LEABHARLANN CHOLÁISTE NA TRÍONÓIDE, BAILE ÁTHA CLIATH | TRINITY COLLEGE LIBRARY DUBLIN
Ollscoil Átha Cliath | The University of Dublin

Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Anti-infective strategies in *Pseudomonas aeruginosa* biofilm formation

A thesis submitted to Trinity College, Dublin for the degree of Doctor of Philosophy

Priya Kapoor, BSc

Department of Clinical Microbiology

2014

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

Signed.....Priya Kapoor.....

TRINITY COLLEGE
5 JAN 2015
LIBRARY DUBLIN

Thesis 10820

Summary

P. aeruginosa infection is one of the main causes of lung function decline in patients with cystic fibrosis (CF). *P. aeruginosa* is associated with biofilm formation which can increase the rate of resistance to antibiotic treatment. Biofilms are communities of cells encased within an exopolysaccharide matrix. The overall aim of the project was to explore the activity of novel therapeutic agents against established *P. aeruginosa* biofilms. The study investigated the phenotypic characteristics of clinical CF *P. aeruginosa* strains such as biofilm forming ability and *in vivo* virulence and the resistance to antibiotic single and combination therapy. In addition, the activity of novel synthesised metal-based complexes and the interactions of *P. aeruginosa* with unusual CF pathogens in a mixed bacterial environment were also explored. The static and dynamic *in vitro* models which were employed to study microbial biofilms provided diversity in terms of biofilm growth, development and the response to antimicrobial therapy.

Biofilm formation was common characteristic of CF *P. aeruginosa* strains; however, the levels differed with the different *in vitro* models. *In vivo* virulence of these strains was more pronounced with *P. aeruginosa* strains that produced the phenazine pigment, pyocyanin. The results demonstrated that antibiotic efficacy was less effective against biofilms than planktonic cells and the synergistic interactions of antibiotic combinations were strain specific. Tobramycin-ceftazidime was the only combination that displayed synergistic interactions against the 24 and 72 hour biofilm of one clinical *P. aeruginosa* isolate. Overall, antibiotic combinations were similar to the activity of either antibiotic used as monotherapy.

The flowchamber biofilm system which was used as part of this study allowed direct visualisation of biofilm structures over a specific period of time. The study also showed that *P. aeruginosa* strains were sensitive to treatment with novel silver-coumarin compounds irrespective of their phenotypic traits. The antimicrobial effects of the agents used in the study may be useful for further study as possible treatments of *P. aeruginosa* infection. Furthermore, the interactions of *P. aeruginosa* and other CF pathogens in a mixed species environment implied that *P. aeruginosa* is capable of remaining the dominant pathogen. *Herbaspirillum* sp. and *Paracoccus yeeii* which did not readily form single species biofilms with the static *in vitro* biofilm model did not interfere with the growth and development of *P. aeruginosa* biofilms. In contrast, it was found that *S. maltophilia* was capable of altering the growth of *P. aeruginosa*. *S. maltophilia* which is capable of forming biofilms is frequently isolated from sputum of cystic fibrosis patients. The study also showed that *S. maltophilia* cell-free supernatants inhibit the growth of certain *P. aeruginosa* biofilms. LC/MS analysis was used to identify the different proteins between *S. maltophilia* strains and findings suggest that specific proteins may be influencing virulence and response to antibiotics during co-infection.

Acknowledgements

Completion of this thesis would not have been possible without the selfless support of others and I would like to take this opportunity to express my sincere appreciation. Firstly, I would like to sincerely thank my supervisor Professor Philip Murphy for offering me the opportunity to study a PhD and for all his patience, support and guidance over the past few years.

I would like to thank Dr. Bernie Creaven and Maeve Sullivan for the gift of silver compounds and Dr. Denis O' Shea for the gift of the copper compounds.

Many thanks to Dr. Thomas Bjarnsholt and his group, especially Anne, from the University of Denmark for allowing me to undertake a research placement in the university to further my research skills.

I would like to thank Jonathan Collins for the CF strains and all his advice and Dr. Emma Caraher for all her guidance. I also acknowledge Dr. Siobhan McClean for all her help with the final part of this thesis and Prof. Sean Doyle and Grainne O'Keeffe from NUI Maynooth for carrying out the LC/MS analysis. To all the other CF researchers and microbiologists in Tallaght hospital for their help over the part few years.

Last but not least I would like to thank my parents, who have been so supportive and understanding throughout my four years and for always pushing me to finish.

Contents

Abbreviations.....12
List of Tables.....17
List of Figures.....19

Chapter 1: Literature Review

1.1 Cystic Fibrosis.....24
1.1.1 Cystic Fibrosis Transmembrane Regulator (CFTR) gene.....24
1.1.2 Mutations in the CFTR gene 25
1.1.3 Inflammation in the CF airways 26
1.1.4 Nitric oxide and CF infection.....27
1.1.5 Diagnosis of CF..... 27
1.1.6 Screening of CF..... 28
1.1.7 Common symptoms associated with CF diagnosis.....29
1.1.8 Diagnostic microbiology.....29
1.1.9 Colonisation of the CF airways with *P. aeruginosa*.....30
1.2 Treatment strategies for CF.....31
1.2.1 Antibiotic susceptibility testing.....31
1.2.2 Antibiotic combination therapy.....31
1.2.3 Aerosolised antibiotic therapy for CF patients.....32
1.2.4 Macrolide antibiotic therapy.....33
1.2.5 Anti-inflammatory agents for CF therapy.....33
1.3 Cystic Fibrosis in Ireland.....34
1.3.1 Statistics for CF in Ireland.....34
1.3.2 Respiratory complications.....36
1.4 Cystic Fibrosis pathogens.....38
1.4.1 *Burkholderia cepacia* complex.....38
1.4.2 *Pseudomonas aeruginosa*.....39
1.4.3 *Staphylococcus aureus*.....40
1.4.4 *Haemophilus influenzae*.....41

1.4.5 Microbiome of the CF airways.....42
1.4.6 Polymicrobial infection.....42
1.4.7 Fungal infection.....43
1.5 Physiology of *Pseudomonas aeruginosa* biofilms.....44
1.5.1 Motility of *P. aeruginosa* biofilms.....45
1.5.2 Quorum sensing in *P. aeruginosa* biofilms.....45
1.5.3 *P. aeruginosa* biofilm formation.....47
1.5.4 Reversible attachment.....48
1.5.5 Irreversible attachment.....48
1.5.6 Maturation.....49
1.5.7 Detachment.....49
1.5.8 Formation of non-surface attached microcolonies.....50
1.5.9 Antimicrobial resistance of biofilms.....51
1.5.10 Reduced penetration of antimicrobials through the EPS matrix.....51
1.5.11 Different growth rates of biofilm forming isolates.....52
1.5.12 Changes to the biofilm structure due to an altered mode of growth.....52
1.5.13 Persister cells.....53
1.6 Biofilm formation in different models.....53
1.6.1 Calgary biofilm device (CBD).....54
1.6.2 Modified Robbins Device (MRD).....55
1.6.3 Colony biofilms.....55
1.6.4 Flow cell system.....56
1.7 Novel agents to control *P. aeruginosa* biofilms.....57
1.7.1 Lactoferrin.....57
1.7.2 Gallium nitrate.....57
1.7.3 Ethylenediaminetetraacetic acid (EDTA).....58
1.7.4 Aspirin.....59
1.7.5 Heparin.....59
1.7.6 Natural products.....59
1.8 Project aims and objectives.....61

Chapter 2: Characterisation and antibiotic susceptibility testing of *P. aeruginosa* strains from patients with cystic fibrosis.

2.1 Introduction.....62

2.2 Materials and Methods.....64

2.2.1 Research ethics.....64

2.2.2 CF patient background.....64

2.2.3 Preparation of Sputasol.....64

2.2.4 Preparation of sputum cultures.....64

2.2.5 Selection of *Pseudomonas aeruginosa* strains.....65

2.2.6 Antibiotics.....67

2.2.7 Growth media.....67

2.2.8 Planktonic growth of *P. aeruginosa* strains.....67

2.2.9 Epsilometer test (E-test).....67

2.2.10 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) determination.....68

2.2.11 Optimisation of biofilm quantification assay.....69

2.2.12 Biofilm growth determination.....69

2.2.13 Determination of virulence of selected *P. aeruginosa* isolates in the *in vivo G. mellonella* model.....70

2.2.14 Biofilm Inhibitory Concentration (BIC) determination.....71

2.2.15 Fractional Inhibitory Concentration (FIC).....71

2.2.16 Effect of antibiotic combinations on viability of *P. aeruginosa* biofilms..72

2.2.17 Statistical analysis.....72

2.3 Results.....73

2.3.1 Diversity of *P. aeruginosa* isolates grown planktonically.....73

2.3.2 Diversity of *P. aeruginosa* isolates grown as biofilms.....74

2.3.3 Characterisation of virulence of *P. aeruginosa* in the *in vivo G. mellonella* model.....78

2.3.4 Optimisation of biofilm growth quantification.....83

2.3.5 Summary of the number of *P. aeruginosa* strains displaying sensitivity to each antibiotic according to the CLSI standards.....86

2.3.6 Antibiotics susceptibility testing of single antibiotics against *P. aeruginosa*.....88
2.3.7 Susceptibility testing of antibiotic combinations.....90
2.3.8 Activity of effective antibiotic combinations against mature (72 hour) *P. aeruginosa* biofilms.....94
2.3.9 The effect of tobramycin, ceftazidime and tobramycin-ceftazidime against *P. aeruginosa* biofilms.....96
2.3.10 The effect of biofilm maturation on the efficacy of antibiotic treatment...99
2.4 Discussion.....104

Chapter 3: Antimicrobial activity of metal-based compounds against *P. aeruginosa* biofilms

3.1 Introduction.....111
3.2 Materials and Methods.....114
3.2.1 Bacterial strains and media.....114
3.2.2 Media for flow cell biofilms.....114
3.2.3 Culture preparation for flow cell experiments.....115
3.2.4 Synthesis of silver-coumarin compounds.....115
3.2.5 Synthesis of copper-benzotriazole compounds.....118
3.2.6 Gallium nitrate.....118
3.2.7Solubility testing and preparation of metal-based compounds for experimental procedures.....119
3.2.8 Effect of synthesised compounds against planktonic cultures of *P. aeruginosa* 119
3.2.9 Effect of synthesised compounds against biofilms grown on polystyrene pegs.....120
3.2.10 Effect of synthesised compounds on viability of *P. aeruginosa* biofilms120
3.2.11 Establishing the flow cell system.....121
3.2.12 Assembly of the flow cell system channels.....121
3.2.13 Sterilisation of the flow cell system.....122
3.2 14 Cultivation of biofilms in the flow cell system.....122

3.2.15 Exposure of biofilms to silver-nitrate and silver-coumarin compounds...123
3.2.16 Fluorescent staining.....124
3.2.17 Microscopy and image processing.....124
3.2.18 Statistical analysis.....125
3.3 Results.....125
3.3.1 Effect of copper-benzotriazole of *P. aeruginosa* planktonic and biofilm formation.....125
3.3.2 Effect of transition metal gallium nitrate on growth of *P. aeruginosa* planktonic and biofilm bacteria.....128
3.3.3 Effect of silver-coumarin compounds against planktonic and biofilm formation of *P. aeruginosa*.....130
3.3.4 Summary of selected clinical CF *P. aeruginosa* strains.....133
3.3.5 Investigation of the effect of silver-coumarin agents on planktonic growth of clinical CF *P. aeruginosa* strains.....134
3.3.6 Inhibition of mature *P. aeruginosa* biofilms by silver-coumarin compounds using two different static models.....137
3.3.7 Investigation of *P. aeruginosa* biofilm formation using the flow cell system.....143
3.3.8 The effect of silver-coumarin compounds on biofilm development in the flow cell system.....145
3.4 Discussion.....148

