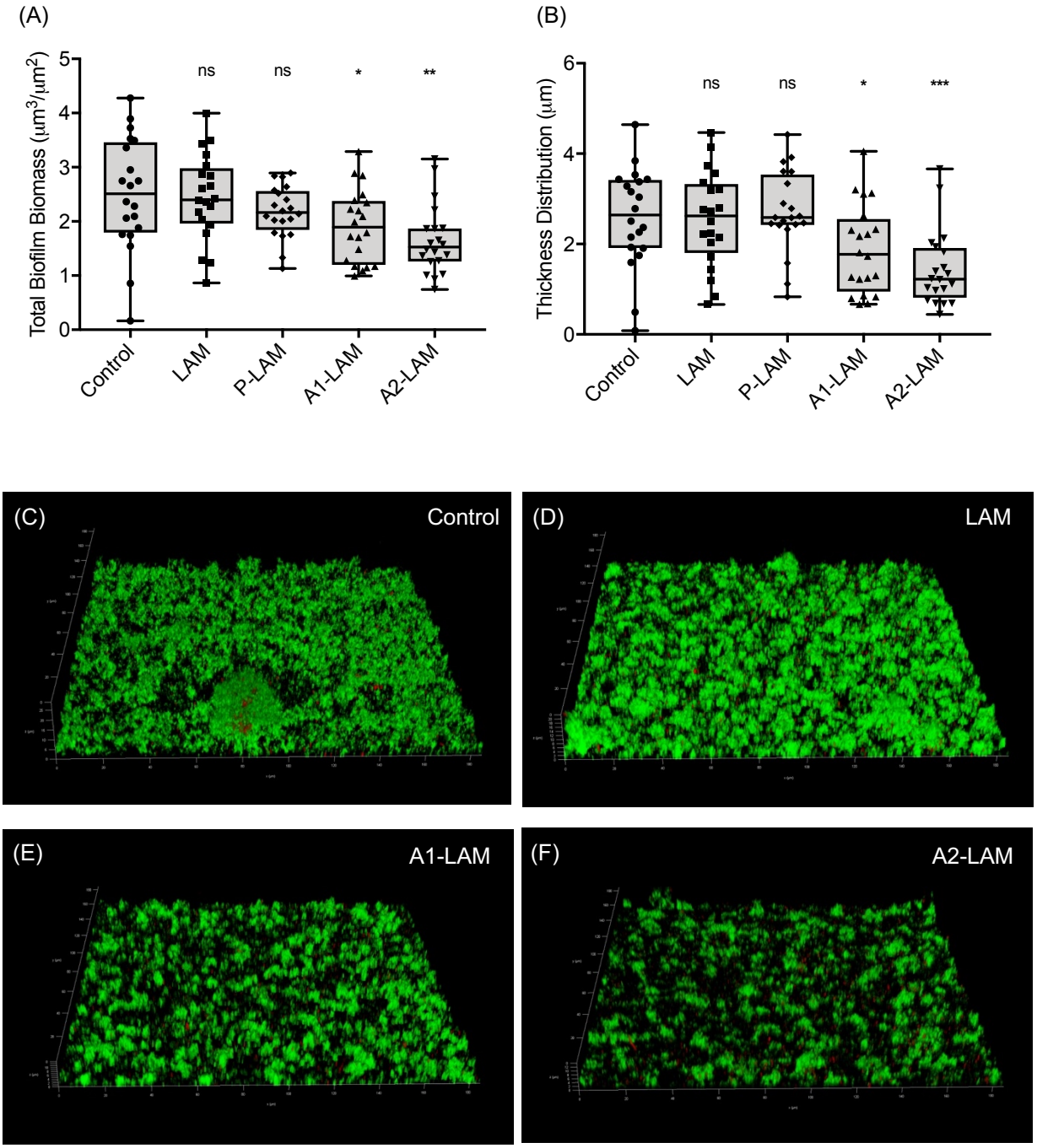


Figure 30. The effects of cephaloram and cephaloram-based NO donor compounds on PA68 biofilms. Biofilms were grown for 24 h and treated for 3 h at 128 μg/ml for all agents, before analysis with CLSM and quantification of images using COMSTAT 2.0. (A) the total biofilm biomass, and (B) the thickness distributions of biofilms. Bars shown the mean and SEM of 20 values, (n=4 from two biological replicates). Welch's T-test was used for statistical analysis, with each treatment group compared to the control. (C-F) representative 3D images of biofilms taken with CLSM: (C) untreated control, (D) treated with cephaloram, (E) treated with AMINOPIP1-cephaloram, (F) treated with AMINOPIP2-cephaloram. Biofilms were stained with SYTO9 and PI.

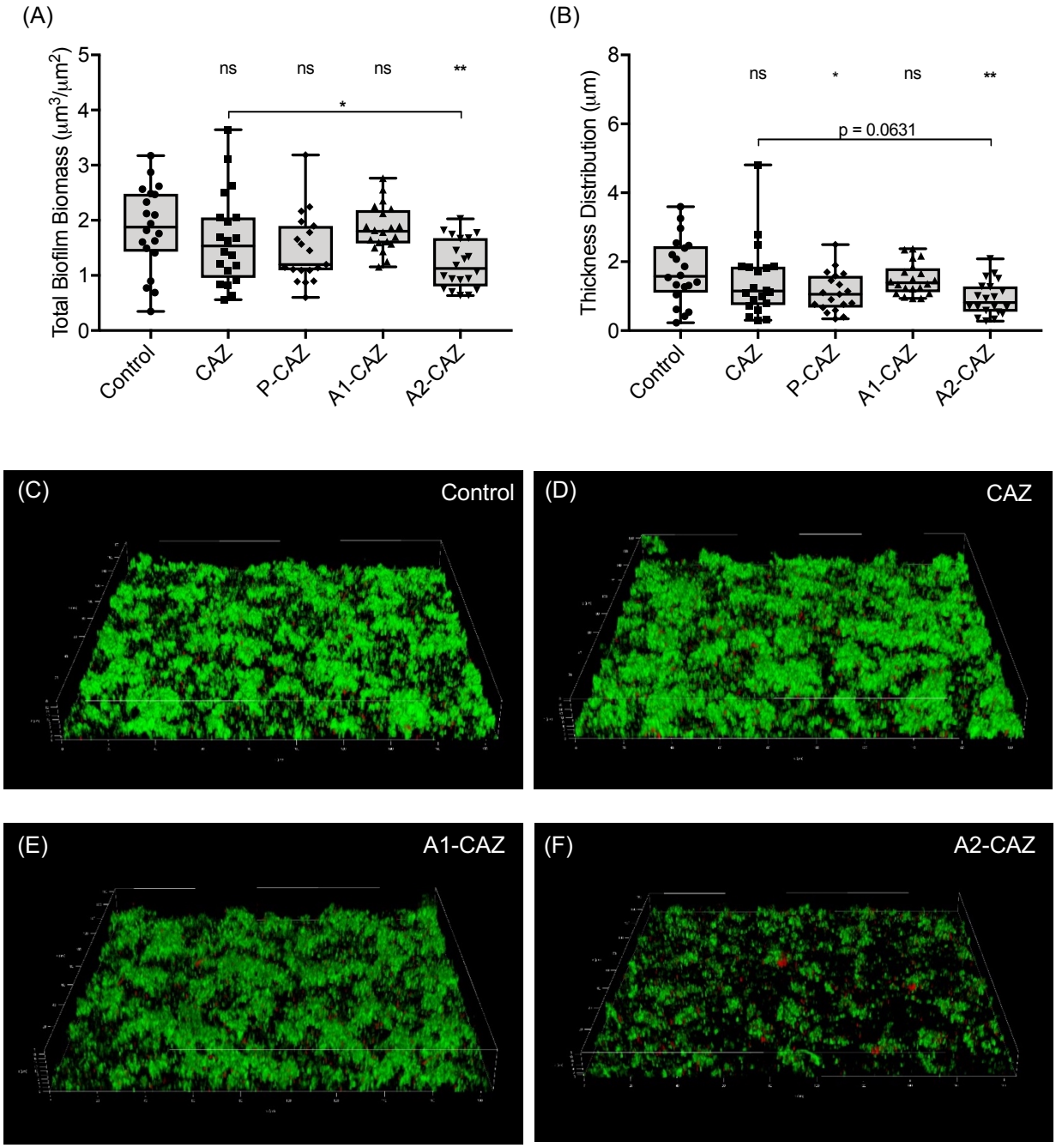


Figure 31. The effects of ceftazidime and ceftazidime-based NO donor compounds on PA68 biofilms. Biofilms were grown for 24 h and treated for 3 h at 128 µg/ml for all agents, before analysis with CLSM and quantification of images using COMSTAT 2.0. (A) the total biofilm biomass, and (B) the thickness distributions of biofilms. Bars shown the mean and SEM of 20 values, (n=4 from two biological replicates). Welch's T-test was used for statistical analysis, isolated stars represent comparison with the control. (C-F) representative 3D images of biofilms take with CLSM: (C) untreated control, (D) treated with ceftazidime, (E) treated with AMINOPIP1-ceftazidime, (F) treated with AMINOPIP2-ceftazidime. Biofilms were stained with SYTO9 and PI.

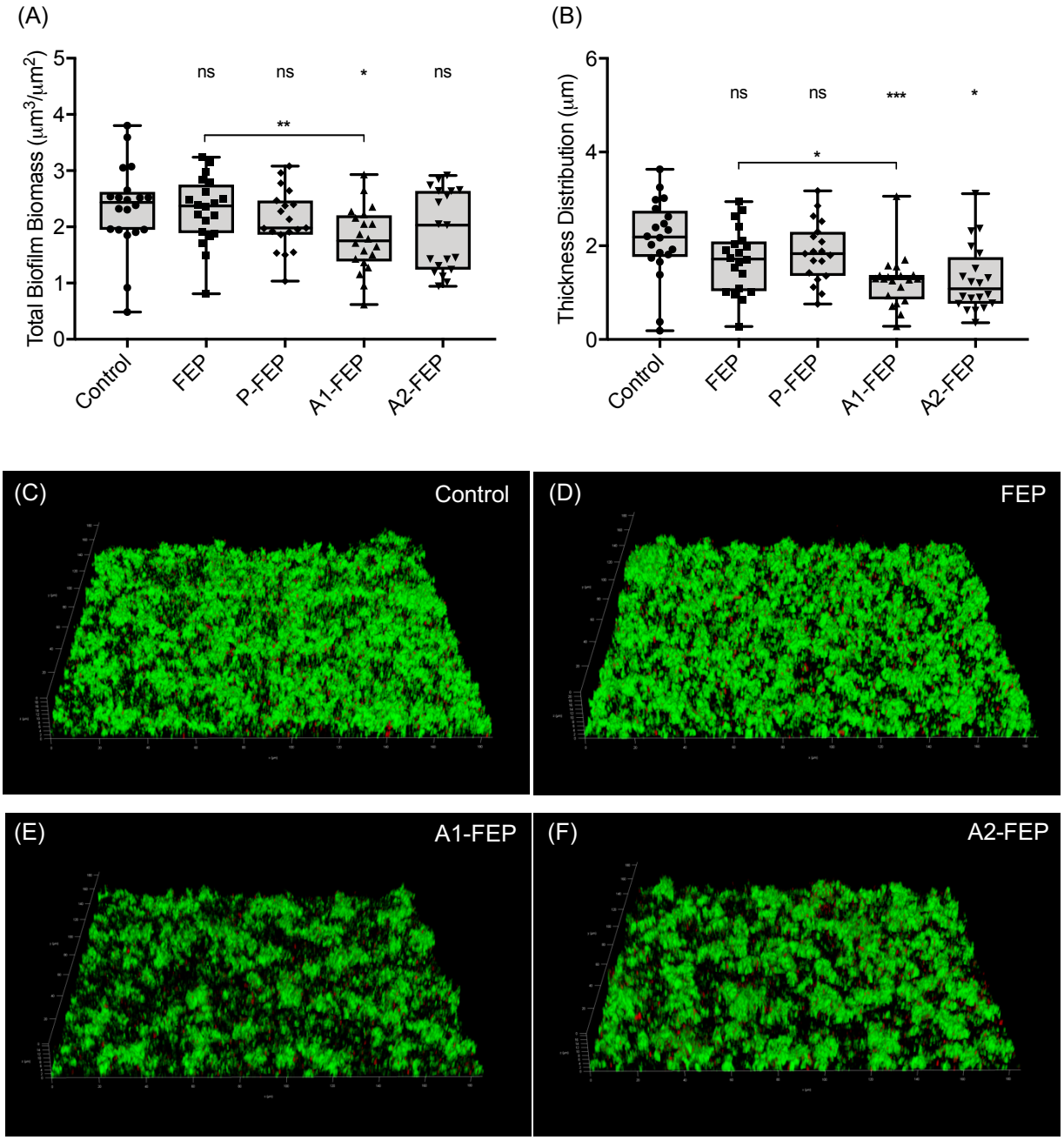


Figure 32. The effects of cefepime and cefepime-based NO donor compounds on PA68 biofilms. Biofilms were grown for 24 h and treated for 3 h at 128 µg/ml for all agents, before analysis with CLSM and quantification of images using COMSTAT 2.0. (A) the total biofilm biomass, and (B) the thickness distributions of biofilms. Bars shown the mean and SEM of 20 values, (n=4 from two biological replicates). Welch's T-test was used for statistical analysis; isolated stars represent comparison with the control. (C-F) representative 3D images of biofilms take with CLSM: (C) untreated control, (D) treated with cefepime, (E) treated with AMINOPIP1-cefepime, (F) treated with AMINOPIP2-cefepime. Biofilms were stained with SYTO9 and PI.

5.2.4 Identification of the most effective C3D against *P. aeruginosa* clinical biofilms

Following the comparative analysis of all six antibiotics and the twelve analogous C3D compounds, four C3Ds were revealed to have caused a statistically significant reduction in biofilm biomass as compared to the untreated control biofilms. All four compounds showed a statistically significantly reduction in biomass when compared to biofilms treated with their respective parent antibiotics. The four compounds were as follows: AMINOPIP1-cephaloram, AMINOPIP2-cephaloram, AMINOPIP2-ceftazidime and AMINOPIP1-cefepime. In **Figure 33**, the mean reduction in total biofilm biomass following treatment with these four compounds is shown. AMINOPI2-cephaloram and AMINOPIP2-ceftazidime caused the greatest reduction in biofilm biomass, at 34% and 34.4% respectively, compared to the untreated control biofilm. AMINOPIP2-cephaloram and AMINOPIP2-ceftazidime were also the two compounds with the largest reduction in mean biofilm biomass compared to their respective parent antibiotics; biofilms treated with AMINOPIP2-ceftazidime had a 26.3% reduction in mean total biofilm biomass compared to biofilms treated with an equivalent concentration of ceftazidime.

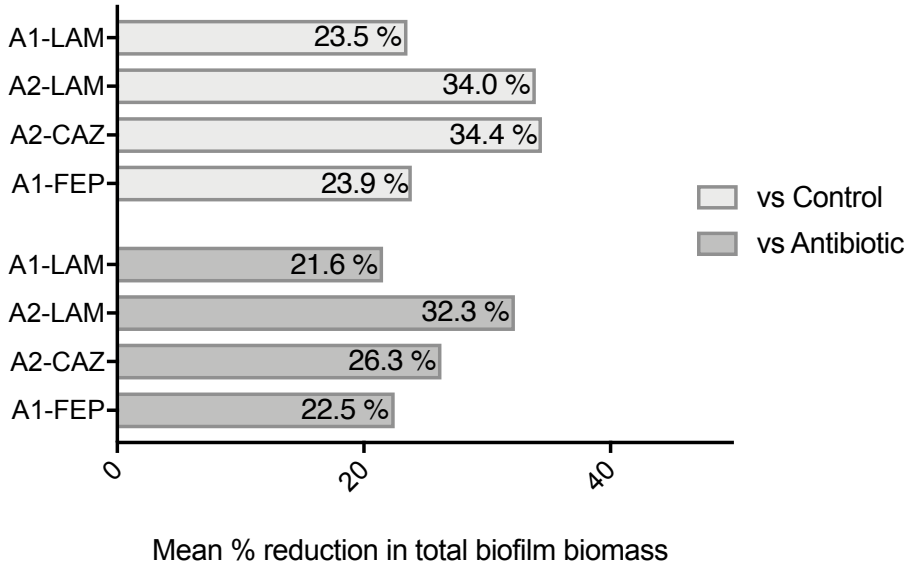


Figure 33. The mean percentage reduction in total biofilm biomass values with PA68 following treatment with the four most effective C3Ds. Mean values compare the treatment groups with the respective untreated control and parent antibiotic groups.

5.2.5 Biofilms formed by a second clinical isolate of *P. aeruginosa* show a response to AMINOPIP2-ceftazidime treatment

The effects of AMINOPIP2-ceftazidime were investigated against biofilms formed by another clinical isolate of *P. aeruginosa*, PA30, also previously demonstrated to form good biofilms *in vitro*. For this strain, treatment with ceftazidime surprisingly caused statistically significant increases in both biofilm biomass and thickness, **Figure 34**. Treatment with AMINOPIP2-ceftazidime showed no significant difference to the control group but significantly reduced the biofilm biomass and thickness compared to the antibiotic treatment group. This was in contrast to the results with PA68, despite the same experimental conditions used for both PA68 and PA30. Interestingly, the effects of the treatments on cell morphology were particularly evident with PA30. Close up images of bacterial cells show the typical bacilli shaped cells within the control biofilm, whereas for biofilms treated with ceftazidime and AMINOPIP2-ceftazidime a large proportion of cells had become elongated.