Chapter 4: Mixed species biofilm infection

4.1 Introduction.....154
4.2 Materials and Methods.....157
4.2.1 Bacterial strains.....157
4.2.2 Planktonic growth of unusual CF species.....158
4.2.3 Single and mixed species biofilm growth on polystyrene pegs.....158
4.2.4 Cell culture biofilm growth assay of single and mixed species.....159
4.2.5 Effect of *S. maltophilia* whole cells and cell-free supernatants on the biofilm forming ability and pre-formed biofilms of *P. aeruginosa* development.....160

4.2.6 Effect of antibiotics on *P. aeruginosa* biofilm formation and pre-formed biofilms with *S. maltophilia* whole cells and cell-free supernatants.....161
4.2.7 Susceptibility of unusual CF isolates to antibiotic treatment.....161
4.2.8 Isolation of proteins from *S. maltophilia* isolates.....162
4.2.9 10% Ammonium persulfate (APS).....162
4.2.10 10% Sodium dodecyl sulfate (SDS).....162
4.2.11 10% Running buffer (1XTGS).....162
4.2.12 SDS-polyacrylamide gel electrophoresis.....163
4.2.13 Preparation of protein bands for liquid chromatography/mass spectrometry (LC/MS) analysis.....164
4.2.14 Statistical analysis.....165
4.3 Results.....166
4.3.1 Growth of planktonic cultures of unusual CF species.....166
4.3.2 Adherence of single and mixed species of *Paracoccus yeeii* and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.....168
4.3.3 Adherence of single and mixed species of *Acinetobacter* sp. and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.....170
4.3.4 Adherence of single and mixed species of *Ralstonia* sp. and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.....173
4.3.5 Adherence of single and mixed species of *Herbaspirillum* sp. and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.....175
4.3.6 Adherence of single and mixed species of *Stenotrophomonas maltophilia* and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.....177
4.3.7 Viability of *P. aeruginosa* PA01 biofilms in conjunction with unusual CF strains.....180
4.3.8 Viability of clinical isolate, *P. aeruginosa* 1826 biofilms in conjunction with unusual CF strains.....184
4.3.9 Effect of *S. maltophilia* cell-free supernatants and whole cells on *P. aeruginosa* biofilm development.....187
4.3.10 Effect of antibiotics on the growth of mixed culture biofilms of *P. aeruginosa* and *S. maltophilia*.....191

4.3.11 Susceptibility testing of cystic fibrosis species to tobramycin.....195
4.3.12 SDS-page and LC/MS analysis of *S. maltophilia* proteins.....197
4.4 Discussion.....203

Chapter 5: General Discussion and Conclusions.....210
Chapter 6: References.....217
Chapter 7: Appendices.....256

Abbreviations

µl	Microlitre
µM	Micromolar
ABC-transporter	ATP-binding cassette transporters
ABPA	Allergic bronchopulmonary aspergillosis
$AgN0_3$	Silver nitrate
AHL	Acyl-homoserine lactone
AI	Autoinducers
AMNCH	Adelaide and Meath Hospital, Incorporating the National Children's hospital
AP-1	Activator protein-1
APH	Aminoglycoside phosphotransferase
APM	Ammonium phosphate mononbasic
APS	Ammonium persulfate
ATCC	American Type Culture Collection
ATP	Adenosine Tri-phosphate
Bcc	*Burkholderia cepacia* complex
BIC	Biofilm inhibitory concentration
BZT	Benzotriazole
BZTM	1H-benzotriazole-1-methanol
C. albicans	*Candida albicans*
CAMP	Cationic antimicrobial peptides
CBAVD	Cogenital bilateral absence or atropy of the vas deferend
CBD	Calgary Biofilm Device
CCa	Coumarin
CDC	Centres for Disease Control and Prevention
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
cGMP	Cyclic guanosine monophosphate

Cl	Chloride
CLSI	Clinical and Laboratory standards institute
CLSM	Confocal laser scanning microscopy
CFRI	Cystic Fibrosis Registry of Ireland
Cu	Copper
$CuSO_4$	Copper sulfate
CV	Crystal violet
dH_2O	Distilled water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonuclease acid
DSF	Diffusible signal factor
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
E-test	Epsilometer test
EPS	Exopolysaccharide
FAC	Ferric ammonium compounds
FEV_1	Forced expiry volume in 1 second
FDA	Food and Drug administration
Fe	Iron
FIC	Fractional inhibitory concentration
FICI	Fractional inhibitory concentration index
FISH	Fluorescent *in situ* hybridization
Ga	Gallium
$Ga(NO_3)_3$	Gallium nitrate
gfp	Green fluorescent protein
h	Hour
H. influenzae	*Haemophilus influenzae*
Hg	Mercury
HSF	Heat shock factor

HSL	Homoserine lactone
H_2O	Hydrogen oxide
IL	Interleukin
KH_2PO_4	Monopotassium phosphate
LA	Luria agar
LB	Luria broth
LC	Liquid chromatography
LES	Liverpool epidemic strain
IRT	Immunoreactive trypsinogen
LPS	Lipopolysaccharide
M	Molar
MBC	Minimal bactericidal concentration
MBZT	5-methyl-1H-benzotriazole
MES	Manchester Epidemic strain
MDR	Multidrug resistance
MHA	Mueller-Hinton agar
min	Minutes
MIC	Minimal inhibitory concentration
ml	Millilitres
mmol	Millimole
MRD	Modified Robbin's Device
MRSA	Methicillin resistant staphylococcus aureus
mRNA	messenger Ribonucleic acid
MS	Mass spectrometry
mw	molecular weight
Na	Sodium
NaCl	Sodium chloride
$Na_2HPO_4.2H_2O$	Disodium hydrogen phosphate
$(NH_4)2SO_4$	Ammonium sulphate
NB	Nutrient broth
ng	Nanogram

nm	Nanometre
°C	Degrees Celsius
OD	Optical density
OP	Oropharyngeal
OTU	Operational taxomonic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGFE	Pulsed-field gel electrophoresis
PI	Propidium iodide
P. aeruginosa	*Pseudomonas aeruginosa*
P. yeeii	*Paracoccus yeeii*
PMA	Polymorphonucleotide activation
PMN	Polymorphonucleotide
QS	Quorum sensing
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rRNA	ribosomal Ribonucleic acid
RSCV	Rugose small colony variants
SA	Salicylic acid
SCV	Small colony variants
S. aureus	*Staphylococcus aureus*
S. maltophilia	*Stenotrophomonas maltophilia*
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
TEM	Transmission electron microscopy
TEMED	N,N,N',N;-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
Th	T-helper
TNF	Tumour necrosis factor
TSA	Tryptone soy agar
TSB	Tryptone soy broth

TSP	Transferable solid phase
TVC	Total Viable Counts
w/w	weight/weight
v/w	volume/weight
v/v	volume/volume

List of Tables

Table 1.1: Pathogens associated with CF infection.....38

Table 2.1: Phenotypic characteristics of *P. aeruginosa* common clones, uncommon clones and reference strains.....76

Table 2.2: E-test (MIC mg/L) results of antipseudomonal antibiotics against *P. aeruginosa*.....85

Table 2.3: Number of antibiotics for which *P. aeruginosa* strains were sensitive (S), intermediate (I) and resistant (R), (*n*=12).....86

Table 2.4: Minimum inhibitory concentration (MIC μg/ml) and Biofilm inhibitory concentration (MIC μg/ml) of single antibiotics against *P. aeruginosa*.....89

Table 2.5: Susceptibility of *P. aeruginosa* planktonic (MIC μg/ml) and 24 hour biofilm-grown isolates (BIC μg/ml) to antibiotic combinations.....92

Table 2.6: Fractional Inhibitory Concentration's (FIC) of antibiotic combinations according to the FIC Index (FICI).....93

Table 2.7: Susceptibility of mature *P. aeruginosa* biofilms grown for 72 hours and treated with effective single and double antibiotic combinations.....95

Table 2.8: Fractional Inhibitory Concentration (FIC) of the most effective antibiotic combinations against mature (72 hour) biofilms.....95

Table 3.1: Molecular weights of synthesised metal-free coumarin ligands.....116

Table 3.2: Molecular weights of synthesised silver-coumarin compounds.....117

Table 3.3: Molecular weights of synthesised copper-benzotriazole compounds and metal-free benzotriazole ligands.....118

Table 3.4: Effect of synthesised copper-benzotriazole compounds against planktonic *P.*

aeruginosa strains.....126

Table 3.5: Effect of synthesised copper-benzotriazole compounds against *P. aeruginosa* biofilms.....127

Table 3.6: Effect of gallium nitrate, $Ga(NO_3)_3$, on antibiotic efficacy of tobramycin...129

Table 3.7: Effect of silver-coumarin compounds against planktonic *P. aeruginosa* strains.....131

Table 3.8: Effect of synthesised silver-coumarin compounds against *P. aeruginosa* biofilms.....132

Table 3.9: Phenotypic characteristics of CF *P. aeruginosa* strains.....133

Table 3.10: Phenotypic characteristics of isolates selected for examination and treatment in the flow cell system.....143

Table 4.1: Cystic fibrosis species used in this study.....157

Table 4.2: Materials for preparation of resolving and stacking gel.....163

Table 4.3: Summary of phenotypic characteristics of selected *P. aeruginosa* reference and clinical isolates.....188

Table 4.4: Comparison of susceptibilities of cystic fibrosis species grown planktonically to aminoglycoside antibiotic, tobramycin (μg/ml).....196

Table 4.5: Summary of identified proteins of *S. maltophilia* by LC/MS analysis.....200

List of Figures

Figure 1.1: CFTR mutations which are separated into classes. There are six classes representing six mutations of the CFTR gene.....26

Figure 1.2: Statistical analysis of the 'median survival' of **(A)** females and **(B)** males with CF in Ireland who were born between 1985-2004. Image adapted from the CFRI.....35

Figure 1.3: Prevalence of CF infections according to age. *P. aeruginosa*, *S. aureus* and *H. influenzae* represent the most common pathogens. Image adapted from the Cystic Fibrosis Foundation, 2006.....37

Figure 1.4: Biofilm formation process depicting stages of reversible attachment, irreversible attachment, maturation and detachment.....47

Figure 1.5: Calgary Biofilm Device (CBD) for biofilm susceptibility testing. **(A)** The tilted table creates shear force for biofilm formation **(B)** The lids containing pegs are designed so that they fit into the tray **(C)** The polystyrene plate contains 96 pins for biofilms to form.....54

Figure 1.6: (a) Schematic diagram of the flow cell system. **(b)** The upper glass of the flow cell on which biofilms are formed can be viewed using a microscope. **2.** Confocal image of a *gfp*-tagged *P. aeruginosa* biofilm grown in this system for 6 days.....56

Figure 2.1: Differences in planktonic growth curves of selected *P. aeruginosa* isolates.74

Figure 2.2: 24 hour biofilm cultures of *P. aeruginosa* clinical CF strains grown on polystyrene pegs.....75

Figure 2.3: Biofilm formation of *P. aeruginosa* strains.....77

Figure 2.4: Evaluation of virulence of selected reference and CF *P. aeruginosa* strains at

different concentrations.....82

Figure 2.5: *P. aeruginosa* pathogenesis in *G. mellonella* larvae relative to the control...83

Figure 2.6: The effect of four different disruption methods against biofilms grown on polystyrene pegs.....84

Figure 2.7: The susceptibilities of *P. aeruginosa* isolates against antibiotics (mg/L) by E-test on Mueller-Hinton agar.....87

Figure 2.8: Optical density (630nm) of *P. aeruginosa* biofilms **(A)** *P. aeruginosa* PA01, **(B)** *P. aeruginosa* ATCC27853, **(C)** *P. aeruginosa* 1826, **(D)** *P. aeruginosa* 4276.3 treated with tobramycin, ceftazidime and tobramycin-ceftazidime at varying concentrations (μg/ml).....98

Figure 2.9: Reduction in biofilm biomass (*P. aeruginosa* PA01) following antibiotic treatment.....101

Figure 2.10: Reduction in biofilm biomass (*P. aeruginosa* 1826) following antibiotic treatment.....103

Figure 3.1: Fully assembled flow cell system showing connecting flow chambers, effluent vessels and connecting tubes. Media was circulated through the bubble traps and flow chambers by a 16-channel periplasmic pump. Waste flows through a series of tubes into flasks.....123