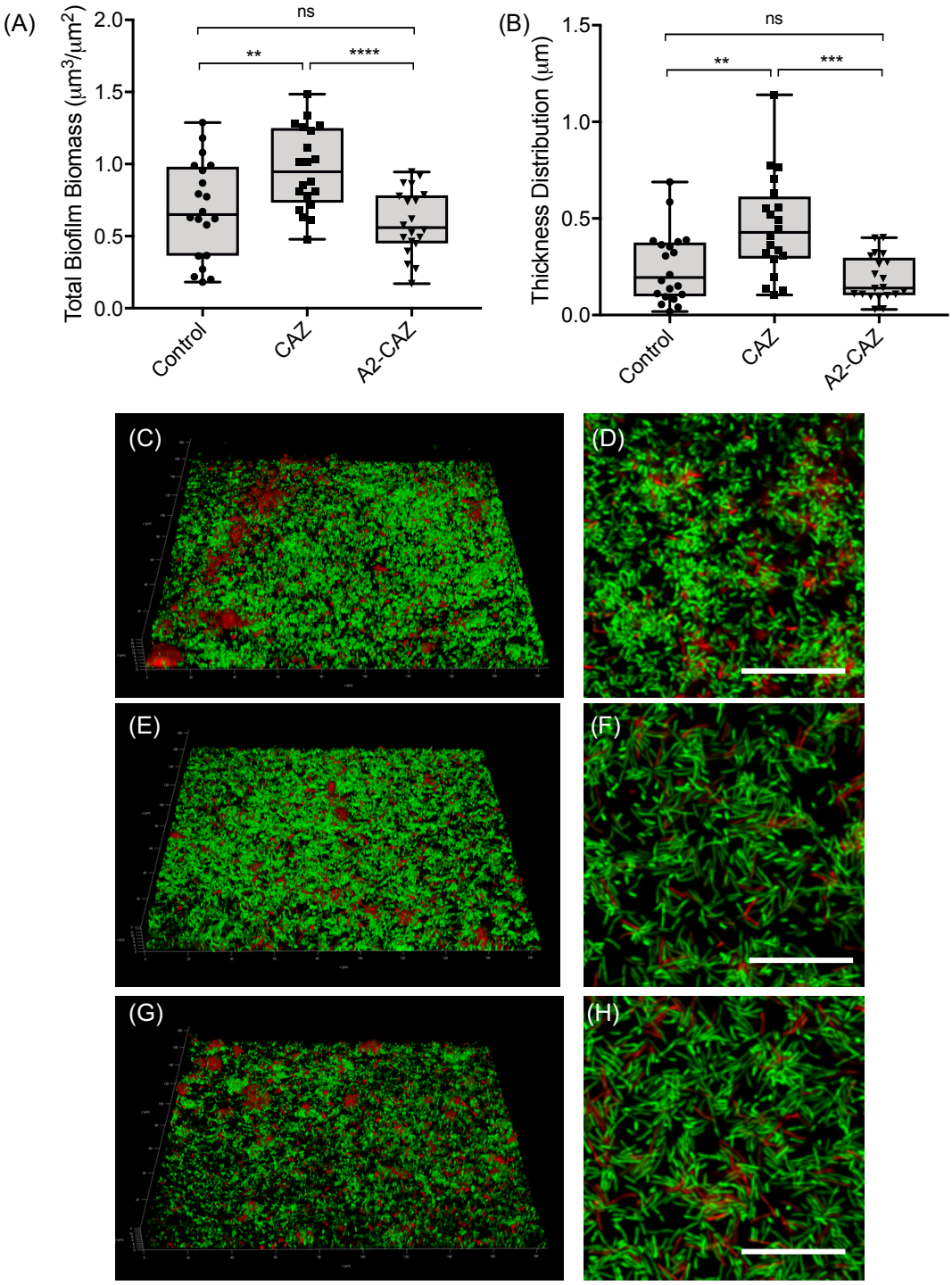


Figure 34. Effects of ceftazidime and AMINOPIP2-ceftazidime on PA30 biofilms. Biofilms were grown for 24 h, treated for 3 h at 128 µg/ml for all compounds, and analysed with CLSM and COMSTAT 2.0. (A) the total biofilm biomass, and (B) the thickness distributions of biofilms. Bars shown the mean and SEM of 20 values, (n=4 from two biological replicates). Welch's T-test was used for statistical analysis. Representative 3D images (axis 186 µm by 186 µm) and close up of a single z-stack (scale bar 20 µm), respectively, for (C and D) control biofilms, (E and F) biofilms treated with ceftazidime, and (G and H) biofilms treated with A2-CAZ. Biofilms were stained with SYTO9 and PI.

5.3 Discussion

5.3.1 NO release from C3D agents

The release of NO from the 12 new C3D compounds containing either pyrrolidine or aminopiperdine based diazeniumdiolates were analysed using a CLD. The six antibiotics with the PYRRO/NO diazeniumdiolate incorporated showed varying NO release profiles with varying quantities of NO released within the first 15 mins post enzyme exposure. From these data, it was evident that the antibiotic base of the C3D affects the rate of NO release following reaction with penicillinase. Cefalexin, cefuroxime, ceftazidime and cefepime antibiotics with the PYRRO/NO diazeniumdiolate attached demonstrated lower NO release compared to the cefozopran and cephaloram antibiotics carrying the PYRRO/NO donor. Therefore, PYRRO-cefozopran and PYRRO-cephaloram appeared to be the most susceptible to β-lactam ring cleavage by the *B. cereus* penicillinase. While this was to be expected with first-generation cephaloram, this is unexpected with fourth-generation cefozopran, supposedly designed to be more tolerant to β-lactam cleavage.

The chemical structure of all cephalosporins consists of a core structure containing a β-lactam ring condensed with a dihydrothiazine ring, with two variable side chains (R1 and R2) (Craig and Andes, 2015). The susceptibility of different cephalosporins to β-lactamases depends on the R1 and R2 side chains. The active site of a β-lactamase enzyme can be subdivided into an R1 site and an R2 site, to accommodate the R1 and R2 substituents on the β-lactam core, respectively (Jacoby, 2009). For cefalexin and cefuroxime, Richmond and Wotton (1976) found similarly found low levels of hydrolysis of these agents with an *E. coli* β-lactamase. Though susceptible to high concentrations of AmpC β-lactamase, ceftazidime is resistant to hydrolysis by most penicillinases (Matagne, Lamotte-Brasseur, and Frere, 1998), consistent with results in this study. Cephaloram has been reported to have a relatively high susceptibility to staphylococcal penicillinase (Goldin, Hawking, and Schnitzer, 1965), again, consistent with this study.

In the case of fourth generation agents cefepime and cefozopran, the R1 side chains of these molecules are similar, with cefozopran having just one extra nitrogen atom in its aminothiadazole ring. The R2 side chains however differ considerably, with cefozopran containing a larger fused aromatic heterocyclic ring system in the R2 side chain (C_6N_3), compared to cefepimes saturated pyrrolidine ring ($C_5H_{13}N$). As the R2 side chain for PYRRO-cefozopran and PYRRO-cefepime are identical, the increased susceptibility of PYRRO-cefozopran to β-lactamase hydrolysis demonstrates the R2 side chain in cefozopran may have been important in conferring its stability against β-lactamases. One potential explanation could be that the larger bi-cyclic R2 group of cefozopran prevented the molecule binding to the active site of the β-lactamase enzyme, whilst the smaller PYRRO/NO group of PYRRO-cefozopran facilitated binding to the β-lactamase active site, enabling hydrolysis and release of NO.

For cephaloram and cefepime antibiotics with aminopiperidine diazeniumdiolates incorporated, NO release rates were increased compared to the equivalent compound carrying the PYRRO/NO group. Again, this suggests that the aminopiperidine side chains confer increased hydrolysis of the molecule in the presence of β-lactamase. However, whilst the NO release of AMINOPIP2-ceftazidime was augmented in comparison to PYRRO-ceftazidime, the NO release for AMINOPIP1-ceftazidime was marginally reduced.

Importantly, the enzyme used to initiate cleavage of the C3Ds in this study was a constitutive periplasmic penicillinase from the Gram-positive bacterium *B. cereus*. This enzyme was selected as it is easily obtainable commercially, and could therefore be used at high concentrations to cause saturation in the reaction with C3Ds, thus leading to maximal release of NO and enabling better comparison between the agents. It must be noted that if C3Ds were utilised as a treatment against *P. aeruginosa*, cleavage could be caused not only by extracellular β-lactamase, but also by periplasmic β-lactamase, and by transpeptidase enzymes (as known as PBPs). Nevertheless, these experiments were crucial to confirm that NO could be released from each of the agents to gain potential insights into which of the agents may have the best anti-biofilm action. PYRRO-cefozopran, AMINOPIP2-cephaloram and AMINOPIP2-cefepime showed the highest amount of NO release, so we speculate these agents would have the best dispersing power and therefore anti-biofilm action.

5.3.2 Anti-bacterial activity of C3D agents

Unsurprisingly, the three non-pseudomonal antibiotics (cephaloram, cefalexin and cefuroxime) all showed little to no activity against all tested isolates of *P. aeruginosa*, and alterations to include either pyrrolidine or aminopiperidine diazeniumdiolates did not improve the antibacterial activity. Also unsurprisingly, ceftazidime, cefepime and cefozopran showed a good level of activity against PAO1 and the 10 clinical isolates. This study found PAO1 MIC values of 1, 0.5 and 1 μg/mL for ceftazidime, cefepime and cefozopran, respectively. Other studies have shown comparable MIC values for these agents against PAO1, validating the experiments in this study. For example, Torrens et al. (2016) and Henrichfreise et al. (2007) found values for ceftazidime of 1 and 2 μg/mL respectively. Drusano et al. (2012) found 1 μg/mL as the PAO1 MIC for cefepime, and Nakae et al. (1999) and Masuda, Sakagawa, and Ohya (1995) found an MIC of 0.78 μg/mL for cefozopran. A variation 2-fold higher or lower is an acceptable variation in MIC testing (EUCAST, 2003).

Addition of the PYRRO/NO group to the 3’ position of ceftazidime, cefepime and cefozopran antibiotics did not improve the bactericidal activity and in fact reduced it. For PYRRO-ceftazidime, PYRRO-cefepime, and PYRRO-cefozopran, MIC values were between 2-fold and 64-fold higher, for all isolates compared to ceftazidime, for 10 of the 11 isolates compared to cefepime, and for 8

of the 11 isolates compared to cefozopran. The three key factors that determine the activity of a β-lactam against Gram-negative bacteria, are: (1) the ability to penetrate the outer membrane, (2) the resistance to enzymatic degradation by β-lactamases, and (3) the affinity of the β-lactam for its target transpeptidases (Fung-Tomc, 1997). This suggests that addition of the PYRRO/NO to the antibiotic was detrimental to one or more of these three factors.

To fully understand why addition of the pyrrolidine diazeniumdiolate resulted in an increase in MIC value to ceftazidime, cefepime and cefozopran, we must first understand more about the chemistry of cephalosporins and their activity against Gram-negative bacteria (Fung-Tomc, 1997). First generation cephalosporins are characterised by good activity against Gram-positive bacteria but limited activity against Gram-negative bacteria due to β-lactamase susceptibility. Subsequently developed cephalosporins generally contain alterations made in the C-7 and C-3 side chains (also known as R1 and R2, respectively) (Craig and Andes, 2015). Some second generation agents have slightly increased Gram-negative activity; cefuroxime was the first agent to feature an α-oxyimino group in the C-7 side chain, which increased resistance to β-lactamase (Fontana et al., 2000). In this study, this is evident as, in contrast to first generation agents cefalexin and cephaloram, an MIC was attainable for cefuroxime for 2 of the 11 isolates examined.

Third generation cephalosporins are characterised by good activity against Gram-negative bacteria due to the aminothiazole ring in the C-7 side chain which enhances molecule penetration through the outer membrane (Perez-Inestrosa et al., 2005)**.** However, ceftazidime (shown in **Figure 35**), unlike other 3rd generation agents has a dimethlyacetic acid group in the C-7 side chain, instead of the methoxyimino group, which has greater steric hindrance and conferst anti-pseudomonal activity (Romano et al., 2015; Zasowski et al., 2015). Fourth generation cephalosporins have the broadest spectrum, active against Gram-positive and Gram-negative bacteria including *P. aeruginosa* (Fung-Tomc, 1997). Fourth generation agents as well as ceftazidime contain a quaternary ammonium group at the C-3 position, giving a positive charge on the C-3 substituent in addition to the negative charge on the C-4 carboxylic group of the cephem nucleus (Garau et al., 1997). The positive and negative charges result in a dipolar ionic (also known as zwitterionic) compound, and this is thought to orient the cephalosporin towards the entrance of the porin channels, facilitating more rapid penetration through the outer membrane of Gram-negative bacteria including *P. aeruginosa*.

Analysing their chemical structure (shown in **Appendix A**) shows that for ceftazidime, cefepime and cefozopran, addition of the pyrrolidine NO donor removes the zwitterionic character of the molecules, thus likely impairing penetration of the outer membrane, leading to an increase in MIC. Interestingly, the MIC values for PYRRO-ceftazidime appear to be slightly lower compared to PYRRO-cefepime and PYRRO-cefozopran; five isolates had a value of 128 μg/mL or higher for PYRRO-ceftazidime, whilst seven isolates had this result for PYRRO-cefepime and PYRRO-cefozopran. Conversely, addition of either aminopiperdine diazeniumdiolate to ceftazidime had

either maintained or, for 6 of the 11 isolates, slightly increased the bactericidal activity. This suggests that the AMINOPIP1- and AMINOPIP2- groups, which can become protonated on their terminal primary amino groups to restore zwitterionic character (in contrast to the pyrrolidine donor), enhanced membrane penetration. However, addition of either of the aminopiperdine diazeniumdiolates to cefepime did not show a similar trend; although MIC values were lower than for PYRRO-cefepime, they were still higher relative to cefepime.

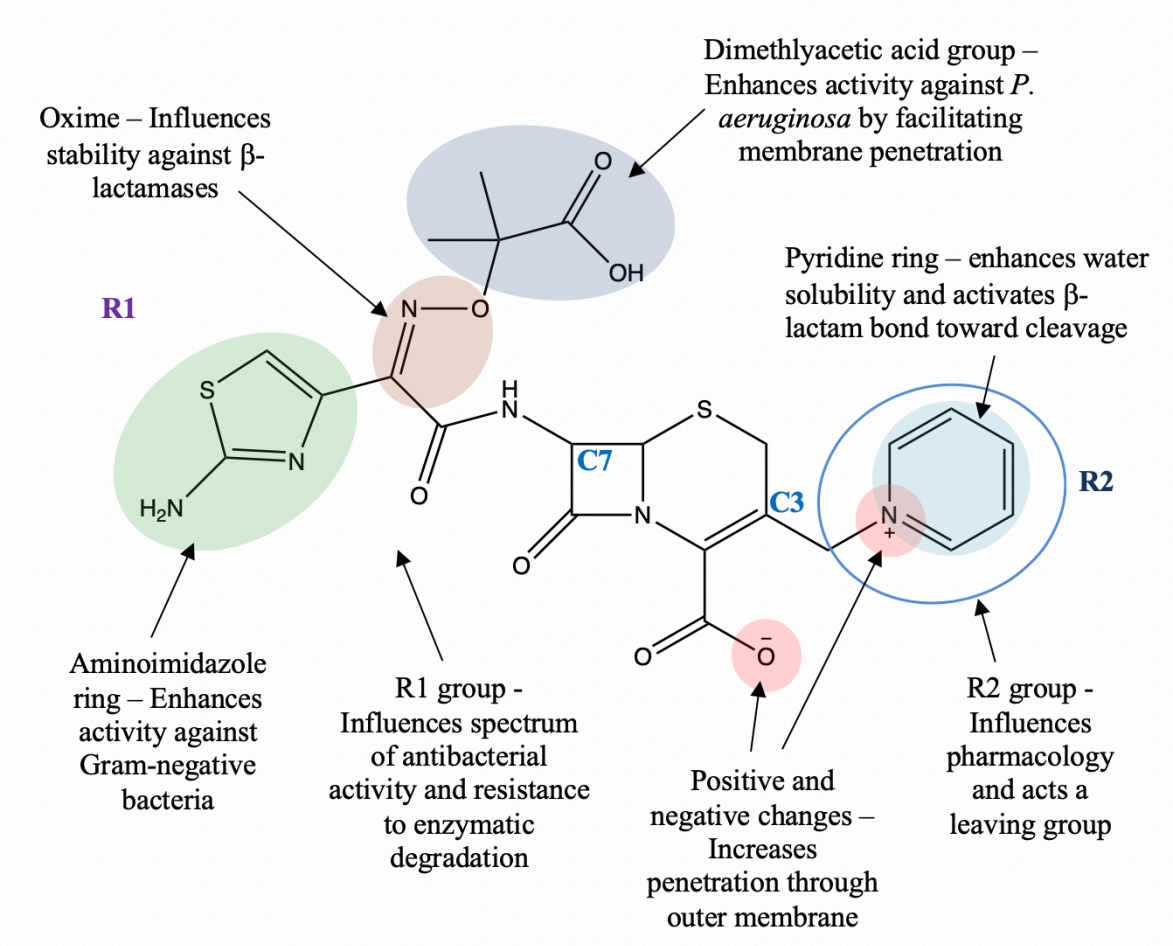


Figure 35. The chemical structure of ceftazidime, with R1 and R2 side chains shown, with C-7 and C-3 labelled. The activities conferred by the various groups are also shown.

Whilst the loss of zwitterionic character and the R2 group is mutual for both PYRRO-ceftazidime and PYRRO-cefepime, AMINOPIP1-ceftazidime and AMINOPIP1-cefepime, and for AMINOPIP2-ceftazidime and AMINOPIP2-cefepime, the R1 groups differ. For the ceftazidime based compounds, the R1 group provides anti-pseudomonal action. For cefepime based compounds, the R1 group is identical to the R1 groups of non-pseudomonal third generation cephalosporins ceftriaxone and cefotaxime, and therefore does not provide any anti-pseudomonal action. This accounts for the lower MICs for all three ceftazidime based C3Ds, compared to the three cefepime based C3Ds, and explains why AMINOPIP1-cefepime and AMINOPIP2-cefepime showed MIC values similar to those of ceftriaxone and cefotaxime.

The most significant finding was that the MICs for AMINOPIP1-ceftazidime and AMINOPIP2-ceftazidime were generally reduced in comparison to ceftazidime for isolates with higher MIC values, such as PA21, PA26, PA37 and PA58. *P. aeruginosa* isolates resistant to ceftazidime are usually because of breakdown by β-lactamases, and resistant isolates often overproduce AmpC (Kos, McLaughlin, and Gardner, 2016). Another point to note is that the isolates resistant to ceftazidime were susceptible to cefepime, and cefepime is known to have better efficacy against *P. aeruginosa* isolates resistant to ceftazidime due to β-lactamase degradation (Garau et al., 1997). Therefore, we postulate that the reductions in MIC seen with AMINOPIP1-ceftazidime and AMINOPIP2-ceftazidime could also be due to an increased resistance to β-lactamases in addition to increased membrane penetration.

In the parent ceftazidime antibiotic molecule, the R2 side chain contains a positively charged 6-membered pyridinium group (C_5H_5N), which is thought to enhance water solubility and activate the β-lactam bond toward cleavage by transpeptidases (Lemke and Williams, 2013). PYRRO-ceftazidime contains a 5-membered saturated pyrrolidine ring (C_4H_9N) with no positive charge, whereas AMINOPIP1-ceftazidime and AMINOPIP2-ceftazidime both contain a 6-membered saturated piperidine rings ($C_5H_{11}N$). Additionally, the AMINOPIP- structures carry a terminal primary amino group that would become protonated and positively charged at physiological pH, conferring zwitterionic character. Based on these observations, we suggest that the presence of a 6-membered ring within the diazeniumdiolate is more favourable over a 5-membered ring and the increased zwitterionic character are likely responsible for the increased bactericidal activity compared to ceftazidime.

5.3.3 Anti-biofilm activity of C3D agents

The anti-biofilm action of the new C3Ds along with the six parent antibiotics were examined using CLSM and by measuring the total biomass and thickness of biofilms formed by a CF isolate of *P. aeruginosa*. PYRRO-cefalexin, PYRRO-cefuroxime, PYRRO-cephaloram, PYRRO-ceftazidime, AMINOPIP1-ceftazidime, PYRRO-cefepime, and AMINOPIP2-cefepime did not demonstrate an anti-biofilm effect against PA68 biofilms. Though a reduction was seen in biofilm thickness compared to the control with AMINOPIP1-ceftazidime and AMINOPIP2-cefepime, we cannot conclude these agents had a significant anti-biofilm action as the biofilm biomass was unchanged, indicating the number of cells had not diminished but there may have been a structural change. Similarly, PYRRO-cefozopran, an agent with one of the highest NO release values, although showed a trend that treatment decreased biofilm biomass and thickness, these data was marginally non-statistically significant, so we cannot conclude that this agent produces an anti-biofilm effect. Promisingly, four C3Ds displayed an anti-biofilm action better than their parent antibiotic: AMINOPIP1-cephaloram, AMINOPIP2-cephaloram, AMINOPIP2-ceftazidime, and AMINOPIP1-cefepime. Of these four, AMINOPIP2-cephaloram and AMINOPIP2-ceftazidime were shown to

have the most effective anti-biofilm action, reducing the biofilm biomass by one third with a single dose.

Interestingly, the compounds shown to release the most NO following cleavage with penicillinase did not correlate well with the anti-biofilm action. However, as previously mentioned, the *in vitro* experiments carried out with isolated and concentrated penicillinase from *B. cereus,* may not accurately represent the release of NO from these agents whilst in contact with whole cell *P. aeruginosa* cultures. In addition to extracellular and intracellular β-lactamases, which can include multiple types of β-lactamase including pseudomonal chromosomal enzyme AmpC and others acquired via horizontal gene transfer, transpeptidase enzymes in the *P. aeruginosa* cell could also result in cleavage of the C3Ds to release NO.

Whilst AMINOPIP1-cephaloram, AMINOPIP2-cephaloram, AMINOPIP2-ceftazidime and AMINOPIP1-cefepime all demonstrated a promising anti-biofilm action against a CF isolate of *P. aeruginosa*, AMINOPIP2-ceftazidime is the only agent of these four to also show a potent anti-bacterial action. Therefore, from the twelve C3D compounds developed and investigated in this study, we conclude that AMINOPIP2-ceftazidime is the best candidate in the search for an agent with dual anti-biofilm and anti-bacterial action.

The anti-biofilm effects of AMINOPIP2-ceftazidime were tested in an additional CF isolate of *P. aeruginosa* to confirm its dual-action. With this second strain, though AMINOPIP2-ceftazidime did not reduce the biofilm biomass or thickness in relation to the untreated control, it was significantly reduced in comparison to the ceftazidime treatment group, which surprisingly promoted biofilm formation. The increase in biofilm growth following a sub-optimal concentration of antibiotic treatment is well known within the field of biofilm science, and has been demonstrated with multiple agents including imipenem and tobramycin (Bagge et al., 2004; Hoffman et al., 2005; Karaman et al., 2013).

Microscopy analysis of individual cells treated with ceftazidime and AMINOPIP2-ceftazidime revealed evidence of cell elongation with both agents that was not observed with the untreated controls. This filamentation occurs when the bacterial cells continue to elongate but division is inhibited (Spratt, 1983). PBP3 has been shown to be essential for cell division in *P. aeruginosa*, similarly to *E. coli* (Chen, Zhang, and Davies, 2017). This is consistent with the mode of action of ceftazidime, shown by Hayes and Orr (1983) to primarily inhibit the PBP3 of both *P. aeruginosa* and *E. coli*, with the aminothiazole ring in the R1 side chain responsible for the high affinity to this particular PBP (Zasowski et al., 2015). Again, this confirms that AMINOPIP2-ceftazidime has an antibacterial action against *P. aeruginosa* via the same mechanism as ceftazidime, whilst also able to release NO.

For both of the CF isolates investigated, AMINOPIP2-ceftazidime showed the same MIC value compared to ceftazidime, suggesting identical bactericidal action, however the anti-biofilm activity

was considerably better than ceftazidime. Therefore, this data supports the continued research into AMINOPIP2-ceftazidime as a potential new treatment for *P. aeruginosa* biofilm infections in CF.

As with DEA-CP, a finite amount of the new generation C3D agents were available to us for microbiological examination, and experiments had to be designed accordingly. Therefore, the key limitation in this chapter was the compromise of carrying out two biological replicates with two technical replicates for assays such as the CLSM analysis to ensure all necessary experiments could be carried out.

5.3.4 Conclusions

In this chapter, the objective was to analyse the effects of twelve new generation C3D compounds on clinical CF isolates of *P. aeruginosa* and identify a compound with dual antibacterial and anti-biofilm action which holds the most promise as a potential new CF therapy. Using CLD analysis, all twelve new generation C3D compounds were shown to release NO in a selective manner, with varying rates of NO release. Assays to investigate antibacterial and anti-biofilm actions were carried out independently. MIC experiments showed that two of the twelve C3D agents, AMINOPIP1-ceftazidime and AMINOPIP2-ceftazidime, had antibacterial activity similar to or better than ceftazidime with 10 of the 11 isolates of *P. aeruginosa*. CLSM showed that four of the twelve C3D agents, AMINOPIP1-cephaloram, AMINOPIP1-ceftazidime, AMINOPIP2-ceftazidime and AMINOPIP2-cefepime, had significant anti-biofilm activity and were more effective than their respective parent antibiotics. Further analysis demonstrated the AMINOPIP2-ceftazidime was the compound with the best antibacterial and anti-biofilm activity and designated as the agent that should be selected for continuing investigations and drug development.

Chapter 6 Investigations into the use of a novel electrochemical NO generator prototype

6.1 Introduction

In addition to SNP and glyceryl trinitite, NO in the form of inhaled gas is also used clinically. It is thought that haemoglobin rapidly scavenges and inactivates NO upon diffusion into the blood, limiting the effects of inhaled NO to the lungs and hence acts as a pulmonary selective vasodilator (Inchinose, Roberts, and Zapol, 2004). Multiple studies have shown that inhaled NO does not cause a measurable change in systemic blood pressure and systemic hypotension has not been reported in trials or clinical use (Weinberger et al., 2001). This is in contrast to systemically administered NO-donors and other vasodilatory agents which cause systemic vasodilation and can lead to arterial hypotension. Currently, inhaled NO (known as INOmax) is FDA approved to treat neonates with hypoxic respiratory failure associated with persistent pulmonary hypertension in newborns (PPHN) (FDA, 2015).

The official recommended dosage of inhaled NO for neonates with PPHN is 20 ppm maintained for up to 14 days or until adequate oxygenation is achieved, however doses of up to 80 ppm can and have been used clinically (FDA, 2015). However, very high doses of inhaled NO may cause side effects such as airway injury from NO_2 and hypoxemia as a result of methemoglobinemia. NO is highly reactive with oxygen and can cause formation of NO_2, an environmental pollutant and potent pulmonary irritant known to be directly toxic to the respiratory tract (Weinberger et al., 2001). This limits human peak exposure to 5 ppm of NO_2. At higher levels, NO_2 can cause epithelial hyperplasia of the terminal bronchioles, diffuse inflammation, fibrosis and pulmonary oedema. Methemoglobinemia occurs when oxyhaemoglobin reacts with NO and can no longer transport oxygen; a 35% methaemoglobin (MetHb) level is symptomatic, whilst 70% is fatal (Taylor et al., 2001). Although MetHb levels rarely have any significant increase at the recommended dosage, it is recommended MetHb levels along with PaO2 and NO2 are monitored during inhaled NO therapy.

Although only clinically indicated in the newborn population, inhaled NO is often used off-label in child and adult patients to treat perioperative pulmonary hypertension associated with cardiac surgery or heart transplantation, and pulmonary hypertension associated with receiving a left ventricular assist device (Inchinose et al., 2004). Inhaled NO has also been used for adults with acute respiratory distress syndrome, however recent studies have shown that although there is a transient improvement in oxygenation, long term mortality rates show no statistically significant change (Bhatraju et al., 2015; Gebistorf et al., 2017).

Other potential clinical uses of inhaled NO are vast; it has been investigated as a novel therapeutic concept for conditions beyond those associated with pulmonary hypertension including malaria, liver transplantation, wound healing, and influenza and bacterial infection. Though positive effects were noted in mice with cerebral malaria, a clinical trial including 180 children with malaria demonstrated inhaled NO was unable to cause changes in measured biological markers, the number of complications or mortality. (Hawkes et al., 2015; Serghides et al., 2011). In some circumstances, inhaled NO has been used as an adjunctive therapy with liver transplantation and demonstrated beneficial effects (Fukazawa and Lang, 2016), with a two-centre RCT with 40 patients showing reductions in the number of complications and enhancement in allograft function (Lang et al., 2014). Though the mechanism by which this occurs is still not entirely clear.

Gaseous NO is recognised as a broad antimicrobial agent. Whilst *in vitro* investigations had shown gNO to be effective against influenza virus, a clinical trial found no reductions in viral load or any clinical benefit to patients with influenza infection following inhaled NO treatment (Darwish et al., 2012; Regev-shoshani et al., 2013). Conversely, the use of gNO against bacterial species appears to have more powerful effects. In a murine wound model, gNO had improved wound healing, accelerated the normalisation of the microcirculations, growth of new microvessels, and decreased inflammation and *S. aureus* infection (Shekhter et al., 2005). Another study examined the bacterial load of rabbit skin infections and showed a mild but significant decrease in *S. aureus* load following treatment of the wound with gaseous NO (Ghaffari et al., 2007).

As previously explained, the benefits of adjunctive low dose inhaled NO therapy (10 ppm) in targeting *P. aeruginosa* biofilm infection in CF patients have recently been demonstrated (Howlin et al., 2017). High dose inhaled NO therapy to target bacterial lung infections is also currently been investigated. *In vitro* experiments utilising 160 ppm NO had shown to result in the killing of planktonic *P. aeruginosa* and *S. aureus* cultures, but biofilm cultures were not considered (Miller et al., 2009). Intermittent treatment periods with this high dosage of gNO were then investigated in ten healthy humans; 163 ppm of NO for 30 mins, 5 times daily for 5 consecutive days was well tolerated, though methaemoglobin showed a significant 0.9% increase during the trial period. A clinical trial is expected to go ahead to examine the effects of high dose NO from pressurized cylinders in CF patients (Clinical Trials.gov, 2015).

The use of inhaled NO for those with bacterial lung infections, and in particular CF patients, clearly warrants further research. However, ultimately, a lower dose is preferable over a higher dose, particularly if administration is likely to be over a long period of time. At 80 ppm NO, formation of 5 ppm NO_2 is expected after 3 mins of contact with air, hence concentrations of NO and NO_2 are measured continuously during clinical administration of inhaled NO.. Therefore, use of a lower dose would be encouraged to limit exposure to NO_2.

6.1.1 Electrical generation of NO

Whilst the potential beneficial effects of gNO for CF patients and non-CF medical conditions has been demonstrated, the issue of gNO delivery remains. Efforts have been made to develop technology to bypass the use of the expensive, inconvenient and the potentially hazardous use of NO from pressurized cylinders. NitricGen Inc., an Amercian based biotechnology company founded by Duncan Bathe, have developed a small portable device to electrically generate NO from room air, termed the 'eNO Generator'. The eNO Generator produces NO from the air by creating plasma from an electronic pulsed arc discharge (spark) between two electrodes in a reaction chamber (NitricGen, 2016). The plasma created then reduces N_2 and O_2 in the air into single ions and the collision of nitrogen and oxygen ions results in the production of NO. A desired concentration of NO is obtained via control of the frequency of the pulsating discharges and duration of each electrical discharge pulse.

A group based in Boston, US, led by Warren Zapol, have also developed a similar device that produces NO from air. This device also electrically generates NO by creating sparks between two electrodes, however, at a rate of hundreds to thousands of pulsed sparks per second (Yu et al., 2015). In contrast, NitricGen's eNO Generator produces a single spark which is then maintained. The high frequency of sparks per second is likely to have an increased power consumption and could lead to complications regarding electromagnetic compatibility (EMC). Electronic medical devices must comply with EMC regulations to ensure electromagnetic interference to not high enough to affect other electronic medical devices from operating. In addition to requiring a lower power consumption, a single maintained spark will result in a decreased level of electromagnetic interference and will be more likely to comply with EMC regulations (Duncan Bathe, personal communication).

The eNO Generator had been initially developed for topical use with diabetic foot ulcers to promote wound healing (NitricGen, 2016). Preclinical data conducted at the Beth Israel Deaconess Medical Centre, US, shows the eNO Generator increases the wound closure rate in diabetic mice (Veves, 2015). However, based on previous and current research interests involving NO and CF, the eNO generator could have an additional clinical application as an adjunctive therapy for CF patients. Hence, in this chapter, the objective was to investigate the use of the eNO Generator against *P. aeruginosa* biofilms *in vitro*, and determine whether this device could be used to treat *P. aeruginosa* infection in CF.

6.2 Results

6.2.1 Preliminary investigations into the NO production rates by the eNO generator

The concentration of NO and NO_2 emitted from the eNO generator, with an NO_2 filter attached, was assessed using a CLD for low set concentrations of NO at variable flow rates. Shown in **Table 11**, the NO_2/NO ratio for all tested parameters was low and with a maximum recorded NO_2 output of 0.2 ppm. However, the inaccuracy of the NO output concentrations were high; with the concentration of NO actually produced between 63% lower and 430% higher than the set NO concentration. This error value is shown to be the greatest for the lower set concentration of 2 ppm, and generally lower as the set concentration is increased. No correlation appears to be present with the NO or NO_2 output values and the flow rate of the eNO generator.