Figure 3.2: MIC and MBC values of **(A)** compound 6 (mw 361.48) and **(B)** compound 8 (mw 327.04) and **(C)** silver nitrate control against clinical CF *P. aeruginosa* strains...136

Figure 3.3: The effect of silver nitrate and compound 8 against 72 hour *P. aeruginosa* biofilms grown in BTrace medium. The activity of all agents was compared to the untreated control. Results are shown as total log counts and represent the average of three

experiments.....139

Figure 3.4: The effect of varying concentrations of compound 6, compound 8 and silver nitrate against mature *P. aeruginosa* biofilms. Biofilms were grown on polystyrene pegs for 72 hours prior treatment. Results represent the mean ± standard deviation of at least two experiments.....142

Figure 3.5: CLSM images of 72 hour *P. aeruginosa* biofilms in 10% glucose supplemented minimal media showing different biofilm structures. Images show sections through the biofilm depicting morphology profiles of the structures.....144

Figure 3.6: Live-dead stained *P. aeruginosa* 1826 grown for 72 hours in minimal medium and treated with 10 x MIC silver nitrate and 10 x MIC compound 8. Images were obtained by CLSM, live cells appear green and dead cells appear red as a result of staining.....146

Figure 3.7: Live-dead stained images of 72 hour *P. aeruginosa* PA01 biofilm treated with 10 x MIC silver nitrate and 10 x MIC compound 8. The results show *P. aeruginosa* PA01 biofilms following 24 hours treatment. Images were obtained by CLSM.....147

Figure 4.1: Optical density (630nm) of planktonic growth of unusual CF species measured after 24 hours incubation.....167

Figure 4.2: Comparison of growth of single and mixed species biofilms with *Paracoccus yeeii*..... 169

Figure 4.3: Comparison of growth of single and mixed species biofilms with *Acinetobacter* sp.....172

Figure 4.4: Comparison of growth of single and mixed species biofilms with *Ralstonia* sp.....174

Figure 4.5: Comparison of growth of single and mixed species biofilms with *Herbaspirillum* sp.....176

Figure 4.6: Comparison of growth of single and mixed species biofilms with *Stenotrophomonas maltophilia*.....179

Figure 4.7: Effect of unusual CF strains on the biofilm growth of *P. aeruginosa* PA01 measured as log CFU/ml based on different colony morphologies on TSA. The controls represent samples that are not in mixed culture.....183

Figure 4.8: Effect of unusual CF strains on the biofilm growth of *P. aeruginosa* 1826 measured as log CFU/ml based on different colony morphologies on TSA. The controls represent samples that are not in mixed culture.....186

Figure 4.9: The effect of *S. maltophilia* cell-free supernatants and whole cells on established biofilms of *P. aeruginosa*. After 24 hours of growth *P. aeruginosa* were exposed to *S. maltophilia* for an additional 24 hours.....188

Figure 4.10: Mixed species biofilms of *P. aeruginosa* and *S. maltophilia* supernatants and whole cells. Bacteria were inoculated at a 1:1 ratio and incubated for 24 hours, 35°C. Control values represent biofilm formation of each single species.....190

Figure 4.11: The effect of antibiotics (tobramycin) against mixed species biofilms. Mixed species biofilms of *P. aeruginosa* and *S. maltophilia* were grown as described in **Section 4.2.5**. Mixed and single bacterial cultures were exposed to various concentrations of tobramycin for an additional 24 hours.....192

Figure 4.12: The effect of antibiotics (tobramycin, ceftazidime) against biofilms of *P. aeruginosa* (PA01, 4505.2) treated with *S. maltophilia* (668, 747) supernatants as described in **Section 4.2.5**. Biofilms were exposed to various concentrations of

tobramycin and ceftazidime for an additional 24 hours.....194

Figure 4.13: Protein analysis of culture supernatants of *S. maltophilia* strains 668 and 747. Proteins were separated by 10% SDS-page and stained with coomassie blue. The migration of molecular weight (kDa) standards is shown on the right of the image.....198

Figure 4.14: Total ion chromatograms from the LC/MS analysis of bands A, B and C of *S. maltophilia* proteins.....199

Figure 4.15: The seven peptides sequenced of *S. maltophilia* proteins identified from bands A, B and C. All peptides that have been matched by LC/MS are highlighted in red.....202

Chapter 1: Literature Review

1.1 Cystic Fibrosis

Cystic Fibrosis (CF) is an autosomal recessive inherited disorder most common in northern Europeans, affecting approximately 1 in 3000 births (Walters *et al.*, 2007) and 60,000 individuals worldwide (Gibson *et al.*, 2003). The disease persists from mutations in the cystic fibrosis transmembrane regulator (CFTR) gene which encodes a chloride (Cl^-) channel in secretary epithelia (Riordan *et al.*, 1989). Mutations in the CFTR gene result in a loss or reduction of chloride secretion. This leads to the production of thick, viscous mucus in the lungs. Mucus prevents cilia beating effectively on normal epithelia, allowing the growth of pathogens in the chronic CF lung (Govan *et al.*, 1996). Mucins which are long, highly glycosylated polymers form a mesh with an optimal pore size for trapping inhaled particles such as bacteria and fungi (Boucher *et al.*, 2007). There are two types of mucins in the CF airways, MUC5AC and MUC5B, which are two of the major components that form the mucus matrix (Groneberg *et al.*, 2002).

1.1.1 Cystic Fibrosis Transmembrane Regulator (CFTR) gene

The CFTR gene consists of 180,000 base pairs located on the chromosome 7 and encodes the membrane protein 1480-amino acid (Riordan *et al.*, 1989; Quinton *et al.*, 2008). The CFTR glycoprotein acts as a regulated chloride channel. CFTR is a part of the adenoseine triphosphate (ATP)-binding cassette (ABC) transporter proteins (Riordan *et al.*, 2008). The process by which CFTR functions involves DNA which is transcribed to mRNA. The mRNA from the nucleus and endoplasmic reticulum (ER) from the cytoplasm cause

translation of polypeptide within the ER. Maturation of the ER results in protein folding within the lipid bilayer (Lukacs *et al.*, 1994; Ward *et al.*, 1994).

1.1.2 Mutations in the CFTR gene

Approximately 1,500 CFTR mutations have been identified. Clinicians select a suitable treatment for CF patients according to mutations which are separated into different classes. Some of the most common mutations among the CF population include misfolded CFTR resulting in degradation of the F508del-CFTR gene prematurely (Van Goor *et al.*, 2006), reduced chloride channel ion transport of R117H (Sheppard *et al.*, 1993), and reduced production of normal plasma membrane due to transcriptional dysregulation as a result of the G551D gene (Lewis *et al.*, 1994).

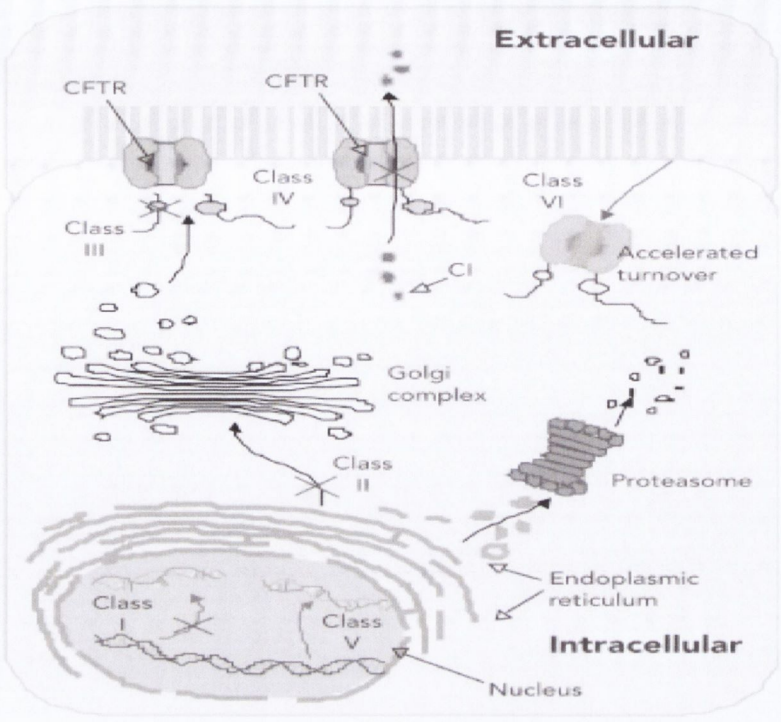


Figure 1.1: CFTR mutations which are separated into classes. There are six classes representing six mutations of the CFTR gene (Rowe *et al.*, 2005).

1.1.3 Inflammation in the CF airways

Inflammatory responses in the CF airways occur due to increases in bacterial concentrations and polymorphonucleocytes. The inflammatory process prevents the spread of colonising bacteria but also leads to tissue damage (Perez *et al.*, 2007). Many studies have documented increases in cytokines regulated by NFkB such as interleukin IL-8, IL-6 and IL1β, tumour necrosis factor (TNF)α, leukotriene B4 and free neutrophil elastase. Airway inflammation can be detected in bronchoalveolar lavage (BAL) fluid samples as early as infancy (Khan *et al.*, 1995). In response to infection, CF patients have higher levels of IL-8 and neutrophils in BAL samples compared to non-CF infected individuals (Muhlebach *et al.*, 1999). Another signalling pathway IL-10, found in low

quantities in BAL samples can reduce the inflammatory response by neutrophil apoptosis and T-cell stimulation (Lentsch *et al.*, 1997; Schottelius *et al.*, 1999). Other studies have described IL-23 and IL-17 as proinflammatory cytokines involved in cell signalling of TH-17 which is enhanced during CF (Dubin *et al.*, 2007; McAllister *et al.*, 2005). The viscous mucus which traps pathogens can trigger the pathway of IL-23 and IL-17 by recruiting neutrophils to the site of infection (Ferretti *et al.*, 2003). These inflammatory responses have shown to decrease after treatment of the pulmonary exacerbation (Konstan *et al.*, 1993; Konstan *et al.*, 1994; Bonfield *et al.*, 1995; Sagel *et al.*, 2001; Colombo *et al.*, 2005).

1.1.4 Nitric oxide and CF infection

Nitric oxide (NO) acts as an antimicrobial in the lungs of individuals without CF. CF patients with reduced levels of NO may experience hypersusceptibility to infection. Increased metabolism of NO to NO_2^- and NO_3^- results in lower NO levels in exhaled breath of CF patients than produced by normal individuals (Mhanna *et al.*, 2001). Reduced sodium (Na^+) has been linked to Na hyperabsorption and Cl^- secretion caused by inhibition interactions between CFTR-ENaC (epithelial sodium channel). Low levels of NO also contribute to a reduction in the activity of guanylate cyclase which is an enzyme that produces cyclic guanosine monophosphate (cGMP) (Jayaraman *et al.*, 1999).

1.1.5 Diagnosis of CF

The diagnosis can be confirmed by a series of tests of abnormal ion concentrations and genotyping of the most common mutations. A series of clinical tests which do not directly

detect defects in the CFTR gene assist in the diagnosis of CF. The procedures involve testing for pancreatic insufficiency or paranasal sinuses (Ratjen and Doring, 2003). Studies have reported that approximately 15% of newborns with CF can be affected with meconium ileus (Evans *et al.*, 2001). Infertility is common among the majority of males caused by cogenital bilateral absence or atrophy of the vas deferend (CBAVD) (Trezise *et al.*, 1993; Dodge *et al.*, 1995). The hypothesis for infertility in CF females is increased viscous production in the cervix which may lead to a reduction in transport of sperm (Hodges *et al.*, 2008).