Table 11. Set and recorded NO and NO_2 concentrations from the eNO generator

Setting		Read Out		NO_2/NO (%)[c]	NO error (%)[d]
NO (ppm)	Flow rate (LPM[a])	NO (ppm)	NO_2 (ppm)[b]		
2	0.5	3.8	0.1	2.6%	+90%
2	1	1.6	0.1	6.3%	-20%
2	1.5	2.1	0.1	4.8%	5%
2	2	10.6	0	0	+430%
4	0.5	4.9	0.1	2%	23%
4	1	1.8	0.1	5.5%	-55%
4	1.5	1.5	0.1	6.7%	-63%
4	2	2.1	0.1	4.8%	-48%
6	0.5	3	0.1	3.3%	-50%
6	1.0	2.3	0.1	4.3%	-62%
6	1.5	4.4	0.1	2.3%	-27%
6	2	4.8	0	0	-20%
10	2	8.7	0.2	2.3%	-13%

[a] LPM = litres per minute
[b] NO_2 concentration read out value was obtained from NO_x read out value minus NO read out value
[c] value = (NO_2/NO) x100
[d] Error between set NO concentration value and actual NO concentration value recorded

6.2.2 Design and development of experimental set up and apparatus to examine the effect of NO gas on *P. aeruginosa* biofilms.

The design and development of the experimental set up to test use of the eNO generator *in vitro* required considerable time and trial and error. The schematic design for the experimental set up is shown in **Figure 36**. Initially, the NO monitor was to be placed within the NO chamber, to measure the NO concentrations directly applied to the sample, however the aerosolization risk of *P. aeruginosa* would have made this difficult to maintain sterility of the monitor and avoid cross contamination. Therefore, the monitor was instead connected to the outlet tubing after a filter was connected. In addition to the system shown in **Figure 36**, an identical system but instead utilising an air pump with an identical same flow rate was substituted for the eNO generator, to provide a control. At first, 5L chambers were utilised, in an attempt to have the volume resemble that of the human lung, however in preliminary investigations, the 5 L chambers were impractically sized and the time required to reach the desired NO concentration was very prolonged. Therefore, the 5 L chambers were substituted for 1.1 L chambers. Photographs of the equipment utilised and the experimental set up are shown in **Figure 37.**

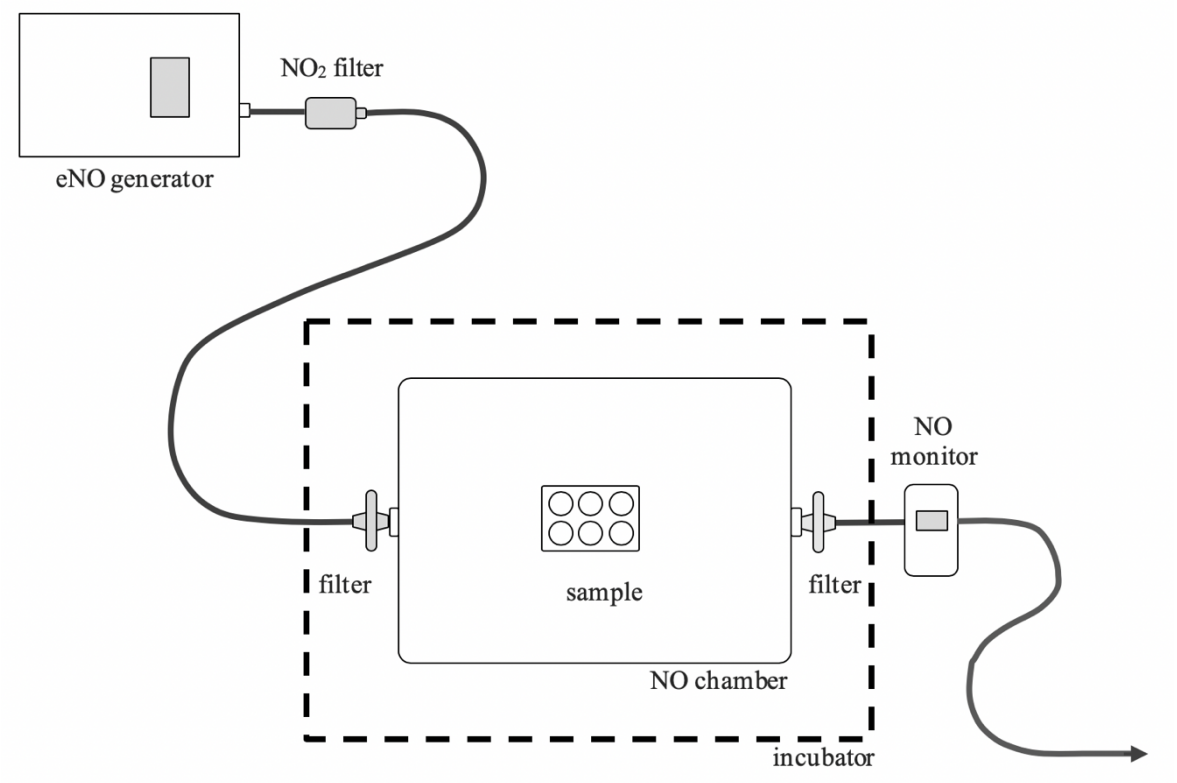


Figure 36. Schematic design for use of NO gas in treating *P. aeruginosa* biofilms *in vitro*. Gas emitted from the eNO generator passes through a NO_2 filter before entering the NO chamber containing the sample. At both the inlet and outlet of the NO chamber are 0.2 μm filters. Finally, the outlet of the chamber is connected to a NO monitor to measure the real-time NO concentration passing through the system.

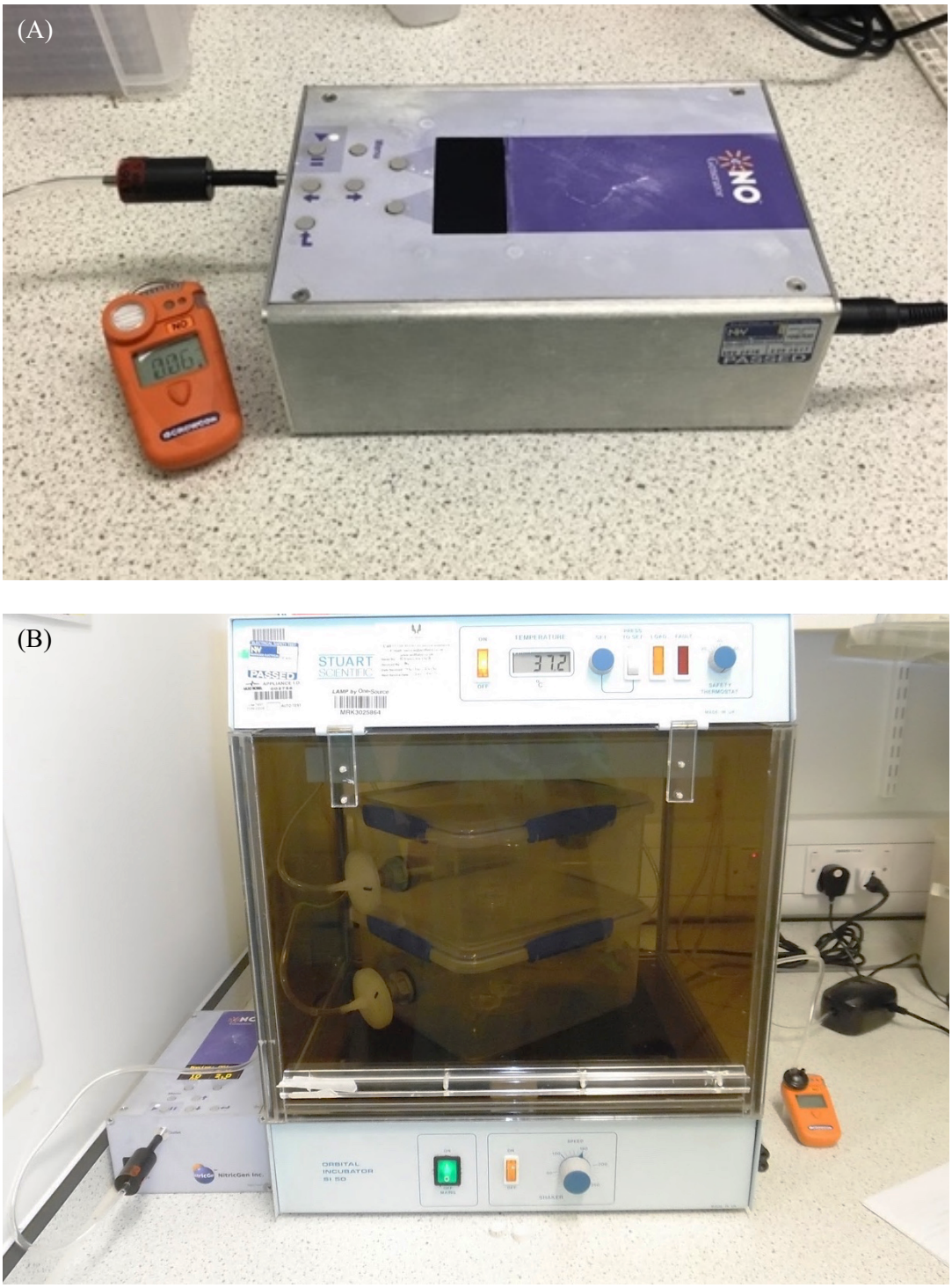


Figure 37. Photographs of (A) the eNO generator and the NO monitor, and (B) the experimental set up using the eNO generator, with 5L NO and air chambers with filters attached inside a shaking incubator.

6.2.3 Absorption of NO in water and biofilm media using the experimental set up

In order to analyse whether NO gas was adequately absorbed and solubilised in liquid media, water and biofilm media were exposed to NO gas and then tested for NO breakdown products. As shown in **Figure 38**, both water and M9 minimal media had tested positive for nitrite and nitrate following exposure with various concentrations of NO gas, and in contrast, exposure to air had shown a negative result. At 20 ppm, approximately 100 μM of nitrite and nitrate was recorded in both water and M9 minimal media samples, though M9 minimal media had shown a marginal and statistically significant increase in nitrite and nitrate values compared to water. At 60 ppm, the recorded nitrite and nitrate had increased; again the nitrite and nitrate concentrations in M9 minimal media were marginally raised in comparison to water. At the highest tested NO gas concentration of 100 ppm, the concentration of nitrite and nitrate in M9 minimal media had increased drastically compared to in water. Overall, a dose dependent effect is noted; a higher gaseous NO concentration results in increased NO absorption into liquid media, and a higher concentration of NO breakdown products is noted in M9 minimal media than in water.

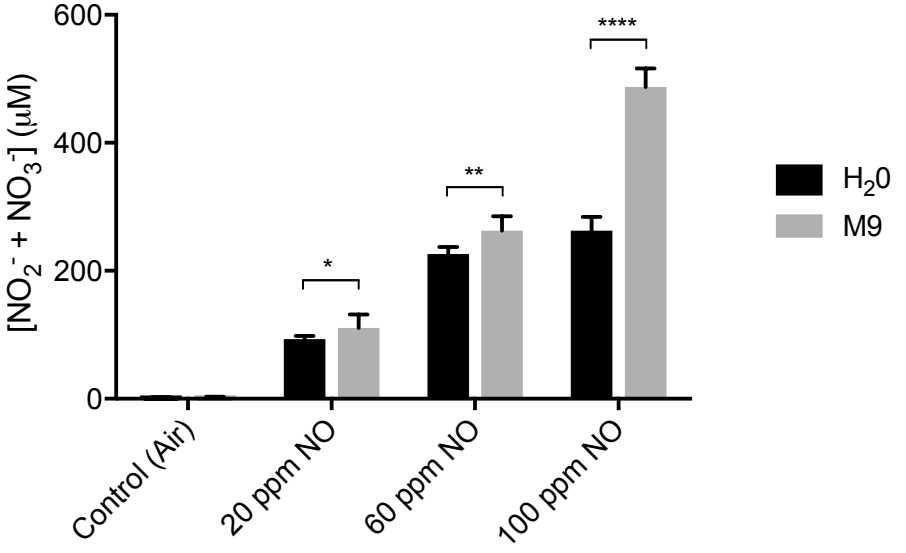


Figure 38. The absorption of NO into water and M9 minimal media, as demonstrated by quantified nitrite and nitrate levels. Water and M9 minimal media samples were exposed to 1 h of NO gas at various concentrations before testing with Griess reagents were immediately carried out to quantify nitrite and nitrate levels. Plotted data represents the mean and SD of 9 technical replicates, with two independent experiments displaying similar results. Welch's t-test was used for statistical analysis.

6.2.4 Treatment of biofilms with NO gas was not able to cause a statistically significant reduction in biofilm

Numerous attempts were made to investigate if utilising NO gas could disperse the biofilms formed by *P. aeruginosa* biofilms. Multiple and gradually increasing concentrations of NO gas were used in experiments. Initially, preliminary experiments were conducted and the effect on the biofilm measured using CLSM and COMSTAT analysis, as described in previous chapters. NO concentrations between 10 and 60 ppm were tested, along with varied treatment durations, and no significant impact was shown to occur on the *P. aeruginosa* biofilms (data not shown). It was then recognised that using CLSM would be too time-exhaustive and financially inefficient for the trial and error approach that was required. As the highest concentration the handheld NO monitor could record was 100 ppm, a final experiment was carried out utilising 100 ppm of NO for two hours at an incubation speed of 50 rpm with biofilms formed by clinical isolate PA68, shown in **Figure 39**. Unfortunately, despite detailed analysis, no effect of the gaseous NO was shown on *P. aeruginosa* biofilms. Total biofilm biomass values and CFU values, for both planktonic and biofilm cultures, showed non-significance between the NO treatment group and control air treatment group (Figure **39A-C**). The functionality of the eNO generator and production of NO gas throughout the duration of each of the independent experiments was verified using the handheld NO monitor (**Figure 39D**). Additionally, the absorption of NO gas into the biofilm media was also verified for each of the independent experiments (**Figure 39E**).

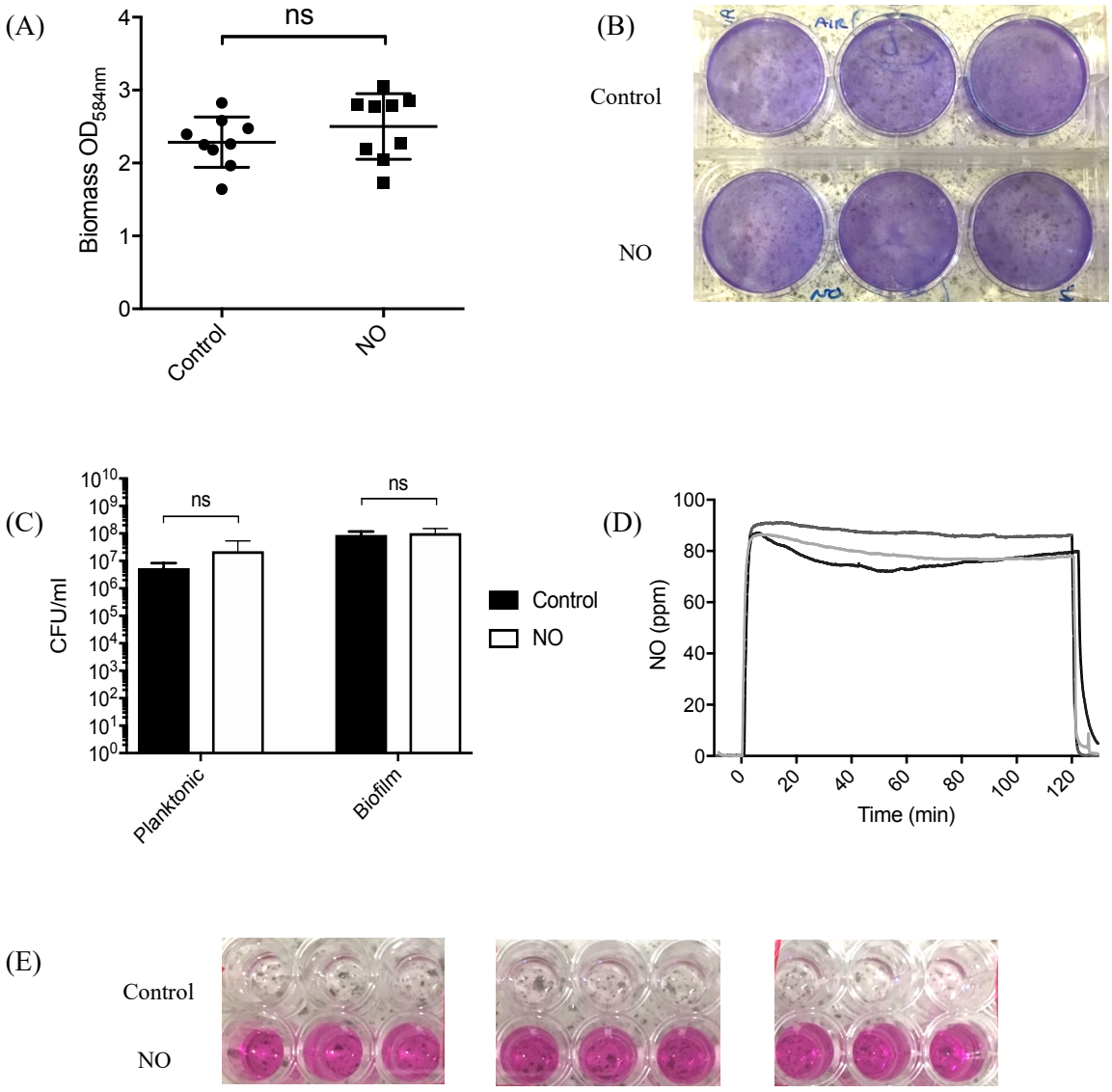


Figure 39. The effects of NO gas on mature *P. aeruginosa* biofilms *in vitro*. Biofilms were grown for 72 hours in 6 well plates and treated with either NO gas at 100 ppm, or the control gas (room air) for 2 h. Results summarise data from 3 independent experiments. (A) the biomass of biofilms treated with control gas and NO gas, quantified by CV staining. The mean and SD of nine technical replicates is shown. (B) an example photograph showing CV staining of biofilms in 6 well plates. (C) CFU counts of corresponding planktonic and biofilm cultures treated with control and NO gas. The mean and SD of nine technical replicates is shown. (D) NO concentrations recorded at the outlet for the duration of the three independent experiments. (E) photographs showing the positive reaction for nitrite from the three independent experiments. Welch's T tests were used for statistical analysis for (A) and (C).

6.3 Discussion

The development of a small-sized readily transportable device able to produce NO from room air provides an exciting opportunity to facilitate the use of gNO as an adjunctive therapy for CF patients with *P. aeruginosa* infection. Whilst the eNO generator prototype is capable of producing NO up to concentrations of 1500 ppm, suited for the treatment of wounds, the concentrations required to disperse *P. aeruginosa* biofilms in the CF lung are presumed to be much lower. Using a CLD, the efficacy, in terms of NO and NO_2 production, was investigated with lower set concentrations. Fortunately, the NO_2 production from the eNO generator fitted with an NO_2 filter was shown to be kept low, and to levels well below the 5 ppm threshold indicated by the Occupational Safety and Health Administration, suggesting lack for safety concerns at this stage.

Unfortunately, however, the error between the set NO concentration and the actual recorded concentration was shown to be high, particularly for lower NO set concentration values. We accept that the eNO generator prototype had originally been developed with the aim of treating wounds and would not be used at concentrations at or below 10 ppm for this purpose. Therefore, our suggestion is that whilst the device demonstrates no safety concerns at this stage, redevelopment would be necessary to produce a device more adept at producing lower concentrations of NO. A key limitation of this assessment was the detection range of the CLD. Higher concentrations of NO (over 10 ppm) could not be accurately measured as the CLD had a limited NO detection range, and a different CLD device would have been required, which, due to the high cost was not a feasible option. Though not as sensitive, the CrowCon NO monitor was utilised in instances to measure NO concentrations at higher values.

6.3.1 Attempts to disperse *P. aeruginosa* biofilms *in vitro* using the eNO generator

Whilst NO donors in solution have been used to successfully disperse *P. aeruginosa* biofilms *in vitro*, and gNO from has been shown to be beneficial to against *P. aeruginosa in vivo*, the use of gNO to disperse *P. aeruginosa* biofilms *in vitro* had not yet been investigated in the literature. Therefore, in this study, a custom-made experimental set up was designed to treat *P. aeruginosa* biofilms with gas. Two identical systems were designed, one with a chamber with NO gas passing through, and one with a chamber with room air passing through as a control. However, a number of issues had to be overcome and were taken into account upon designing of the system. One of the key issues was maintaining sterility; as such, inlet and outlet holes were made in the two gas chambers and fitted with autoclavable 0.22 μm filters and tubing. As previously mentioned, a key limitation was the narrow and small detection range of the CLD. Hence, an alternative and less sensitive device, the CrowCon handheld NO monitor, had to be utilised as part of these assays. Whilst the CrowCon NO monitor was initially designed to be placed within the chamber with NO gas, ensuring sterility of the monitor post-experiment could not be achieved or guaranteed without

compromising the electrical components. Therefore, the NO monitor was connected to the outlet tubing from the NO chamber; though this provided readings slightly different to those within the chamber itself, it provided confirmation that the experiment was correctly set up and no leaks were present.

Additional issues were noted with regards to sample preparation. For example, during preliminary investigations, aspiration of the biofilm culture media resulted in dehydration of the biofilm following treatment with gNO, most likely compromising cell viability and biofilm structure. Despite this, concerns were that a thick layer of media over the biofilm within the culture dish would prevent gNO penetrating down to the biofilm. Therefore, multiple different volumes of media were trialled, and a volume just high enough to prevent dehydration of the biofilm following exposure to the gas flow was selected.

Crucially, the absorption of NO into uninoculated liquid media was assessed using Griess reagents to test for NO breakdown products nitrite and nitrate following exposure to gNO. Results in this study demonstrate increased levels of nitrite and nitrate in water and M9 minimal media as the concentration of gNO was increased. This bears similarity to result by (Ghaffari et al., 2005); Griess reagents were utilised to measure NO breakdown products in saline and Dulbecco's Modified Eagle Medium (DMEM) are showed an increase in values as the exposure time to a consistent gNO concentration was increased. For lower concentrations of gNO, a marginal increase in the concentration of nitrite and nitrate in M9 minimal media compared to water was seen, whilst at the highest tested concentration of gNO this increase was more notable. Reasons for this are not entirely clear but likely relate to interaction of NO with components in the M9 minimal media, such as ammonium chloride and transition metals which are present in small amounts but can affect NO breakdown.

However, despite conformation of NO absorption into biofilm media, a conclusive dispersal action could not be achieved with gNO and *P. aeruginosa* biofilms. Many possible reasons could account for this, firstly, the penetration of NO through the liquid media could be brought into question. As the half life of NO is merely 1.5 seconds, whether the NO molecule was able to dissolve into solution and reach the biofilm at the bottom of the well before disassociating can be queried. In order to examine whether the biofilm had any exposure to NO or if the molecule had broken down before direct exposure to bacterial cells occurred, the use of NO specific probes would be necessary. NO specific probes were not used in this study due to technical difficulties in their incorporation into the NO chamber set up; most likely redevelopment of the NO chamber set up would be necessary to enable the use of NO specific probes and the measurement of NO at the bottom of the culture well. (Miller et al., 2009) had utilised gNO to treat planktonic cultures and demonstrated an effective kill rate with continuous or intermittent delivery at 160 ppm of NO. However, within a planktonic culture, bacterial cells are more evenly distributed through the liquid solution; this is in stark contrast to the biofilm culture utilised in this study where all cells are

located at the bottom of the well. This supports the notion that although NO can be absorbed into solution, it dissociates before it is able to diffuse through the liquid in the biofilm culture and reach the biofilm cells.

Another reason for why *P. aeruginosa* biofilms were not dispsersed by gNO in this study could relate to the age and growth conditions of the biofilm. Mature biofilms were grown (cultured for 72 h) in attempt to resemble the biofilm formation occurring the in CF lung; future work should seek to investigate if better results could be achieved with immature biofilms. Another complication relating to the use of gNO is the conversion of NO to NO_2 in the presence of oxygen. The NO chamber set up was not executed under completely anoxic conditions, due to both the technical difficulties of achieving this, and to ensure it was not dissimilar to the inhalation of NO by CF patients. It is worth considering however that the presence of oxygen has huge implications into the biofilm formation of *P. aeruginosa*, as demonstrated in this study in **Appendix G**. Though the vast majority of *in vitro* experiments in the current literature with *P. aeruginosa* are carried out in aerobic conditions, the CF lung has microaerophilic and anoxic niches. In comparison to growth in aerobic conditions, in this study, *P. aeruginosa* biofilm growth in microaerophilic conditions demonstrated enlarged clumped structures and a higher dead cell to live cell ratio. Whilst in anaerobic conditions (supplemented with potassium nitrate), the biofilm growth and structure were in stark contrast to biofilms grown in anaerobic and microaerophilic conditions; growth was slower and multiple densely packed, isolated cell aggregates were formed as opposed to a single large structure, with the cells demonstrating good viability. These huge differences in the resultant biofilm in aerobic, microaerophilic and anaerobic conditions may explain why in this study we were unsuccessful in dispersing the biofilm. To investigate the effects of gNO on biofilms cultured in all three conditions would have been of interest, however time was the key limiting factor in this instance, in addition to the labour intensity and expense of CLSM use. These experiments would be a priority for future work.

Following the failure of gNO, even at the highest concentrations, to disperse the *P. aeruginosa* biofilms, an alternative approach was investigated. As SNP is known and has previously been used in this study to disperse biofilms, an attempt was made to equate the concentration of NO released by SNP for a concentration successful in dispersing the biofilm to a concentration of gNO. This could be achieved by exposing biofilms to a range of SNP concentrations, deciding which of these concentrations was the most effective in dispersing the biofilm, measuring the nitrite and nitrate in solution, and then investigating by trial and error what concentration of gNO would result in a similar nitrite and nitrate value in solution. Hence, in theory, this concentration of gNO should therefore be effective in biofilm dispersal.

Though this approach was attempted, it was not successful; primarily due to the differences in optimal treatment duration between SNP and gNO. Multiple studies, current one included, have demonstrated that SNP is most effective with long term treatment durations and this was an

additional limiting factor. In this study, treatment durations for gNO with the eNO generator were attempted for 2 h or 6 h. A treatment duration for longer than 6 h was not attempted with the eNO generator due to the recommendation by NitricGen that the generator be constantly monitored whilst in use. Additional time required for the assembly of the experimental system, biofilm culture preparation, biofilm and nitrite/nitrate analysis following treatment, and disassembly and sterilisation of the experimental system at the end prevented an exposure time longer than 6 h being feasible. Yet treatment of biofilms for 2 h or 6 h with multiple concentrations with SNP did not result in dispersal; increasing the concentration had resulted in reductions in cell viability as opposed to biofilm dispersal, most likely due to the toxic by-products of SNP (preliminary experiments, data not shown). As a suggestion for future work, either the treatment duration with gNO could be increased to 8 h, the recommended sleep duration for adults, or further optimisation with different faster-acting NO donors could be carried out.

6.3.2 Potential of the eNO generator as a novel therapy for CF patients

Whilst in this study we have failed to demonstrate the ability of gNO produced from the eNO generator to disperse *P. aeruginosa* biofilms *in vitro,* previous *in vitro* evidence and data from the proof-of-concept clinical trial (Howlin et al., 2017) indicate the potential for this device to be utilised as a novel therapy for CF patients. Based on the unsuccessful results from *in vitro* experiments, we suggest an *in vivo* experiment could yield better and more clinically relevant results. The Miller group had followed up their 2009 publication using gNO on planktonic cultures of *P. aeruginosa* with a *in vivo* study examining the effect of gNO in a murine pulmonary infection model, with the aim of using high concentrations to exert a direct antibacterial effect (Miller et al., 2013). Lungs of mice infected with *P. aeruginosa* and treated with 160 ppm of NO for 30 mins every 4 h, resulted in a 1 log reduction in *P. aeruginosa* CFU count after 12 h of treatment and a 2 log reduction after 24 h. If a similar *in vivo* experiment were to be executed using the eNO generator and lower gNO concentrations, and results were positive, this would demonstrate that high gNO concentrations are not needed and lower gNO concentrations can be utilised to tackle the infection via molecular signalling and dispersal.

6.3.3 Conclusions

In this chapter, the objective was to investigate the ability of the eNO Generator to disperse *in vitro P. aeruginosa* biofilms, and to determine if this novel device has potential to be used by CF patients as an adjunctive treatment. Initial testing demonstrated that at low concentrations of NO the accuracy of the NO output varied greatly, however the levels of toxic by-product NO_2 were below set safety standards. Unfortunately, whilst NO produced by the eNO generator was shown to be absorbed into solution of laboratory media, we were unable to show this caused dispersal of *P. aeruginosa* biofilms *in vitro.* However, we conclude that these data do not demonstrate that the

eNO generator is not suitable for use in CF, but that further, and most likely *in vivo*, investigations are needed.

Chapter 7 Conclusions and Future Research

For CF patients, the acquisition of infection with *P. aeruginosa* is a clinical event with serious implications as treatment with current antibiotics cannot prevent or adequately combat *P. aeruginosa* biofilm growth. The 2006 discovery of NO as an anti-biofilm dispersal agent led to an influx of research and provided the potential for a new NO based anti-biofilm therapy to target *P. aeruginosa* in CF. Multiple sources have described the dispersal effects of NO on *P. aeruginosa* biofilms, though most publications have exclusively utilised PAO1 or other non-clinically relevant strains of *P. aeruginosa*.

7.1 NO has varying effects on clinical CF isolates of *P. aeruginosa*

In this study, we examined the effect of NO on CF isolates of *P. aeruginosa* and, importantly, presented evidence that this effect is not identical amongst different CF isolates. Whilst some clinical isolates were dispersed by NO, other isolates had biofilm formation promoted with a long treatment period with NO donor SNP. Phenotypic and genotypic variations between CF isolates and other *P. aeruginosa* isolates, and between different CF isolates, are well recognised, due to the selective pressures in the CF lung and the adaptation required for the bacterium to survive. This study corroborated this by demonstrating the phenotypic distinctions amongst the panel of CF isolates, showing vast variations in colony morphology, mucoidy, pigment production, growth rates, and AMS. Interestingly, a correlation was found whereby isolates with a slower biofilm growth were less susceptible to NO induced dispersal. Cai (2018) supported this finding by showing some isolates of *P. aeruginosa* were not able to be dispersed by shorter acting NO donor Spermine NONO-ate, and one isolate had an increased number of cells within its cell aggregates. Additionally, Zhu et al. (2018) found that PAO1 biofilms were not susceptible to NO induced dispersal if the cells were previously cultured with NO, supposedly due to an increase in expression of NO scavenging proteins.

C-di-GMP has been implicated in the mechanism for NO induced dispersal; with levels thought to be reduced by the action of PDEs with GGDEF and EAL domains. In this study, we showed that even with isolates where biofilm dispersal had not occurred following NO treatment, the c-di-GMP level was reduced in a similar manner to isolates which were dispersed by NO. This suggests that the increase in biofilm formation following NO treatment for selected isolates is independent of the c-di-GMP pathway.

We suggested that the increase in biofilm formation of some clinical CF isolates following treatment with NO relates to their adaptation to the environment of the CF lung. This is further supported by the correlation that non-dispersing strains were slower growing, and slow growth is a known adaptation to the lung. As such, these strains are likely to be more adept to anaerobic

respiration and denitrification, calling into question whether NO itself or its breakdown products could be utilised as nutrient sources by the bacterium as part of the denitrification process. This theory was supported by the fact that with a long term treatment period, biofilm formation was promoted, but with a short term treatment period, though dispersal was not noted, promotion was not seen either. A longer treatment duration correlates with an increased concentration of breakdown products. An alternative theory suggested relates to the exposure of the bacterium in the lung to NO produced by immune cells during the response to infection. Adaptation to the low NO concentrations produced by these cells may have resulted in a modulated dispersal response to NO. Efforts were made to investigate if genetic based 'resistance' to the NO dispersal effect could be induced *in vitro* via a passaging experiment, however the results were not conclusive enough to make a conclusion. Further investigations are needed to discover the reason for the increased biofilm formation with SNP treatment and to confirm whether this is from a direct result of NO or indirectly caused by the accumulation of NO breakdown products *in vitro*.

7.2 Lead C3D compound DEA-CP is an effective *P. aeruginosa* biofilm dispersal agent against clinical CF isolates

In order to tackle the issue of the lack of adequate anti-biofilm therapies in CF, this study investigated the use of C3Ds. Lead compound, DEA-CP, was investigated first, and was shown to successfully release NO in a selective manner following exposure to bacterial specific enzymes, suggesting this compound to be a well-designed targeted therapy, minimising potential adverse effects to the host. Results in this study were consistent with those in the literature in that DEA-CP could successfully disperse PAO1 biofilms, and additionally, resulted in the dispersal of biofilms formed by clinical CF isolates of *P. aeruginosa*. Biofilm dispersal was confirmed via two techniques, a common general biofilm staining method and a most robust microscopy-based analysis allowing for 3D visualisation and quantification of biofilm parameters.

Excitingly, the combination of DEA-CP and two common anti-pseudomonal antibiotics were effective against the biofilms. Though only an additive effect was seen with tobramycin, a more synergistic effect was noted between DEA-CP and colistin. The synergy between DEA-CP and colistin could be explained by the effect of colistin on the Gram-negative bacterial cell membrane. In addition to β-lactamases, PBPs have also been theorised to cleave C3Ds. Therefore, the membrane perturbation and disruption caused by colistin could allow DEA-CP to cleaved by previously inaccessible PBPs in the periplasm, resulting in an enhanced and even more localised NO release. Therefore, we conclude that DEA-CP could become a viable novel therapy for use in CF, particularly if it is combined with colistin.

7.3 New generation C3D compound AMINOPIP2-ceftazidime has both anti-bacterial and anti-biofilm effects on clinical CF isolates of *P. aeruginosa*

Despite the effectiveness of DEA-CP, this compound had no direct anti-bacterial activity against *P. aeruginosa*, and therefore attention turned to whether a C3D could be developed with both anti-bacterial and anti-biofilm actions. Twelve new generation C3Ds with varying cephalosporin bases (cephaloram, cefalexin, cefuroxime, ceftazidime, cefepime and cefozopran) were examined and all were shown to have selective NO release. As suspected, the agents with 1st and 2nd generation cephalosporin bases did not show any antimicrobial activity against *P. aeruginosa* isolates. The addition of aminopiperidine based diazeniumdiolates were shown to be superior compared to the pyrrolidine based diazeniumdiolates, particularly with ceftazidime as the antibiotic core. We concluded that this was a result of the chemical structure of the aminopiperdine based diazeniumdiolate, and that more specifically the 6 membered ring and the amino groups were able to reinstate the zwitterionic character of the molecule and provide increased membrane penetration compared to both the original ceftazidime antibiotic and the pyrrolidine based ceftazidime C3D.