1.1.6 Screening for CF

Prenatal screening allows identification of CF before birth while newborn screening can detect the condition after birth. Screening protocols generally involve two steps, phenotypic tests that measure immunoreactive trypsinogen (IRT) in dried blood spots followed by a repeat immunoreactive trypsinogen test if there is uncertainity about the result of the sweat test (Ranieri *et al.*, 1994). According to the NCCLS guidelines (2000), a sweat test involves stimulation of sweat by iontophoresis of a cholinergic drug, pilocarpine nitrate to a selected area of the skin. This is followed by a quantitative or qualitative analysis of sweat to determine electrolyte concentration, conductivity or osmolarity for CF diagnosis (LaGrys *et al.*, 1996). Elevated sweat chloride levels are related to abnormal CFTR function. Infants with sweat chloride concentrations of 40-59 mmol/L are likely to be diagnostic of CF and require repeat sweat testing and follow-up tests. A level of > 60 mmol/L sweat chloride by a second sweat test signifies diagnosis of CF (NCCLS, 2000). DNA analysis of a blood sample can also detect ≥ 1 CFTR specific

mutation. Infants without a mutation but a highly elevated IRT value are also classified as being at risk for CF as there may be other mutations that have not been detected (Wilfond *et al.*, 2005). List below shows the common clinical symptoms of CF.

1.1.7 Common symptoms associated with CF diagnosis (adapted from Ratjen and Doring, 2003).

- Chronic cough
- Bacterial colonisation in the airways
- Clubbing
- Airway obstruction
- Sinusitis
- Nasal polyps

1.1.8 Diagnostic microbiology

Sputum samples are the preferred source of airway secretions and an accurate indicator of microorganisms in the lower airways of CF patients (Thomassen *et al.*, 1984; Henig *et al.*, 2001). Other procedures including throat swabs, oropharyngeal cultures (OP) and broncho-alveolar lavage fluids (BAL) are preferred for paediatric patients that cannot readily expectorate sputum (Armstrong *et al.*, 1996; Rosenfeld *et al.*, 1999). BAL samples which are collected from the lower respiratory tract are a more sensitive means of measuring infection. This procedure can be more invasive for the patient and generally requires a general anaesthetic (Henig *et al.*, 2001). While standard culture testing can identify different microorganisms present in samples, molecular techniques allow direct

detection of bacteria, fungi and viruses (Fredricks *et al.*, 2005; Khot *et al.*, 2008; Rabodonirine *et al.*, 1997; Gutierrez *et al.*, 2001; Gilchrist *et al.*, 2011). Willner *et al.*, (2012), concluded reproducibility of these assays varies depending on the DNA extraction method since the volume of BAL obtained from younger patients can be low (Shields *et al.*, 2000).

1.1.9 Colonisation of the CF airways by *P. aeruginosa*

P. aeruginosa isolated from the CF lung displays morphological diversity to environmental isolates (Govan *et al.*, 1996). Inflammation in the CF lungs as a result of *P. aeruginosa* colonisation leads to high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which are generated by neutrophils as part of the innate immune response. ROS and RNS lead to mutations that enable *P. aeruginosa* to colonise the CF airways (Ciofu *et al.*, 2005). *P. aeruginosa* isolates in the CF lung are often mucoid, biofilm-formers, lack flagella and pili and are highly resistant to antibiotics. Many *P. aeruginosa* strains have been described as hypermutable as they display a higher spontaneous mutation frequency than wild-type bacteria and contribute to increased clonal expansion (Oliver *et al.*, 2000). Dominant mutations such as the conversion of non-mucoid to mucoid colony morphology are caused by an over-production of alginate and this can affect the clinical outcome of the patient (Ramsey *et al.*, 2005). Mutations within the *mucA* gene, encoding a member of the extracytoplasmic function of anti-sigma factors causes a change to the mucoid phenotype. Mutations in *algW*, *mucC* and *mucD* genes can also lead to conversion to the mucoid phenotype (Hasset *et al.*, 2009).

1.2 Treatment strategies of Cystic Fibrosis

1.2.1 Antibiotic Susceptibility testing

Previous studies have suggested pulmonary exacerbations have been associated with new microorganisms or a change in the bacterial density of colonising isolates (Regelmann *et al.*, 1990; Smith *et al.*, 1988; Wat *et al.*, 2003; Van Ewijk *et al.*, 2005). Chronic bacterial infection can be maintained with antibiotics such as tobramycin, ceftazidime, colistin, piperacillin/tazobactam, meropenem and ciprofloxacin, commonly administered for *P. aeruginosa* and *B. cepacia* (Banerjee *et al.*, 2000). CF patients infected with *P. aeruginosa* and suffering from acute exacerbations of chronic multiresistant lung disease are often treated with potential synergistic combinations of anti-pseudomonal antibiotics (Aaron *et al.*, 2002). Cephalosporins, macrolides and tetracycline are often administered for staphylococcal infection. Due to bacterial resistance, flucloxacillin can be administered as it is more appropriate at minimising this effect (Hodson, 1995). Beardsmore *et al.*, (1994), suggested that prophylactic therapy can reduce the frequency of *S. aureus* isolates in sputum samples compared with intermittent therapy.

1.2.2 Antibiotic combination therapy

Bacterial mutations enable resistance to develop in response to the majority of antibiotics. Tolerance is defined as a reduction in the killing effect of an antibiotic against a bacterial population (Tomaz *et al.*, 1985). Many studies have focused on exploring the activity of antibiotic combinations against CF bacteria. Synergistic therapies allow reductions in the quantities of the agents used (Harrison *et al.*, 2008). Zhai *et al.*, (2010), showed lower

concentrations of polymyxin B and fluconazole exhibited fungicidal activity against a range of fluconazole-resistant *Candida albicans* isolates and moulds. *In vitro* studies by Seibert *et al.*, (1992), reported synergistic interactions between cefpirome and vancomycin against several enterococci and the majority of MRSA strains by the checkerboard assay. In addition, Hermann *et al.*, (2010), found colistin and tobramycin inhibited the mature biofilm of *P. aeruginosa* by inhibiting different components of the structure. Additionally, the study by Moskowitz *et al.*, (2005), reported that no specific antibiotic combination was effective against the majority of *P. aeruginosa* isolates and the results vary depending on each individual strain.

1.2.3 Aerosolised antibiotic therapy for CF patients

Numerous studies have examined the relationship between aerosolised antibiotics and morbidity of CF patients. Antibiotic inhalation enables the delivery high doses of antibiotics directly to the site of infection (Geller, 2009). Recent studies focusing on aerosolised antibiotics in conjunction with regular oral antibiotic treatment found significant improvements in pulmonary function with reduced bacterial colonisation in CF sputum. Wall *et al.*, (1983), found CF patients receiving 80 mg tobramycin by inhalation and 1 g ticarcillin orally experienced reduced exacerbations. The study by Hoiby *et al.*, (2005), found improved lung function of patients treated with daily use of oral ciprofloxacin and inhaled colistin. Antibiotics such as colistin and tobramycin can be inhaled more effectively due to improvements in particle size, distribution and duration of treatment (Heijerman *et al.*, 2009; Geller, 2009). Many commonly administered antibiotics may be developed as liposomal formulations as research has shown they may

be more effective at penetrating the biofilm matrix. One possible disadvantage of these formulations is bacterial resistance due to prolonged exposure; however, this effect can often be dependent on the concentration present within the CF airways (Geller, 2009).

1.2.4 Macrolide antibiotic therapy

A previous study involving 29 *P. aeruginosa* chronically infected patients found the macrolide antibiotic, roxithromycin resulted in an 80% reduction in sputum viscosity, `speculated to be linked to chloride transport being restored (Dupont *et al.*, 1990). Saiman *et al.*, (2003), found a CF patient group receiving 250 mg or 500 mg of oral azithromycin 3 days a week showed improvements in FEV_1% and experienced fewer exacerbations than the placebo group. Jaffe *et al.*, (1998), found CF children at end-stage lung disease, colonised with *P. aeruginosa* and treated with long-term azithrmycin also had higher median FEV_1% values. These studies have shed light on the use of macrolides as a long term therapy for CF patients. In addition, Gillis *et al.*, (2004), found sub-MIC concentrations of azithromycin reduced biofilm development of *P. aeruginosa* cultivated in the flow cell system by delaying initial adherence.

1.2.5 Anti-inflammatory agents for CF patients

Anti-inflammatory agents may be used for CF therapy to the deal with lung disease progression due to inflammation in the CF airways. Analysis of BAL is the most common method used by clinicians for assessing inflammation as well as infection in the lungs. Neutrophils are the main inflammatory cells in the CF lungs in which DNA and filamentous actin increase the viscosity of the CF sputum reducing the clearance of mucus (Perks *et al.*, 2000; Tang *et al.*, 2005). Anti-inflammatory treatments that restore

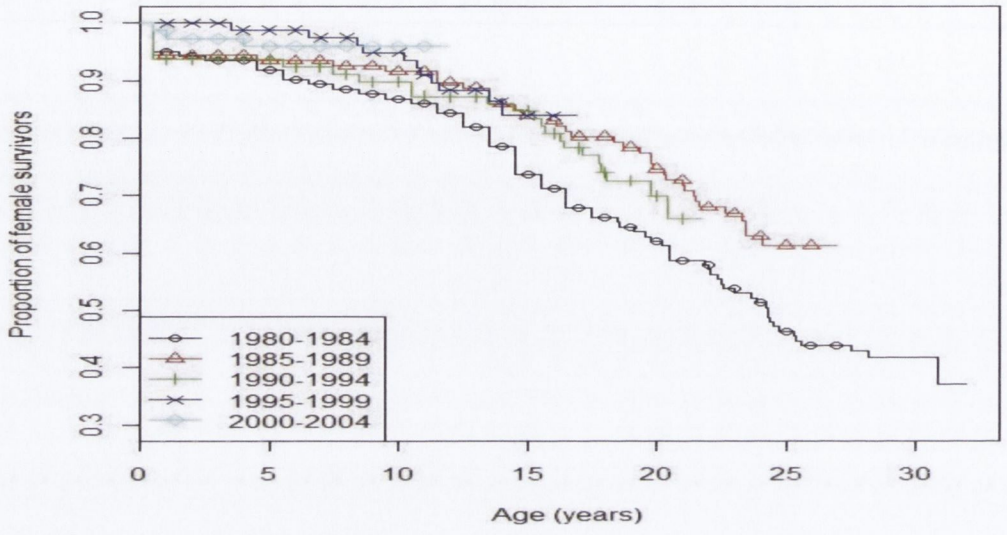
airway surface liquid and interfere with the function of the CFTR proteins have shown more promise. Corticosteroids have the ability reduce mucus and interfere with the inflammatory mediator action. Aspirin and ibuprofen have been shown to have effective anti-inflammatory action against neutrophils by inhibiting neutrophil migration. At higher concentrations the agents can inhibit the action of the main proinflammatory transcription agents, NFkB and AP-1 or HSF (Kopp *et al.*, 1994; Dong *et al.*, 1997; Jurivich *et al.*, 1995).

1.3 Cystic Fibrosis in Ireland

1.3.1 Statistics of CF in Ireland

According to the Cystic Fibrosis Registry of Ireland (CFRI), Ireland has approximately 1 in 1600 infants born with this inherited condition per annum which is an average of 30-40 births each year. The 'median survival' is defined as following an entire CF population until half have died. As of 2011, the CFRI has estimated that the median survival of males and females is 24.3 and 29.4 years, respectively. During the same year more deaths have occurred in male patients than in females. A higher decline rate was observed for males born between 1985-1994; however, the results presented in **Figure 1.2** show females exhibit similar birth cohort survivorship curves throughout the years. Respiratory or cardiac failure accounts for the largest proportion of all deaths of the Irish CF population.