Of the twelve new generation C3Ds, four were identified as having anti-biofilm activity superior to their respective parent antibiotic. Of these four agents, just one, AMINOPIP2-ceftazidime, also had substantial anti-bacterial effects. When examined with CF isolate PA68, AMINOPIP2-ceftazidime caused a statistically significant reduction in biofilm biomass compared to both the untreated control and ceftazidime treated biofilms. Against a second CF isolate, PA30, AMINOPIP2-ceftazidime did not reduce the biofilm biomass compared to the untreated control, though was significantly better than the ceftazidime antibiotic which promoted biofilm growth. As such, from the twelve new generation C3Ds, we concluded AMINOPIP2-ceftazidime is the compound with the best dual-action, and this compound should be investigated further for its potential to be developed as a novel CF therapy.

7.4 *In vitro* experiments did not demonstrate the effects of gNO but the eNO generator remains a potential novel therapeutic device for CF patients

Despite a recent clinical trial demonstrating the effectiveness of low dose NO in CF patients, the sole method of delivering gNO remains via gas cylinders, which is hazardous,

expensive and requires specialist training. A novel NO delivery device, the eNO generator, is able to produce NO from room air by creating sparks between two electrodes. This device is readily transportable due to its small size, does not require specialist training and is more economically sustainable than using NO gas cylinders. Analysis of the gas produced by the eNO generator revealed that at lower set concentrations of NO the accuracy varied immensely, suggesting that the novel prototype device may need some optimisation. However, the low levels of NO_2 produced by the eNO generator are reassuring and are below the set safety standards. Whilst we were able to demonstrate the absorption of NO gas into water and liquid biofilm media, we were unable to conclude that biofilm dispersal had occurred with gNO treatment *in vitro*. Numerous possible reasons exist for this, including insufficient penetration of NO through the liquid layer to reach the biofilm cells. However, despite this, we re-iterate that the eNO generator could still a viable option for CF patients as a potential novel adjunctive therapy and suggest that *in vivo* investigations may be more worthwhile to further explore this option.

7.5 Follow on research and plans

In this study, we have shown that whilst NO has previously been demonstrated to be an effective anti-biofilm molecule, its effects on clinical CF isolates of *P. aeruginosa* vary. This is an extremely important note to consider if research into NO-based anti-biofilm strategies is continue with the aim of delivering novel therapies to patients in a clinical setting. The correlation between biofilm growth and response to NO is another important point to consider. This indicates that whilst we may accept that certain isolates of *P. aeruginosa* may not respond and disperse as well as other isolates do, there is a potential to predict how isolates may respond, leading the way to a more personalised therapeutic approach. For example, patient sputum samples could be analysed and the phenotype or genotype of the infection characterised to determine whether NO would be a beneficial adjunctive therapy. Alternatively, follow on research or clinical trial data could reveal that a subset of patients, for example younger patients at risk of acquiring *P. aeruginosa* infection or those recently infected, may benefit more from NO treatment than other patient subsets, such as those chronically infected. A new and soon to be launched Strategic Research Centre (SRC), again funded by the UK CF Trust and bringing together the research teams in Southampton and Imperial College London, will investigate *P. aeruginosa* infection in CF, this time with a focus on personalised medicine, nicknamed ‘PAPA’ (Personalised Approach to *P. aeruginosa*). This research theme links well to the potential follow-on research plans from this study, and therefore could form the basis of the Southampton-based PhD project in the new SRC.

More specifically, we suggest the next stages of research should look towards 'omic' investigations. For example, comparison of the genomes between isolates dispersed by NO and those that had biofilm promotion following NO treatment could provide some insight and understanding. Whole genome sequencing could be carried out with focus on genes associated with NO sensing, dispersal, denitrification, anaerobiosis, and, in particular *lasR*. However, of higher priority may be transcriptomic analysis; RNA-sequencing of a select number of isolates with and without NO treatment would be key to understanding how the bacterium responds to NO, and how the response differs with different isolates. Furthermore, in the laboratory, additional experiments that may be worth conducting include using either nitrite, nitrate or SNP exposed to light for an extended period of time, to treat *P. aeruginosa* clinical biofilms and elucidate if the biofilm effect is due to NO or NO breakdown products. We also suggest that work should move towards exploring the effects of NO in multi-species or multi-isolate containing biofilms, and that assays be developed to properly investigate the effects of NO on biofilms grown in microaerophilic and anaerobic conditions.

This study also served as part of the pre-clinical drug development of C3Ds. We identified a single compound, AMINOPIP2-ceftazidime, from a group of analogues as the most promising 'all-in-one' agent, demonstrating both anti-bacterial and anti-biofilm function against multiple clinical isolates of *P. aeruginosa in vitro*. As a direct result of this, our colleagues in Australia are in the process of commissioning next stage *in vivo* experiments using a murine pulmonary infection model to compare the efficacy of AMINOPIP2-ceftazidime against the currently used CF antibiotic ceftazidime. The compounds investigated in Chapter 5 are also currently being investigated *in vitro* against other clinically relevant pathogens, including *E. coli*, *S. aureus*, *K. pneumoniae*, and *Neisseria gonnorheoae* by other research groups, both in Southampton and globally across multiple institutions.

Whilst we were unable to demonstrate the effectiveness of using gNO produced from the eNO generator against *P. aeruginosa* clinical biofilms grown *in vitro,* we recommend that *ex vivo* or *in vivo* experiments may be better suited to assess the effectiveness of the eNO generator against *P. aeruginosa* biofilms, as discussed in Section 6.3.2. Our research group in Southampton are also investigating the use of NO, but within 'microbubbles' as a way of delivering NO to the site where its required, and the eNO generator has been utilised to generate NO filled microbubbles. In addition, future work in our group will include looking at the effects of NO on wound relevant pathogens in *ex vivo* pig and human skin models; in addition to the use of NO donors in solution, the eNO generator could be

utilised in these experiments to investigate the effects of NO gas on biofilm dispersal and wound healing.

Appendix A - Chemical structures of C3D compounds

Table 12. Chemical formula, molecular weight and chemical structures of DEA-CP potassium salt and DEA-CP carboxylic free acid

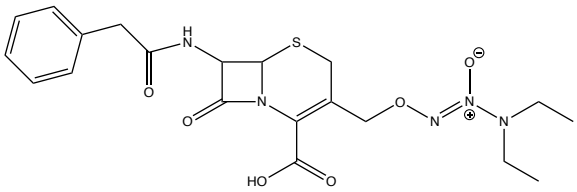
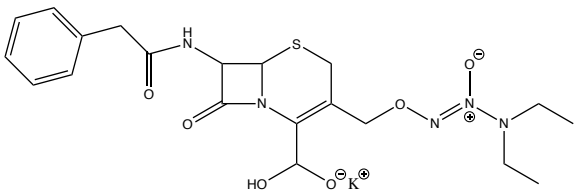
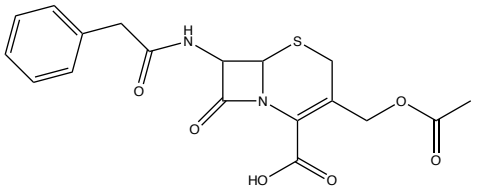
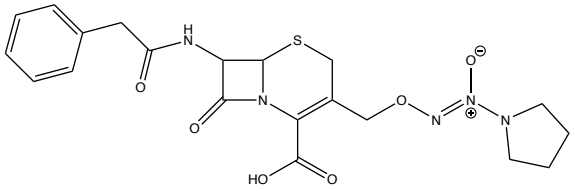
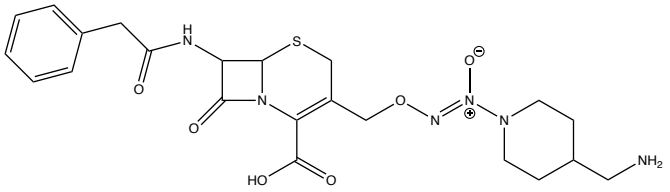
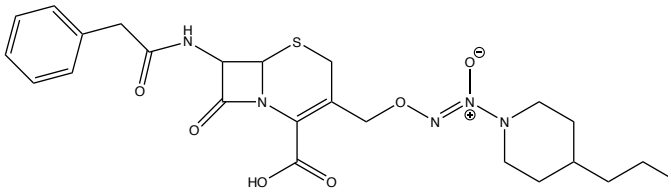
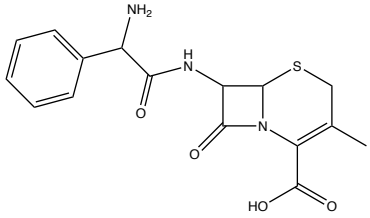
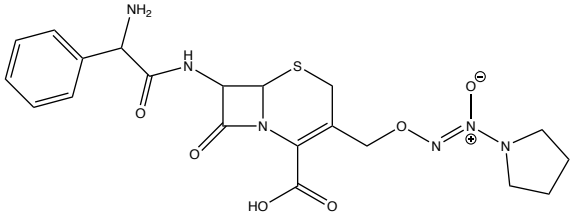
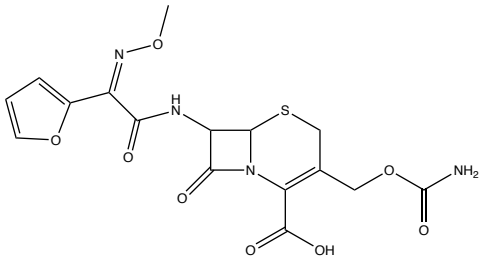
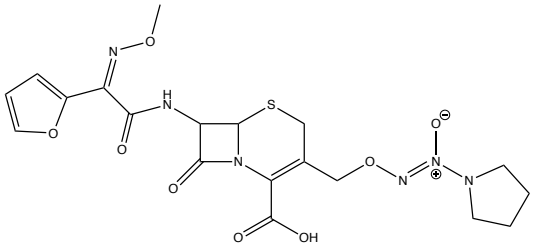
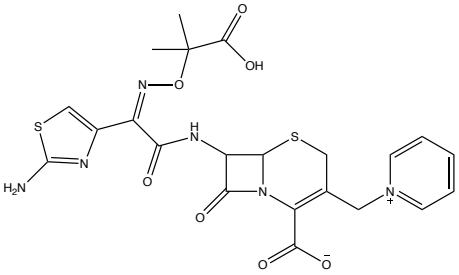
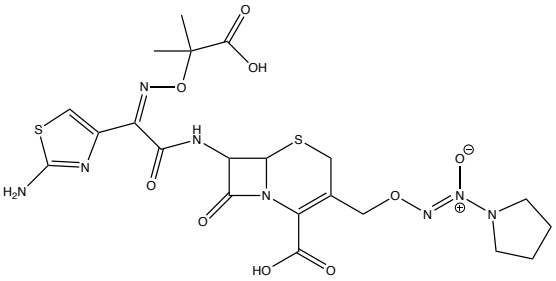
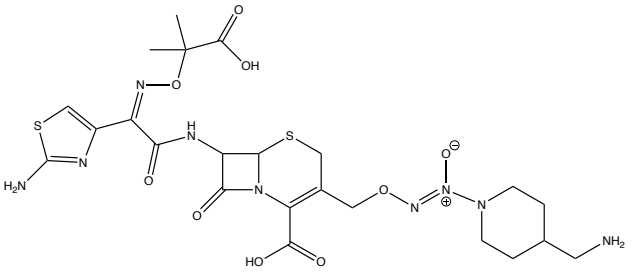
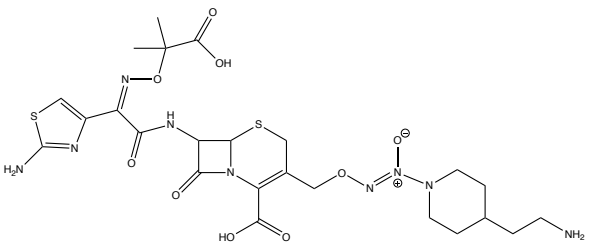
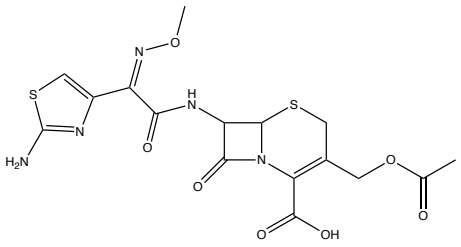
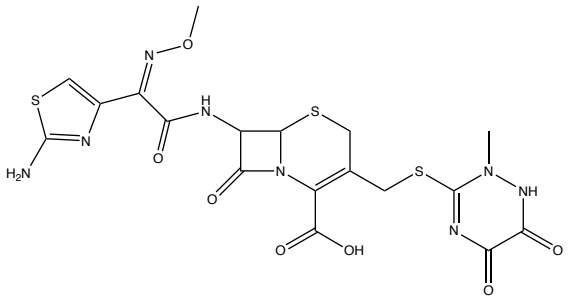
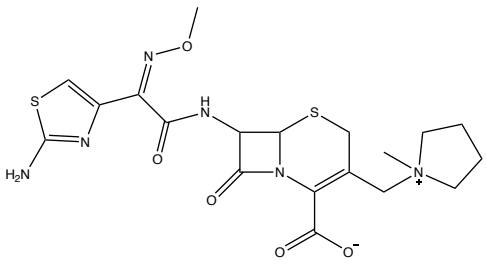
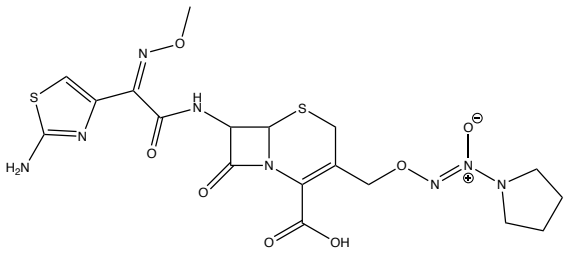
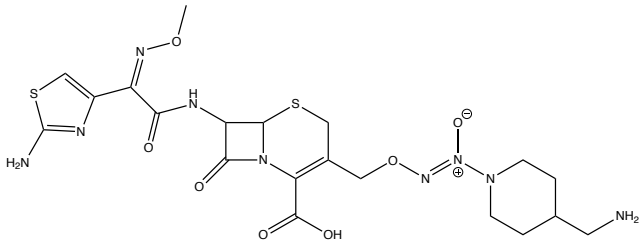
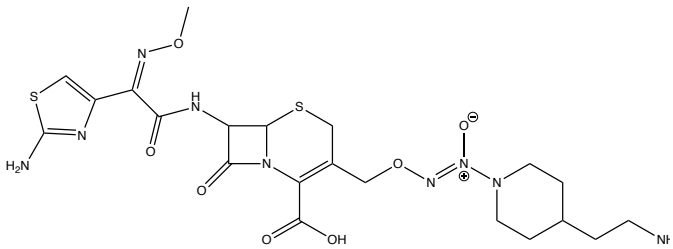
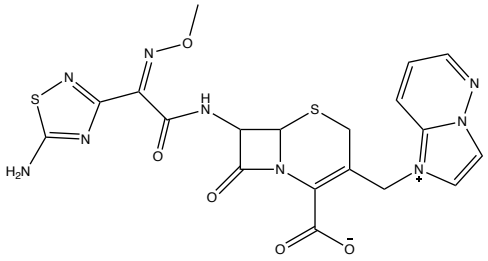
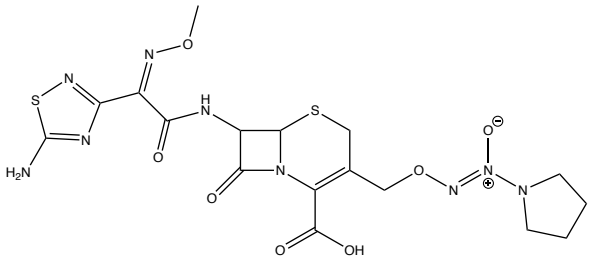
Compound	Chemical Formula	Molecular Weight (g/mol)	Chemical structure
DEA-CP carboxylic free acid	$C_{20}H_{25}N_5O_6S$	463.51	
DEA-CP potassium salt	$C_{20}H_{23}KN_5O_6S$	501.60	

Table 13. Chemical formula, molecular weight and chemical structures of nine antibiotics and twelve new generation C3D compounds examined in Chapter 5

Compound (Abbr.)	Chemical Formula	Molecular Weight (g/mol)	Chemical structure
LAM	$C_{18}H_{18}N_2O_6S$	390.41	
P-LAM	$C_{20}H_{23}N_5O_6S$	461.49	
A1-LAM	$C_{22}H_{28}N_6O_6S$	504.56	
A2-LAM	$C_{23}H_{30}N_6O_6S$	518.59	
CEX	$C_{16}H_{17}N_3O_4S$	347.39	
P-CEX	$C_{20}H_{24}N_6O_6S$	476.50	

Compound (Abbr.)	Chemical Formula	MW (g/mol)	Chemical structure
CXM	$C_{16}H_{16}N_4O_8S$	424.38	
P-CXM	$C_{19}H_{22}N_6O_8S$	494.47	
CAZ	$C_{22}H_{22}N_6O_7S_2$	546.58	
P-CAZ	$C_{21}H_{26}N_8O_9S_2$	598.60	
A1-CAZ	$C_{23}H_{31}N_9O_9S_2$	641.68	
A2-CAZ	$C_{24}H_{33}N_9O_9S_2$	655.70	

Compound (Abbr.)	Chemical Formula	MW (g/mol)	Chemical structure
CTX	$C_{16}H_{17}N_5O_7S_2$	455.47	
CRO	$C_{18}H_{18}N_8O_7S_3$	554.58	
FEP	$C_{19}H_{24}N_6O_5S_2$	480.56	
P-FEP	$C_{18}H_{22}N_8O_7S_2$	526.54	
A1-FEP	$C_{20}H_{27}N_9O_7S_2$	569.61	
A2-FEP	$C_{21}H_{29}N_9O_7S_2$	583.64	

Compound (Abbr.)	Chemical Formula	Molecular Weight (g/mol)	Chemical structure
CZOP	$C_{19}H_{17}N_9O_5S_2$	515.52	
P-CZOP	$C_{17}H_{21}N_9O_7S_2$	527.53	

Appendix B - Supplementary data for phenotypic experiments in Chapter 3

Table 14. MIC values for tobramycin, ceftazidime, and colistin against PAO1 and twenty CF isolates

Strain	TOB MIC (all n=1)	CAZ COMB. * = from Ch3 and n=3	COL COMB. *=from Ch3 and n=1
PAO1	1	1*	2*
PA05	0.5	1*	1*
PA08	2	32*	1
PA10	2	1*	1*
PA15	4	16	2
PA20	2	1	2
PA21	4	16*	2*
PA26	4	64*	2*
PA30	2	4*	2*
PA31	8	0.5	8
PA37	2	128*	2*
PA39	1	2	2
PA44	4	4*	4*
PA47	4	0.5	8
PA49	1	32	2
PA55	8	2	4
PA56	0.5	4*	2*
PA57	0.5	4*	2
PA58	16	16*	2*
PA66	4	128*	2
PA68	2	4*	2*

TOB = tobramycin; CAZ = ceftazidime; COL = colistin. All in μg/ml.

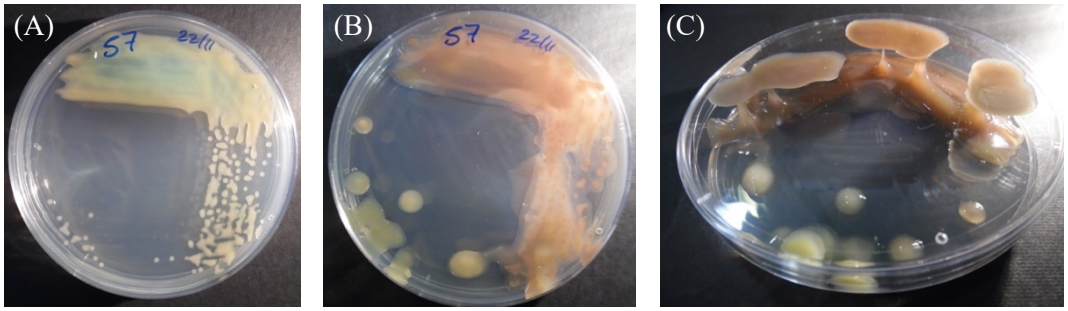


Figure 40. Photographs showing the hyper-mucoid phenotype of clinical CF isolate PA57. (A) with two days incubation at 37°C, (B) and (C) with 7 days incubation at 37°C.

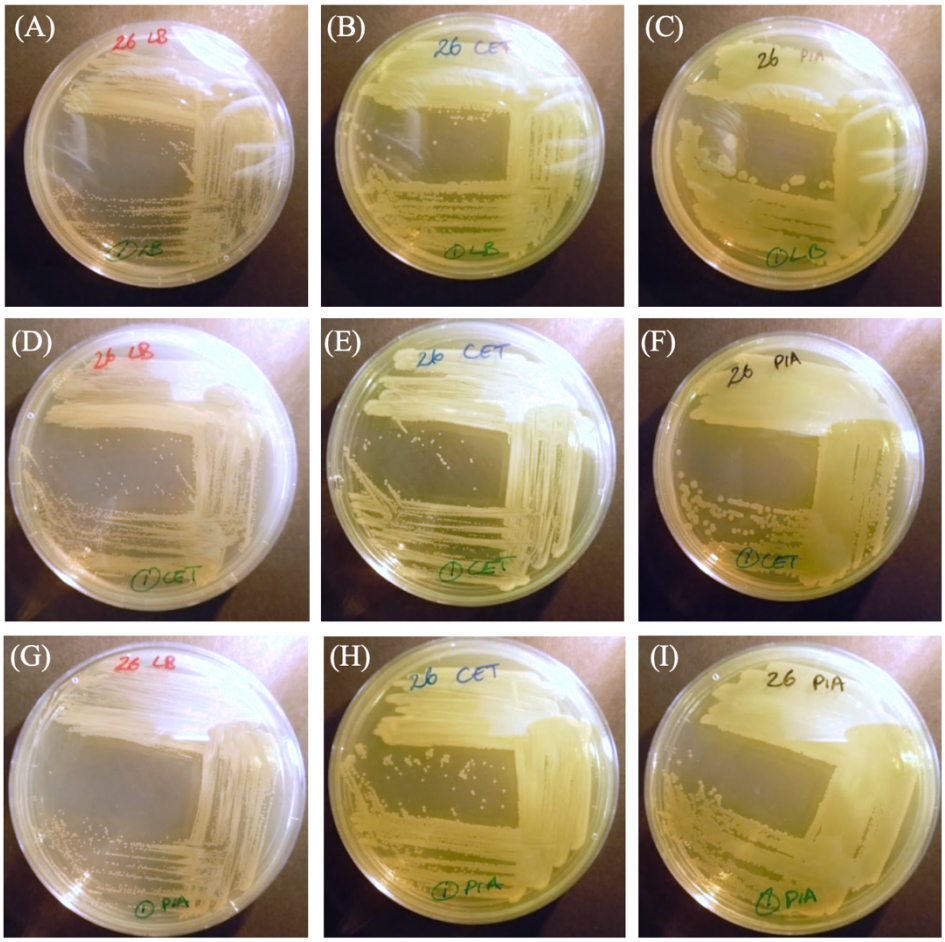


Figure 41. Photographs showing the colony size and phenotype of clinical CF isolate PA26 is consistently dependent on the agar used for culturing. (A), (B) and (C), PA26 culture restreaked from LB agar onto LB, cetrimide agar and PIA respectively. (D), (E) and (F), PA26 culture restreaked from a cetrimide agar onto LB, cetrimide and PIA respectively. (G), (H) and (I), PA26 culture restreaked from a PIA agar onto LB, cetrimide and PIA respectively.

Appendix C - Additional data for *P. aeruginosa* biofilms treated with SNP

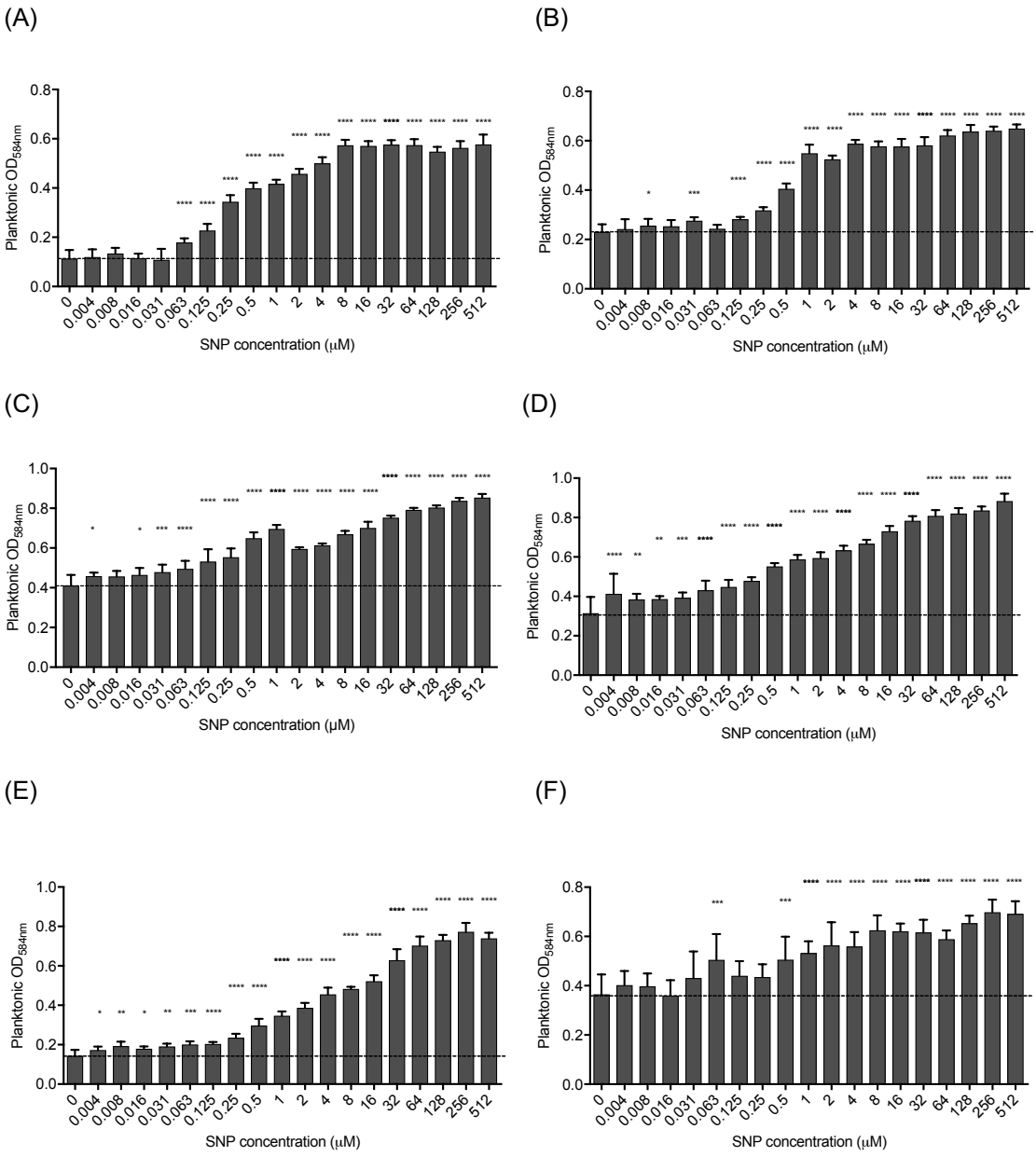


Figure 42. Planktonic growth values for *P. aeruginosa* biofilms treated with SNP concentrations ranging from 4 nM to 512 μM; (A) PAO1, (B) PA10, (C) PA21, (D) PA26, (E) PA30 and (F) PA68. One-way ANOVA statistical testing with treatment groups compared to untreated control group.

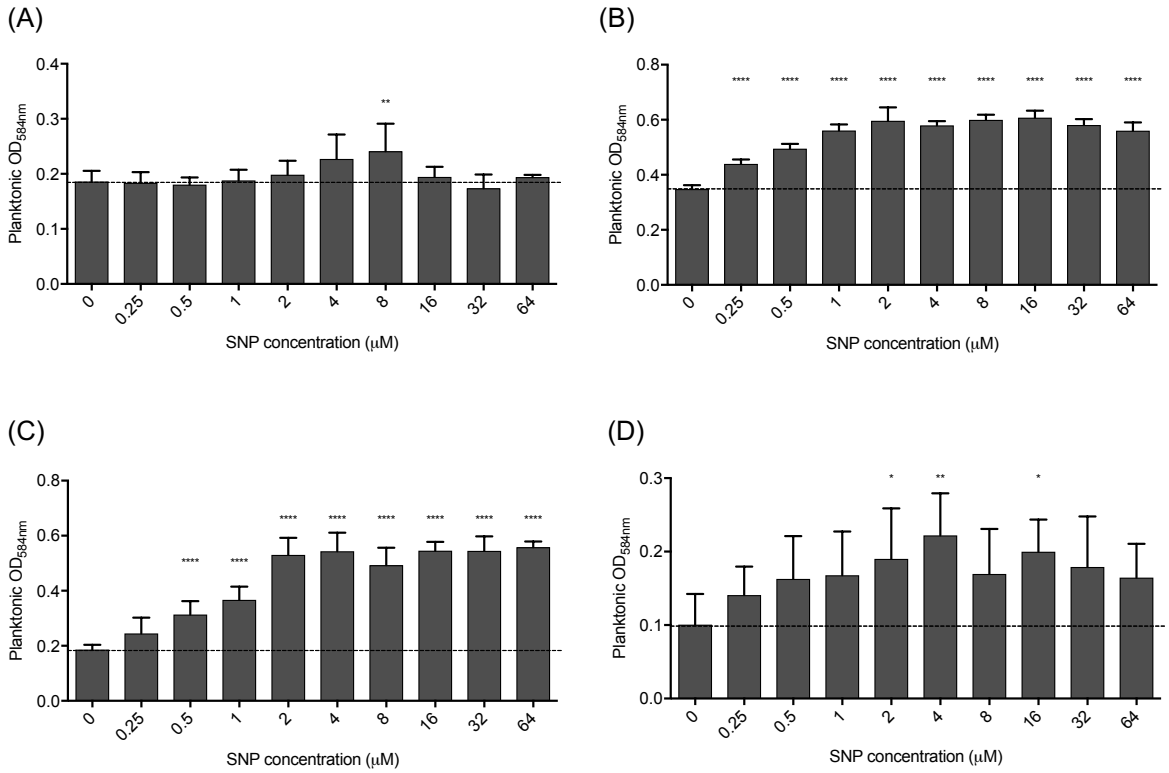


Figure 43. Planktonic values for *P. aeruginosa* biofilms treated with SNP concentrations ranging from 250 nM to 64 μM for (A) PA20, (B) PA47, (C) PA56, and (D) PA66. One-way ANOVA statistical testing with treatment groups compared to untreated control group.

Appendix D - Preliminary passaging experiment

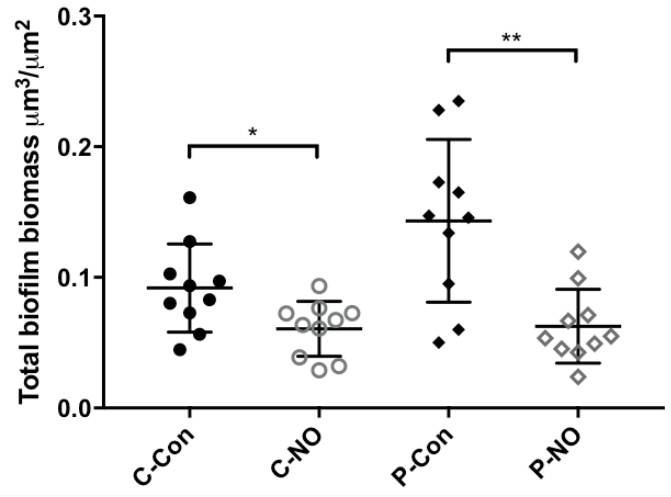


Figure 44. Total biomass of biofilms treated with and without NO; passaged 5 times planktonically then biofilms grown for 24 h then treated with 1 mM SNP. C-passaged naturally, P- passaged with 1 nM SNP. No loss in dispersal effect with NO after 5 passages.

Appendix E - Additional data for biofilm assays with DEA-CP potassium salt

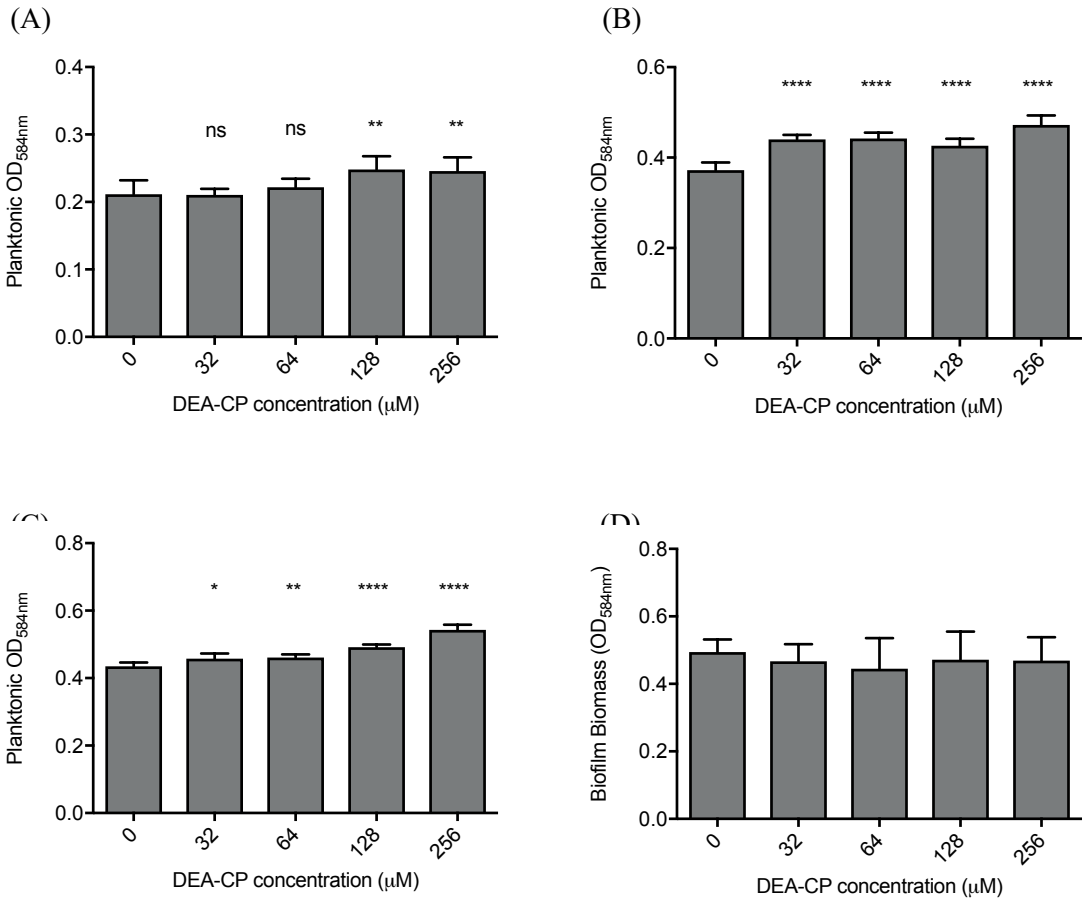


Figure 45. Planktonic measurements of biofilm dispersal experiments (results in **Figure 22)**. Graphs show mean value and SD; based on 6 technical replicates. (A) PAO1, (B) PA21, (C) PA30 and (D) PA68. Welch's T test were used for statistical analysis.