(A)



(B)

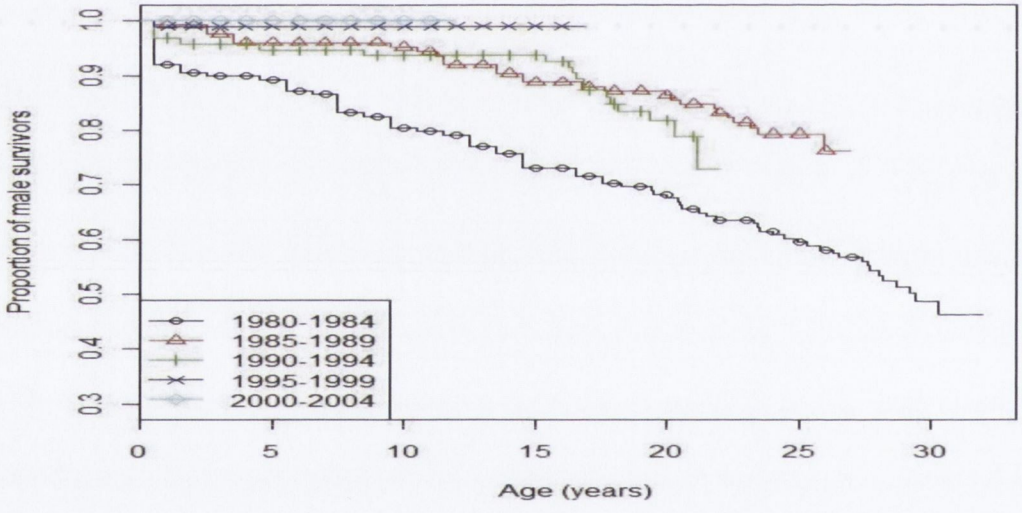


Figure 1.2: Statistical analysis of the 'median survival' of **(A)** females and **(B)** males with CF in Ireland who were born between 1985-2004. Image adapted from the CFRI.

1.3.2 Respiratory complications

The CFRI has reported that respiratory failure accounts for 70% of all deaths due to patients contracting deadly infections in the hospital environment. This rate has declined steadily in recent years from approximately 69.5% in 2009 to 69.3% in 2011. Respiratory complications occur more frequently in the adult CF population than among infants. *P. aeruginosa* was the most commonly contracted organism among Irish CF patients in 2011. A recent study by Van deale *et al.*, (2005), found patients were more susceptible to colonisation of more than one *P. aeruginosa* genotype due to patient-to-patient transmission.

Other organisms that persist in the CF lungs include *B cepacia*, accounting for a significantly lower proportion of lung infection (**Figure 1.3**). Govan *et al.*, (1993), indicated patient-to-patient spread is more problematic with *B. cepacia* strains. Studies by Vandamme *et al.*, (1997), and Clode *et al.*, (2000), indicated *B. cenocepacia* was the most dominant species of the *B. cepacia* complex amongst the CF population, followed by *B. multivorans* and *B. cepacia*. Many CF centres segregate *B. cepacia* infected patients particularly those with transmissibility marker-positive *B. cenocepacia* strains to avoid cross infection. According to LiPuma *et al.*, (2002), segregation does not prevent naturally acquiring the species from the environment.

The CFRI reports *S. aureus* infections have risen steadily in recent years with approximately 32.2% of adults with CF infected in 2011. A study by Elizur *et al.*, (2007), found staphylococcus infection increased from 22% to 27% over three years in one CF centre. Previously, Campana *et al.*, (2007), found 6.7% of sputum samples were

methicillin-resistant *Staphylococcus aureus* (MRSA) infected form one CF centre. Bacterial resistance has been reported as a common occurrence after quinolone treatment which is considered as a high risk factor when treating MRSA infections (LeBlanc *et al.*, 2006). SCV variants of *S. aureus* have demonstrated reduced clinical and microbiological responses even after prolonged antibiotic treatment (Seifert *et al.*, 2003).

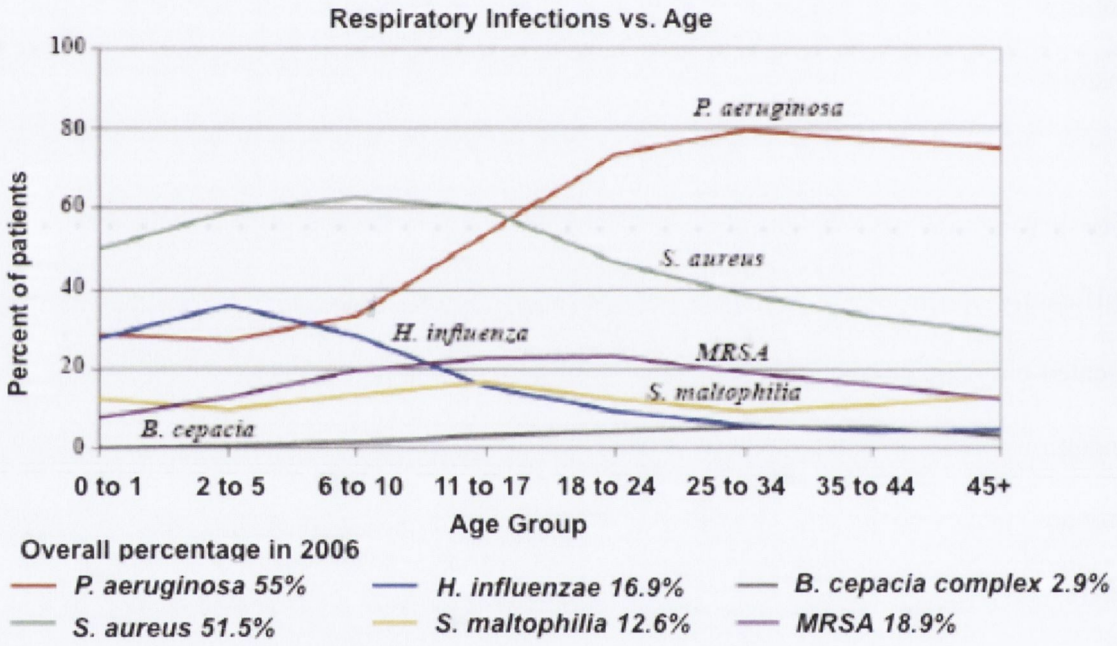


Figure 1.3: Prevalence of CF infections according to age. *P. aeruginosa*, *S. aureus* and *H. influenzae* represent the most common pathogens. Image adapted from the Cystic Fibrosis Foundation, 2006.

1.4 Cystic Fibrosis pathogens.

Several of the pathogenic organisms commonly found in the CF lungs are shown in **Table 1.1.**

Table 1.1: Pathogens associated with CF infection

Group	**Organism**
Gram-negative bacteria	*Pseudomonas aeruginosa* *Burkholderia cepacia* complex *Stenotrophomonas maltophilia* *Haemophilus influenzae*
Gram-positive bacteria	*Staphylococcus aureus* MRSA
Fungi	*Aspergillus fumigatus* *Candida albicans*

1.4.1 *Burkholderia cepacia* complex (Bcc)

Burkholderia cepacia is a gram-negative bacterium commonly found in the environment, associated with increased morbidity and mortality in CF patients. There are 17 closely related species associated with the *B. cepacia* complex (LiPuma *et al.*, 2010). Overall, the *Burkholderia* genus is comprised of approximately 43 phenotypically different species (Vial *et al.*, 2007). *Burkholderia multivorans* and *Burkholderia cenocepacia* are the more

prevalent groups among the CF population (LiPuma *et al.*, 2001; Speert *et al.*, 2002). The quorum sensing system of Bcc controls biofilm development (Eberl *et al.*, 2001) and the rate of resistance to antimicrobials (Chernish and Aaron, 2003). Numerous studies have indicated that once acquired, Bcc is rarely eradicated from the site of infection. A transmissible, multiresistant epidemic *B. cepacia* strain known as ET-12 has been detected in numerous CF centres in the UK (Pitt *et al.*, 1996). During the end stages of infection with Bcc, lung transplantation remains the most promising therapeutic option for patient survival (Heath *et al.*, 2002).

17 species of the *Burkholderia cepacia* complex (LiPuma *et al.*, 2010).

B. cepacia
B. multivorans
B. cenocepacia
B. stabilis
B. vietnamiensis
B. dolosa
B. amblifaria
B. anthina
B. pyrrocinia
B. latens
B. diffusa
B. arbores
B. metallica
B. seminalis
B. contaminans
B. lata
B. ubonensis

1.4.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a motile, gram-negative bacterium, found in soil and the aqueous environment. It is an aerobic bacterium which is able to grow under anaerobic

conditions in the presence of nitrate (Govan *et al.*, 1993). Infections with this pathogen are common in immunocompromised individuals and are one the most prevalent species associated with CF respiratory tract infection. Costerton *et al.*, (1999), described the prevalence of infection to vary according to the different age groups. The report commented that approximately 65-80% of cases are age related. Other reports suggest that CF patient groups may contract distinct strains such as Manchester epidemic strain (MES) and Liverpool epidemic strains (LES) (Cheng *et al.*, 1996). A previous survey published in 2004 in the UK of CF clinics in England and Wales revealed LES was the most common CF clone with 11% of CF patients reportedly infected with the strain (Scott *et al.*, 2004).

More severe chronic lung infection in CF patients is caused by mucoid *P. aeruginosa*. This species produces numerous toxins and virulence factors which are known to enhance colonisation and persistence in the respiratory tract forming biofilms (Aaron *et al.*, 2000). Virulence factors of *P. aeruginosa* include elastase, phospholipase C, exotoxin A, exoenzyme S, rhamnolipids. Phenazine pigment known as pyocyanin and pyoverdine are also secreted by *P. aeruginosa*. Additionally, flagella, pili and lipopolysaccharide are frequently identified during biofilm development of *P. aeruginosa* (Wagner *et al.*, 2008; Lyczak *et al.*, 2000).

1.4.3 *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive facultative anaerobic bacterium associated with the normal flora of the skin and nasal passage. *S. aureus* has multiple virulence factors

which contribute to severe disease progression. SCV's are often associated with persistent infection and higher antibiotic resistance levels (Beiser *et al.*, 2007). *S. aureus* contains a quorum sensing system known as the *agr* system which regulates cell-surface proteins, exotoxins and hydrolytic enzymes (Chan *et al.*, 2004). Under certain *in vitro* conditions the *agr* system may influence attachment and biofilm formation of *S. aureus* (Yarwood *et al.*, 2004). The production of polysaccharide intracellular adhesion (PIA) is necessary for multi-layered cell cluster formation (Vuong *et al.*, 2000). A recent finding by Boles *et al.*, (2008), suggested the *agr* system regulates the conversion of planktonic to biofilm formation of *S. aureus*. Numerous hyper-mutable strains containing a defective DNA mismatch repair system have been isolated from CF patients. Studies have shown these strains are capable of adapting to certain selective pressures (Oliver *et al.*, 2010; Prunier *et al.*, 2003; Prunier *et al.*, 2005).

1.4.4 *Haemophilus influenzae*

Haemophilus influenzae is another common bacterium associated with severe CF lung infection. Studies have shown that CF patients are infected during childhood with this bacterium and any inflammation and damage caused to the airways may enable *P. aeruginosa* to colonise the lungs more effectively (Smith *et al.*, 1997).

1.4.5 Microbiome of the CF airways

The studies of polymicrobial infections have gained much interest among researchers in recent years since numerous microorganisms exist within the respiratory tract of CF patients (Kiska *et al.*, 1996; Shelly *et al.*, 2000; Saiman *et al.*, 2003). Many authors have suggested that these infections can be problematic when selecting suitable treatments. The different species may have different growth rates and nutritional requirements. Recently studies on the CF airway microbiome have been conducted by several research groups to provide a detailed account of the pathogens within the airways. This allows a more detailed identification of CF pathogens that would otherwise not be noticed with standard laboratory culturing. Organisms are identified by 16S rRNA sequencing of sputum samples (Sibley *et al.*, 2011). CF reports on paediatric patients have documented some of the highest levels of different bacterial species. Harris *et al.*, (2007), found a total of 65 pathogens existed within a selected group of CF patients. Recently, Sibley *et al.*, (2011), identified 2,015 strains using partial sequence of the 16S rRNA which was separated into 110 operational taxonomic units. The operational taxonomic units (OTU) were classified into 33 families from five phyla. Tunney *et al.*, (2008), described the detection of high numbers of anaerobic species in sputum samples equal to or greater than *P. aeruginosa* by molecular methods.

1.4.6 Polymicrobial infection

There are limited studies on the interactions between bacterial species commonly found in the CF lung. Recently, Allison *et al.*, (2000), reported synergistic interactions in mixed species biofilms may form thicker and more stable structures than single species. Other

studies have described bacteriocins which are toxins produced by certain species may inhibit the growth of other species (Kreth *et al.*, 2005). Soong *et al.*, (2006), found that a synergistic relationship existed between *P. aeruginosa* and the influenza virus. The virus possesses neuroaminidase activity which could increase bacterial adherence post viral infection. Another study by Yang *et al.*, (2011), found co-culturing *S. aureus* and *P. aeruginosa* resulted in the formation of colonies with different morphology to each monospecies and that *P. aeruginosa* was able to influence the growth of *S. aureus in vitro*.

1.4.7 Fungal infection

Fungal isolates are frequently recovered from respiratory secretions of CF patients (Mortensen *et al.*, 2011; Paugam *et al.*, 2011). *Aspergillus fumigatus* is the predominant fungal pathogen with a prevalence rate of approximately 60% (LiPuma *et al.*, 2010), followed by *Aspergillus terrus* as the second most common species (Pihet *et al.*, 2009). Colonisation of *A. fumigatus* generally results in allergic bronchopulmonary aspergillosis (ABPA) which is characterised by impaired mucociliary clearance, frequent bronchial obstruction and respiratory exacerbations. Generally, 10-15% of patients will develop ABPA which mainly occurs in older children and adults (Stevens *et al.*, 2003). Spore germination and the release of antigens may be caused by excess mucus which could stimulate the T-helper cell type (Th) 2-biased responses leading to ABPA. Inflammation in the CF lungs as a result of *A. fumigatus* colonisation may be caused by the antibodies IgG and IgE. Patients may become infected with more than one strain, although single strains may become dominant over the course of infection (Cimon *et al.*, 2001; deValk *et al.*, 2009; deValk *et al.*, 2005). Rarely, *Aspergillus fumigatus* may present as a fungal ball

known as an aspergilloma which can develop within the lung parenchyma and may be an indication of lung transplantation in CF patients (Zmeili *et al.*, 2007; Ryan *et al.*, 1995). Another form of pathology has been recognised as non-allergic fungal bronchitis. Denning *et al.*, (1995), described fungal bronchitis as *Aspergillus* infection confined to the tracheobronchial tree in which there is evidence of bronchial inflammation and excess mucus production with *Aspergillus* as the only pathogen.

Few studies have reported on the frequency of resistance *Aspergillus* sp. to antifungal agents. A CF fungal study by Mortensen *et al.*, (2011), found 4.5% of *A. fumigatus* isolates from CF patients were resistant to azoles, all of which were treated with azoles. Recently, Burgel *et al.*, (2011), found 4.6% of *A. fumigatus* isolates recovered from one CF centre were resistant to itraconazole. Studies have found the TR/L98H resistance mechanism associated with *A. fumigatus* azole resistance is prevalent in CF patients from many countries including the United Kingdom, Denmark and the Netherlands (Snelders *et al.*, 2008).

1.5 Physiology of *Pseudomonas aeruginosa* biofilms

Biofilms are communities of bacterial cells growing on a surface encased within an exopolysaccharide matrix. It has been estimated that approximately 65% of bacterial infections are biofilm related (Costerton *et al.*, 2003), such as infections associated with cystic fibrosis patients, dental diseases, wounds and infections associated with indwelling

devices such as catheters, orthopedic devices and heart valves (Singh *et al.*, 2003).

1.5.1 Motility of *P. aeruginosa* isolates

Three types of motility are associated with *P. aeruginosa*: swimming, swarming and twitching. Swarming motility involves the movement of cells across a hydrated, viscous, semi-solid surface while swimming motility involves movement across a low-viscous surface (Deziel *et al.*, 2003; Kohler *et al.*, 2000; Rashid *et al.*, 2000). Twitching motility enables the formation of microcolonies and facilitates bacterial interactions, forming cell clusters and strengthening the attachment of cells to a surface (Sauer *et al.*, 2002). Deligianni *et al.*, (2010), observed isolates capable of twitching motility produce towering structures, while others are associated with the formation of thick lawns. Molecular studies by Caiazza *et al.*, (2007), identified mutations in the *SadB* gene participate in inverse regulation of biofilm formation and motility. Flagellar motility is important for *P. aeruginosa* surface attachment while type-IV pili facilitate microcolony formation (Henrichsen *et al.*, 1972).

1.5.2 Quorum sensing in *P. aeruginosa* biofilm

Quorum sensing (QS) is defined as the communication between bacteria which leads to the development of specific bacterial structures. *P. aeruginosa* contains two QS systems, *las* and *rhl* systems. The *las* system is comprised of the transcriptional activator *lasR* and an AI synthase enzyme *lasI* and the *rhl* system is comprised of the transcriptional activator *rhlR* and the AI synthase enzyme *RhlI* (Latifi *et al.*, 1996; Pesci *et al.*, 1999). Both of the systems are autoinducer synthases which catalyse the formation of

homoserine lactone (HSL) known as autoinducer N-(3-oxododecanoyl)-homoserine lactone (Pearson *et al.*, 1994). Siderophore production and the formation of mature biofilms of *P. aeruginosa* are dependent on the N-3-oxododecanoyl homoserine lactone ($3OC_{12}$-HSL). Recent studies found both QS systems existed within the sputum of CF patients that had been colonised with *P. aeruginosa* (Parsek *et al.*, 2000; Singh *et al.*, 2000; Davies *et al.*, 1998). According to the study by Davies *et al.*, (1998), the *las* quorum sensing system enables biofilms to develop into towering-shaped structures. Whiteley *et al.*, (1999), suggested approximately between 3 to 10% of the *P. aeruginosa* genome is controlled by the QS system.

The QS regulatory system of *B. cepacia* relies on two different proteins, *CepI* which controls the synthesis of N-octanoylhomoserine lactone (C8-HSL) and N-hexanoylhomoserine lactone (C6-HSL) and *cepR* which after binding with C8-HSL either activates or represses transcription to certain target genes (Eberl *et al.*, 2001). Studies have documented that *B. cepacia* strains defective in the *cep* QS system develop biofilms that differ from the wild-type. In addition, the ability of such bacteria to swarm across an agar plate has been linked to the activity of this system (Allison & Hughes, 1991). Lewenza *et al.*, (2001), found the *cep* gene was an important regulator of protease production and repressor of the synthesis the siderosphore ornibactin.

1.5.3 *P. aeruginosa* biofilm formation

The biofilm formation process consists of a number of formation steps (O'Toole *et al.*, 2000; Sauer *et al.*, 2002).

These stages are listed below:

- Reversible attachment involves free-floating bacterial cells adhering to a surface.
- Irreversible attachment involves growth and aggregation of cells into microcolonies.
- Maturation involves biofilm growth into the typical mushroom and tower-shaped structures.
- Detachment involves dispersal of surface bacterial cells to new locations.

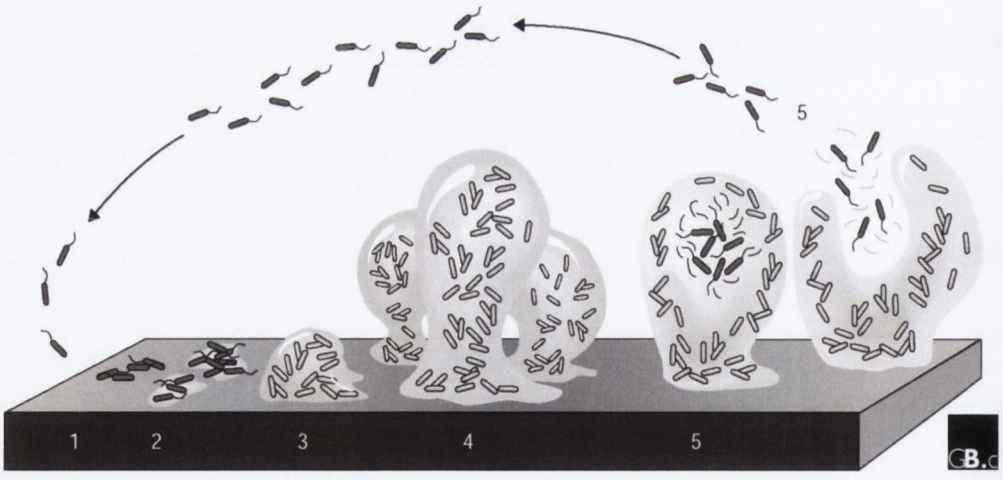


Figure 1.4: Biofilm formation process depicting stages of reversible attachment, irreversible attachment, maturation and detachment (Costerton *et al.*, 2002).

1.5.4 Reversible attachment

The first step of the biofilm formation process involves initial reversible attachment to a conditioning surface (Marshall *et al.*, 1985; Boland *et al.*, 2000). Attachment of the microorganisms occurs by specific interactions such as van der Waals forces, electrostatic and hydrophilic interactions or a combined effect of the different forces between the surface on which the cells and molecular groups that have adhered (Van Loodsdrecht *et al.*, 1990; Carpentier *et al.*, 1993; Zottola and Sasahara, 1994; An *et al.*, 2000). A small amount of EPS is produced which surrounds the biofilm (O'Toole *et al.*, 1998). At this point the structure is not stable enough and is capable of dispersion into planktonic cells by external forces. Biofilm architecture is facilitated by the direction of cell growth which has been described as horizontal, vertical or a combination of both directions (Stoodley *et al.*, 1999).

1.5.5 Irreversible attachment

The exopolysaccharide matrix facilitates the conversion from reversible to irreversible attachment by surrounding the individual cells, forming a community with unique physiological characteristics (Allison *et al.*, 2000; Costerton *et al.*, 1994; Costerton *et al.*, 2001). Cells begin to distribute evenly forming multi-layered structures containing void sections through which fluid can flow (Costerton *et al.*, 1994). Glucose, fructose, mannose, galactose, pyruvate and uronic acid residues of D-mannuronic and L-guluronic acid are present within the EPS matrix (Brisou *et al.*, 1995; Franklin *et al.*, 1993). EPS forms the bulk of biofilm architecture at 75 - 90% (Chang *et al.*, 2007). Davies *et al.*, (1993), found the expression of alginate genes may be critical to biofilm formation by *P.*

aeruginosa. Nivens *et al.*, (2001), showed that *O*-acetylation of alginate was essential for biofilm maturation. The production of EPS may be required for initial structure composition, not just for attachment to abiotic surfaces (Danese *et al.*, 2000; Prigent-Combaret *et al.*, 2000).

1.5.6 Maturation

A recent study by Davey *et al.*, (2003), indicated that the development of mature biofilm structures is mainly facilitated by quorum sensing. Bacterial motility which is caused by type-IV pili and flagella forces cells to become distributed (O'Toole *et al.*, 1999; Klausen *et al.*, 2003). Biofilms increase in thickness forming mushroom or pillar shaped structures containing an array of active and dormant cells with void spaces and channels (O'Toole *et al.*, 2000; Sauer *et al.*, 2002). Any voids that appear may decrease in size as the structures mature (Venugopolon *et al.*, 2005). Quorum sensing influences the concentration of a diffusible autoinducer. This provides a regulatory signal in response to cell density which is essential for the formation of mature *P. aeruginosa* structures (Davies *et al.*, 1998). The production of biosurfactants, known as rhamnolipids, by *P. aeruginosa* has been shown to maintain all channels surrounding the microcolonies (Davey *et al.*, 2003). If the nutrient supply is limited, biofilms can expand or migrate in order to search for nutritional or carbon sources (Stoodley *et al.*, 1999).

1.5.7 Detachment

When biofilms reach a particular mass, cells begin to detach from the outer layers of the matrix and re-colonise at new locations (Korber *et al.*, 1989; Sternberg *et al.*, 1999). The

sole purpose of biofilm detachment is to prevent starvation of the cells (Rice *et al.*, 2005). Cells located within the interior of the biofilm structure have less access to nutrients and studies have suggested a large volume of metabolic waste can accumulate within this area (Sternberg *et al.*, 1999). Boyd *et al.*, (1995), and his group described a less polymerized exopolysaccharide forms a less dense matrix, facilitating the detachment of cells. Additional studies have shown that alginate lysae; a degradative enzyme cleaves the polymer matrix into short oligosaccharides increasing the detachment process (Boyd *et al.*, 1995). Research by Sauer *et al.*, (2002), and Hoiby *et al.*, (2002), reported that cells removed from the biofilm may retain some of the same mechanisms of antimicrobial resistance as attached cells.