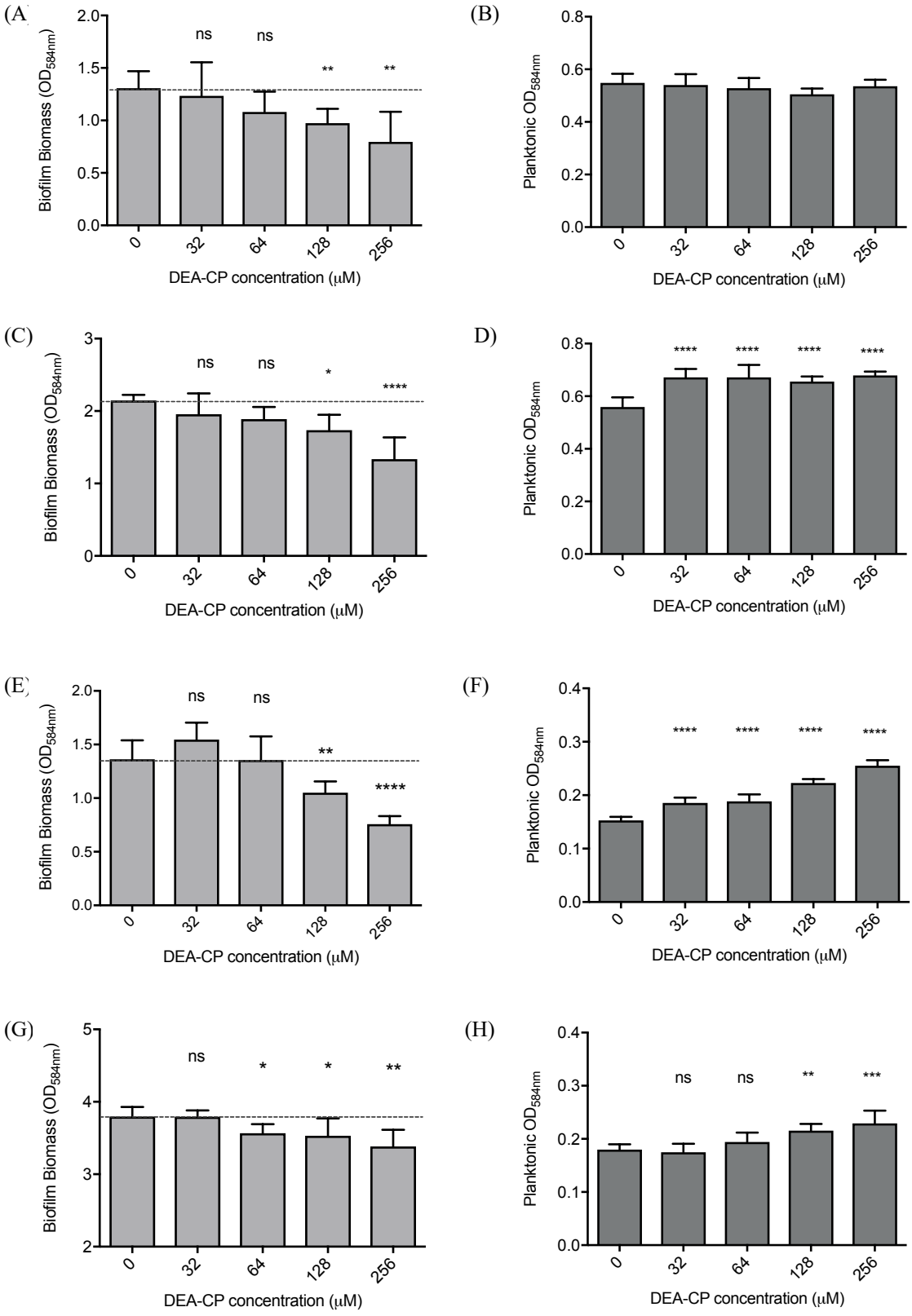


Figure 46. Biological replicate results of experiments detailed in **Figure 22.** Biofilm biomass values and planktonic measurements shown respectively for (A) and (B) PAO1, (C) and (D) PA21, (E) and (F) PA30, and (G) and (H) PA68. Welch's T test were used for statistical analysis.

Appendix F - Additional COMSTAT analysis of biofilms treated with DEA-CP and tobramycin/colistin

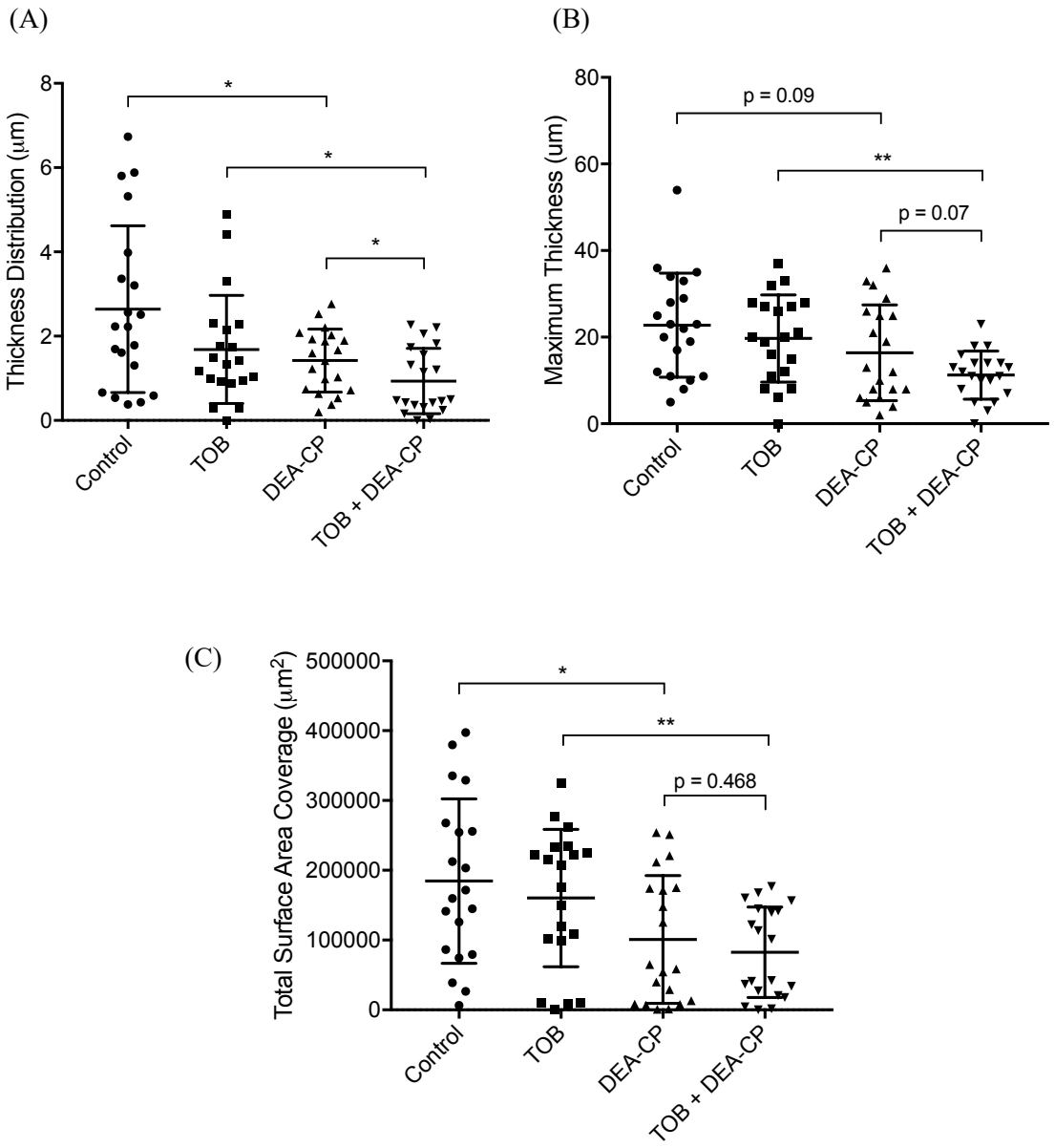


Figure 47. Scatter plots of data featured in **Table 7**; (A) biofilm thickness distribution, (B) maximum thickness, and (C) surface area coverage of PA68 biofilms treated with tobramycin and DEA-CP.

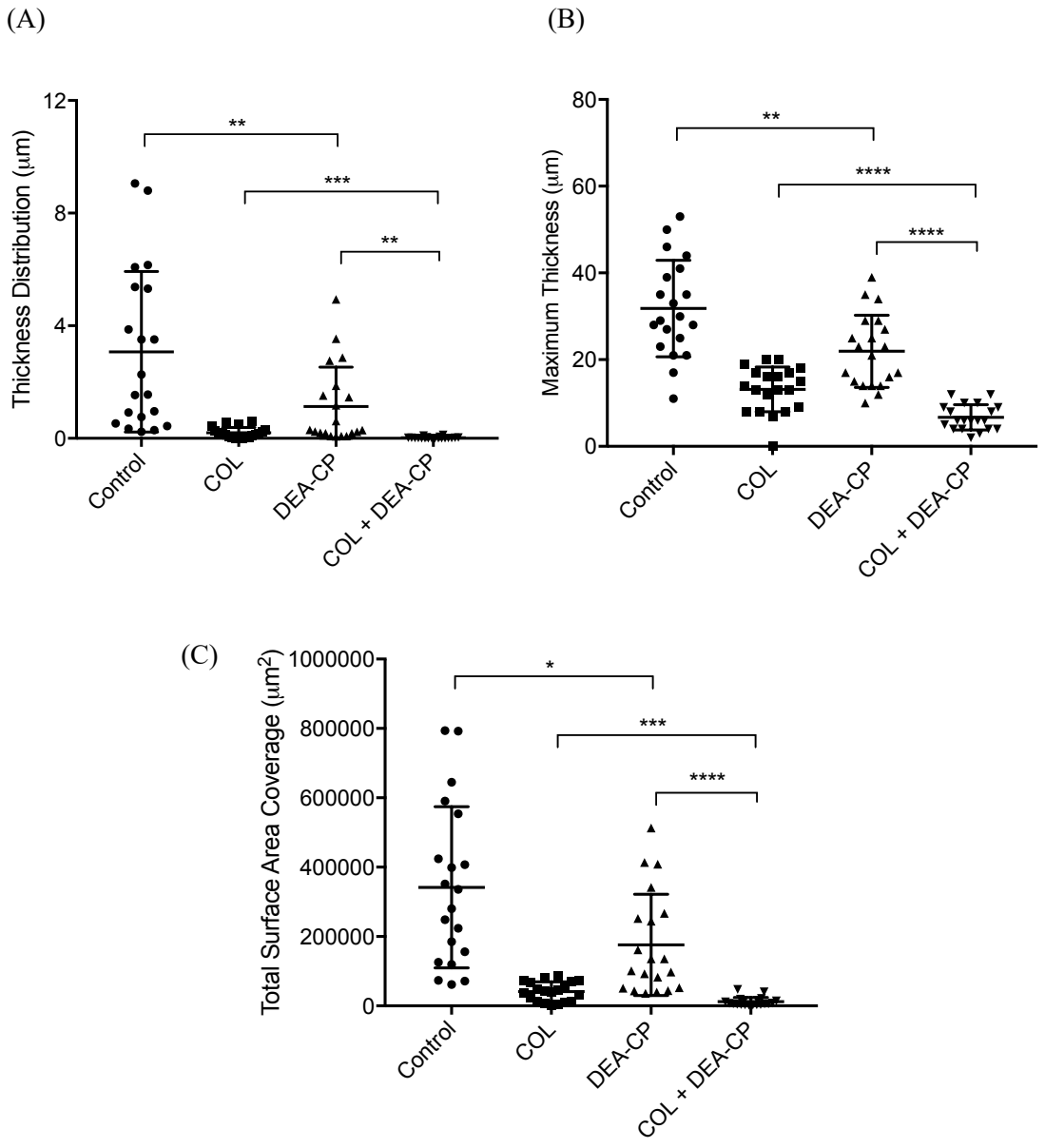


Figure 48. Scatter plots of data featured in **Table 8**; (A) biofilm thickness distribution, (B) maximum thickness, and (C) surface area coverage of PA68 biofilms treated with colistin and DEA-CP.

Appendix G - Biofilm formation with different oxygen concentrations

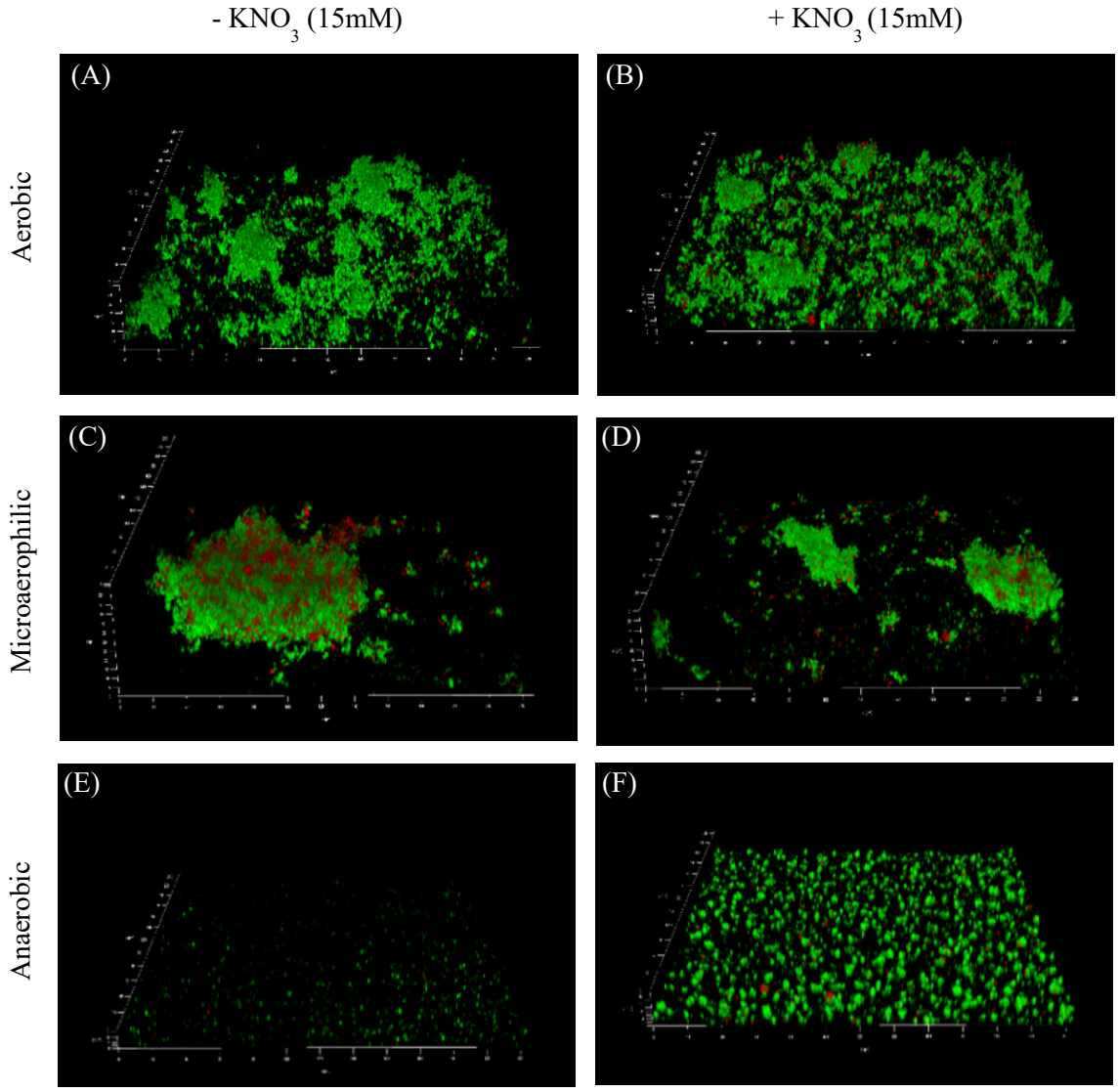


Figure 49. PA68 biofilms grown in varying oxygen concentrations; aerobic (A and B), microaerophilic (C and D) and Anaerobic (E and F). Biofilms were grown for 24 h in M9 minimal media at pH 6.5, with or without potassium nitrate supplementation (15 mM), before staining with SYTO9 and PI, and analysis using CLSM.

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