1.5.8 Formation of non-surface attached microcolonies

During the biofim development process bacteria may develop as microcolonies that are not attached to any particular surface. These structures may differ from biofilms that attach to surfaces enclosed within a mucous membrane. Recent clinical studies have found samples recovered from infectious sites in the human body contain microorganisms that do not directly attach to the site of colonisation. Biofilm clusters of chronic wounds that harbour *P. aeruginosa* tend to be larger and have a longer healing process than other strains (Alhede *et al.*, 2011; Gjodsbol *et al.*, 2006). Barclay *et al.*, (1996), described detachment of bacterial cells as an important strategy if species are to survive in a stressful environment.

Previously, Costerton *et al.*, (1987), described the microcolonies of different species may encase within their own unique exopolysaccharide matrix. The larger microcolonies

which are surrounded by a matrix are more protected to a stressful environment (Morris *et al.*, 1997). Limited studies have compared phenotypic characteristics of bacteria that are not attached to the typical biofilm structures that adhere to a surface. Recently, Alhede *et al.*, (2011), reported non-attached bacterial aggregates have been associated with infections in the chronic CF lungs, otitis media and wounds.

1.5.9 Antimicrobial resistance of biofilms.

According to Costerton *et al.*, (2001), there are several mechanisms which are responsible for biofilm resistance to antimicrobial agents listed below:

- Reduced penetration of antimicrobial agents through the EPS matrix.
- Growth rates of biofilm-forming isolates are different for each strain.
- Changes to the physical biofilm structure due to different growth conditions in comparison to planktonic cells.

1.5.10 Reduced penetration of the antimicrobial agent through the EPS matrix.

Previous studies have reported that *P. aeruginosa* exopolysaccharide matrix can slow the diffusion of antibiotics into the biofilm. Many antibiotics are capable of diffusing through the EPS matrix of the biofilm but may lose their efficacy during this process. These processes can enhance resistance to low concentrations of aminoglycoside antibiotics, such as tobramycin (Brooun *et al.*, 2000). Wild-type strains may be able to reduce the efficacy of antibiotics at the outer layers of the biofilms more rapidly than the rate at

which they diffuse into the biofilm matrix (Kumon *et al.*, 1994; Shigeta *et al.*, 1997).

1.5.11 Different growth rates of biofilm-forming isolates.

Cells growing within a biofilm undergo phenotypic changes which are associated with surface attachment and antibiotic resistance. Multidrug resistant pumps (MDR) are thought to play a role in the resistance of *P. aeruginosa* to antimicrobial agents. The four efflux pumps of *P. aeruginosa* are MexAB-oprM, MexXY-oprM, MexCD-oprJ and MexEF-oprNIO (Poole *et al.*, 2001). DeKievit *et al.*, (2001), indicated that two MDR efflux pumps of *P. aeruginosa*, MexAB-OprM and MexCD-OprJ are heterogeneous throughout the biofilm population, with greater expression occurring in cells located at the substratum. According to Allison *et al.*, (2000), *P. aeruginosa* strains that do not have the MexAB-OprM efflux system are more resistant to fluoroquinolones such as ciprofloxacin.

1.5.12 Changes to the biofilm structure due to an altered mode of growth.

Bacteria within biofilms may employ specific mechanisms to resist the activity of antimicrobial agents. *P. aeruginosa* strains that produce rugose small colony variants (RSCV) demonstrated resistance to kanamycin, amikacin, carbenicillin, gentamicin, tobramycin and tetracycline (Drenkard *et al.*, 2002). Zhang *et al.*, (1996), reported that accumulation of acidic waste products might lead to pH differences between the bulk fluid and biofilm interior which could antagonise the activity of antibiotics, particularly against the aminoglycoside agents. Tuomanen *et al.*, (1986), mentioned accumulation of agents by some organisms might cause them to enter a non-growing state in which they

are protected from antimicrobial agents. The study by Tack *et al.*, (1985), revealed that aminoglycosides are less active against the same strain in anaerobic rather than aerobic conditions.

1.5.13 Persister cells

Studies have documented that a group of cells known as 'persisters' is responsible for *P. aeruginosa* high tolerance to antimicrobial agents (Lewis and Speoring, 2001). Persisters are phenotypic variants of wild-type cells that produce a similar amount of persister cells upon re-inoculation (Keren *et al.*, 2004). They were first described by Joseph Bigger in 1944 that discovered penicillin could not inhibit a culture of *staphylococcus* (Bigger *et al.*, 1944). Lewis *et al.*, (2001), proposed that persister cells ensure the survival of a cell population in the presence of other inhibitory agents. The gene expression profile of persister cells of *E. coli* showed that their formation was caused by chromosomally encoded toxin-antitoxin molecules. Overexpression of toxins *Re1E* and *HipA* increases the production of multidrug tolerant persister cells to aminoglycosides such as tobramycin and kanamycin (Keren *et al.*, 2004).

1.6 Biofilm formation in different model systems.

Biofilms are dynamic and organised communities of cells and can form in static or flowing systems (Costerton and Stewart, 2001). The literature supports the use of numerous models with different biofilm forming abilities. The flowing system enables the internal structure of biofilm to adapt to the flow velocity at which they grow. This

alters the rate of the internal mass transfer and microbial activity (Singh *et al.*, 2003; Beyenal and Lewandowski, 2002; Goodman and Geesey, 2001). The flow cell system for studying biofilms allows individual cells to be monitored over time (Singh *et al.*, 2002) but does not allow an array of studies to be conducted at any one time. More rapid approaches for the study of biofilms include the microtitre plate assay (Moskowitz *et al.*, 2004) and the 'colony biofilm assay'. The 'colony biofilm assay' involves a high density of bacteria applied as a spot onto an agar plate which allows communities of cells to develop on polymer membranes (Anderl *et al.*, 2000).

1.6.1 Calgary Biofilm Device

The Calgary Biofilm Device (CBD) consists of a 96-well plate and peg lid on which biofilms are grown. The pegs are designed to sit in the reaction vessel and the bottom of the channel which allows medium to flow across the pegs and create a shear force. The device is placed onto a shaker table and after a sufficient amount of time cells adhere to the pegs. Biofilms that are formed can be treated with various antimicrobial agents to determine the susceptibilities of different strains (Ceri *et al.*, 1999).

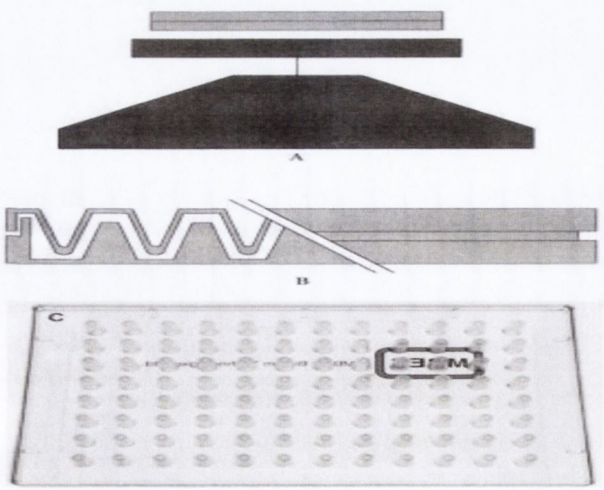


Figure 1.5: Calgary Biofilm Device (CBD) for biofilm susceptibility testing. **(A)** The tilted table creates shear force for biofilm formation. **(B)** The lid containing pegs is designed so that it fits into the tray. **(C)** The polystyrene plate contains 96 pins for biofilms to form (Ceri *et al.*, 1999).

1.6.2 Modified Robbins Device (MRD)

In vitro biofilms can be formed under shear force on diverse substrata under controlled flow conditions by the modified Robbins Device. This device operates by pumping inoculums through a channel in which removable studs are set against a lumen surface. The studs where the biofilm develops, as a result of shear force, are able to be removed and studied (Stoodley *et al.*, 1999; Nickel *et al.*, 1985). Unlike other *in vitro* model systems simultaneous biofilm formation of different microbial species can be conducted on different media and substrata. The discs can be removed from the MRD and various antimicrobials can be tested for antibiofilm activity (Coenye *et al.*, 2007).

1.6.3 Colony biofilms

The colony biofilm assay involves growing bacteria on the top of polymer membranes that have been placed onto TSA agar plates. Any colonies that develop are transferred to fresh TSA agar plates and monitored for 24 hours. This method allows penetration of antimicrobial agents to be measured form all sections of the biofilm (Anderl *et al.*, 2000). Transmission electron microscopy (TEM) results by Walters *et al.*, (2002), have shown that bacteria can grow in an evenly distributed manner throughout the colony.

1.6.4 Flow cell system

More advanced bacterial culture systems are emerging for studying the stages of biofilm development in a non-destructive way (Allegrucci *et al.*, 2006). The studies involving flow cells frequently found in the literature allow biofilms to be studied under controlled conditions and enable direct observation using a microscope. Continuous flow provides a steady supply of nutritional requirements, enhancing the expression of various genes for greater biofilm growth (Sauer *et al.*, 2004). The concentration of nutrients supplied has been reported to influence the architecture of developing biofilms (Stoodley *et al.*, 1999). Typically anaerobic species are analysed with this model but studies have extended to aerobic bacterial cultures (O'Sullivan *et al.*, 2009). The system enables biofilms to develop under hydrodynamic conditions. Visual analysis of the biofilms which have developed may be carried out without stopping flow which could disturb biofilm growth (Manz *et al.*, 1999).

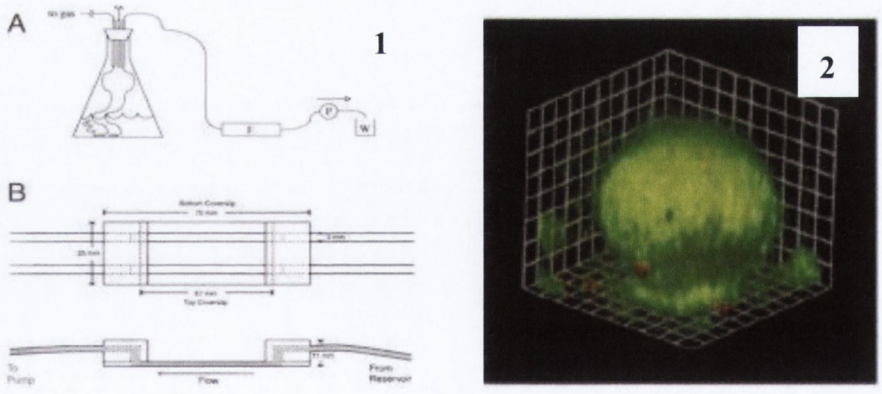


Figure 1.6: 1. (a) Schematic diagram of the flow cell system. **(b)** The upper glass of the flow cell on which biofilms are grown can be viewed using a microscope (Drake *et al.*, 2002). **2.** Confocal image of a *gfp*-tagged *P. aeruginosa* biofilm grown in this system for 6 days (Banin *et al.*, 2006).

1.7 Novel agents to control *P. aeruginosa* biofilms

1.7.1 Lactoferrin

Lactoferrin is an iron-binding protein of the innate immune system, found on mucosal surfaces (Harbitz *et al.*, 1984). Recent findings by Ammons *et al.*, (2009), demonstrated the importance of lactoferrin in blocking biofilm formation of *P. aeruginosa.* Other studies have shown that lactoferrin displays bacteriostatic activity through the ability to sequester iron (Bullen *et al.*, 1972). Additionally, the agent is able to bind to lipopolysaccharides and disrupt bacterial membranes (Ellison *et al.*, 1994; Leitch *et al.*, 1999). Synergistic interactions of lactoferrin with other agents such as xylitol, a rare alcohol sugar commonly found in fruit and vegetables, have also been reported against *P. aeruginosa* biofilms. As single agents, lactoferrin and xylitol weakened the biofilm structure of *P. aeruginosa*, however, biofilms were almost completely disrupted with both agents in combination (Ammons *et al.*, 2009). Another study by Caraher *et al.*, (2007b), found human lactoferrin increased the susceptibility of Bcc biofilms to many antibiotics. Overall, the studies mentioned suggest iron chelators may be useful for disrupting bacterial biofilms in the CF lung.

1.7.2 Gallium nitrate

Singh *et al.*, (2007), on studying the effect of transition metal gallium nitrate against *P. aeruginosa* indicated that organisms are unable to distinguish between iron and gallium. The availability of iron enhances virulence, promotes biofilm formation and enhances the cell cluster development and maturation of biofilms into three-dimensional structures. In

addition, Singh *et al.*, (2007), suggested gallium may prevent *P. aeruginosa* biofilm formation when applied to sites of high lactoferrin by enhancing the activity of lactoferrin. Another possible suggestion may involve a direct relationship between iron acquisition genes and pathogenesis indicating low levels of iron in the CF lungs is essential for *P. aeruginosa* virulence. The *PvdS* gene which controls pyoverdine production is regulated in a low iron environment and the *PvdS* protein is synthesised only under conditions of reduced iron availability (Singh *et al.*, 2007). Additionally, Musk *et al.*, (2005), found ferric ammonium compounds (FAC) inhibited *P. aeruginosa* biofilm formation in a dose-dependent manner indicating that *P. aeruginosa* biofilm formation can be influenced by the concentration of iron.

1.7.3 Ethylenediaminetetraacetic acid (EDTA)

The most specific mechanism of action of EDTA against gram-negative bacteria involves the disruption of the lipopolysaccharide structure in the outer membrane which leads to a more permeable membrane (Marvin *et al.*, 1989). As a result, many researchers have explored the interactions of EDTA with other antimicrobial agents. The study by Lambert *et al.*, (2004), reported a possible avenue of combined treatment with EDTA and ampicillin and other antipseudomonal antibiotics for the treatment of patients chronically colonised with *P. aeruginosa*. Recent findings by Al-Bakri *et al.*, (2009), described synergistic interactions of aspirin and metal chelator, EDTA involved altering the mechanisms involved in membrane permeability, virulence factor production and a reduction in extracellular polysaccharide and prostaglandin production.

1.7.4 Aspirin

Farber *et al.*, (1992), demonstrated salicylic acid (SA), a major metabolite of acetylsalicylic acid, inhibited adherence, growth and biofilm production of *S. epidermidis*. Steptanovic *et al.*, (2004), explored the activity of aspirin against *Candida* species biofilm formation revealing a significant decrease in biofilm growth at concentrations $\geq$ 1.73 mM. Anti-adhesion properties of aspirin were reported by Demirag *et al.*, (2007), when the agent was used to treat graft infections caused by coagulase-negative *staphylococcus*. Other studies found salicylic acid was involved in down-regulation of several virulence factors of *P. aeruginosa* (Prithiviraj *et al.*, 2005).

1.7.5 Heparin

Researchers have explored the ability of anticoagulants to disrupt biofilms since most antibiotics do not possess this mode of action. Gotz *et al.*, (2002), described heparin to alter the processes that involve attachment and adhesion for biofilm formation. In combination with teicoplanin and ciprofloxacin, heparin displayed strong antibiofilm activity against *P. aeruginosa* and *S. aureus*. A study by Heilmann *et al.*, (1996), indicated heparin enhances detachment of biofilms that produce biofilm matrix polysaccharide poly-N-acetylglucosamine/polysaccharide intercellular adhesion of *S. aureus, S. epidermidis, S. lugdunensis* and *S. carnosus.*

1.7.6 Natural products

Natural extracts derived from plants have provided medicinal properties for treating infectious diseases for thousands of years. A number of recent studies have investigated

the activity of plant extracts on specific pathogens. Rasmussen *et al.*, (2005), demonstrated extracts of garlic inhibit quorum sensing and polymorphonucleotide activation (PMA) in *P. aeruginosa* and reduce antibiotic resistance to tobramycin. The active component of garlic, allicin is effective against many gram-negative pathogens (Ankri *et al.*, 1999). In other studies, ginseng has shown to prevent *P. aeruginosa* biofilm formation (Hoiby *et al.*, 2011), by reducing signalling of quorum sensing systems (Song *et al.*, 2010). Ginseng has shown to stimulate and develop the TH1 immune response allowing clearance of severe infection (Song *et al.*, 1997).

1.8 Project Aims and Objectives:

1) To phenotypically characterise clinical *P. aeruginosa* isolates from the cystic fibrosis lung.

2) To determine the effects of antibiotics singly and in combination against a range of clinical CF and reference strains.

3) To assess the ability of novel metal-based agents to inhibit established *P. aeruginosa* biofilms. To determine the effects of selected synthesised compounds against biofilms grown in the flow cell system.

4) To investigate the growth and development of *P. aeruginosa* biofilms in a mixed culture with other identified CF pathogens and to explore any interactions between these species. To analyse the protein profiles of CF strains that may be interfering with the growth of *P. aeruginosa* with the aim of identifying proteins that may be contributing to virulence during co-infection.

Chapter 2: Characterisation and Antibiotic Susceptibility testing of *P. aeruginosa* strains from patients with cystic fibrosis.

2.1 Introduction

P. aeruginosa biofilms are difficult to eradicate with standard antibiotic therapy and isolates recovered from CF patients undergo mutations that are likely to become highly antibiotic resistant (Ogle *et al.*, 1988). Antibiotic susceptibility testing allows rapid identification of the susceptibility profile of certain microorganisms using standardised laboratory methods (Saha *et al.*, 2001). The most common way of assessing the activity of antimicrobial agents is to determine the minimum inhibitory concentration (MIC) and synergism between antimicrobial combinations is determined by the fractional inhibitory concentration index (FICI) (Horrevorts *et al.*, 1987).

Biofilm quantification may be analysed using a variety of different techniques. Moskowitz *et al.*, (2004), developed the biofilm inhibitory concentration (BIC) assay based on the assay by Ceri *et al.*, (1999), for a more rapid and reliable means of screening various antimicrobial agents against biofilms. This technique allows biofilms to develop by attaching to the surfaces of polystyrene pegs. Masten *et al.*, (1981), described the importance of this *in vitro* model in a CF clinical setting as it acts as an initial guide when selecting suitable concentrations of antibiotics for CF patients, since many CF pathogens are able to form biofilms.

Previous studies have suggested results obtained from *in vitro* models do not correlate to

the outcome of infection in an *in vivo* environment (Perdreau-Remington *et al.*, 1998). According to the study by Saiman *et al.*, (1999), there has been no record of the most accurate *in vitro* model for antibiotic susceptibility testing of CF species. Aaron *et al.*, (2002), described biofilms formed on polystyrene pegs as less mature than the typical multi-layered biofilm structures. Burton *et al.*, (2007), noted that following crystal violet staining, the dye may interact with negatively charged cells and does not reflect the total amount of cells that are viable and non-viable. Other studies that have quantified viable bacteria by removing cells from the surfaces of the pegs suggest there is a possibility not all cells are dislodged effectively after biofilm disruption (Aaron *et al.*, 2002).

The use of *in vivo* models as a means to investigate virulence of complex bacterial species and their response to treatment has been used by many researchers. Nematodes such as *Galleria mellonella* and mice and insects such as Drosophila have all been employed as alternative models to determine the virulence of bacterial species (Rambaugh *et al.*, 1999). *G. mellonella* has been described as more accurate than other infection models (Seed *et al.*, 2008), and shares similar structural and functional homology to the innate immune systems of mammals (Hoffmann *et al.*, 1995). Phenotypic characteristics of isolates have been shown to play an important role in the rate and severity of disease progression and response to antimicrobial agents within these models (Oliver *et al.*, 2000).

The aim of this chapter was to determine phenotypic characteristics of *P. aeruginosa* clinical CF and reference strains using *in vitro* and *in vivo* models. This section also explored the activity of single and combinations of antibiotics against *P. aeruginosa*

planktonic and biofilm forming isolates.

2.2 Materials and Methods

2.2.1 Research Ethics.

Ethical approval was not required for the research carried out in this section.

2.2.2 CF sputum samples.

Previously, an Irish CF clinical study was undertaken from 2004 – 2005. Sputum samples were collected from patients who were chronically colonised with *P. aeruginosa* from 12 different Irish CF clinics. All strains were stored in glycerol beads at -80°C for further study.

2.2.3 Preparation of Sputasol.

An equal volume of Sputasol (v/v) (Oxoid, Basingstoke UK) was added to the sputum samples. A quantity of 7.5 mls of sterile distilled water was added to 7.5 mls Sputasol and added to 92.5 mls sterile water. All prepared reagents can be used for up to 48 hours.

2.2.4 Preparation of sputum cultures.

Prepared sputum samples were agitated gently for approximately 10 seconds and incubated for 15 minutes at 35°C with gentle agitation to assist homogenisation. A quantity of 10 µl of the homogenised sputum was added to 5 mls sterile distilled water and 1 µl of this dilution was inoculated onto Columbia blood agar (CBA), centrimide agar, *Burkholderia cepacia* selective agar (BCSA) and McConkey agar (Fannin, Ireland).

Plates were incubated for 24 - 48 hours at 35°C and total viable counts (TVC) were calculated. Aliquots of homogenised sputum samples were added to glycerol and stored at -80°C. Any bacterial strains that were gram-negative, mucoid or produced a blue/green pigment on Pseudomonas selective agar were identified as *P. aeruginosa.* Any strains that could not be identified were analysed using the Vitek 2 system (bioMerieux) in the microbiology laboratory, AMNCH.

2.2.5 Selection of *Pseudomonas aeruginosa* strains.

All clinical isolates of *P. aeruginosa* used in this study were obtained from 12 different Irish cystic fibrosis clinics and screened in Adelaide and Meath Hospital Incorporating the National Children's Hospital (AMNCH), Tallaght hospital. All strains were typed using pulsed-field gel electrophoresis (PFGE) in the CF reference laboratory in AMNCH. *P. aeruginosa* strains that represented dominant clones and several clones that were not dominant among patients with cystic fibrosis from the different clinics were used for further study. *P. aeruginosa* ATCC27853 and *P. aeruginosa* PA01 were used as reference strains. All strains were added to glycerol and stored at -80°C. When conducting experimental procedures, strains were grown on Tryptone Soy Agar (TSA) plates and inoculated in nutrient broth for 24 hours at 35°C.

Sources of *P. aeruginosa* strains used in this study.

***P. aeruginosa* common clones.**

Species:	**Source:**
P. aeruginosa 1826	CF lung (Irish CF clinic 1)
P. aeruginosa 4276.3	CF lung (Irish CF clinic 2)
P. aeruginosa 81599	CF lung (Irish CF clinic 4)
P. aeruginosa 1438	CF lung (Irish CF clinic 5)
P. aeruginosa 5605	CF lung (Irish CF clinic 3)

***P. aeruginosa* uncommon clones.**

Species:	**Source:**
P. aeruginosa 4505.2	CF lung (Irish CF clinic 2)
P. aeruginosa 4649	CF lung (Irish CF clinic 2)
P. aeruginosa 5714	CF lung (Irish CF clinic 3)
P. aeruginosa 88716	CF lung (Irish CF clinic 4)
P. aeruginosa 6279	CF lung (Irish CF clinic 6)

Reference strains.

Species:	**Source:**
P. aeruginosa ATCC27853	Reference strain
P. aeruginosa PA01	Reference strain

2.2.6 Antibiotics.

The antibiotics used were tobramycin, ceftazidime, meropenem, ciprofloxacin and colistin and were obtained from the pharmacy at AMNCH. Stock solutions were prepared for tobramycin (40 mg/ml), ceftazidime (50 mg/ml) and meropenem (50 mg/ml) in sterile water and stored at -80°C. A stock solution of colistin (20 mg/ml) was prepared in sterile water and stored at -20°C. Ciprofloxacin solution (2 mg/ml) was stored at room temperature. All reagents were filter sterilised through 0.45 μM pore filters (Fisher Scientific) before use. The final concentration of each antibiotic added to the media was 256 μg/ml. This was the final concentration used for all experimental procedures.

2.2.7 Growth media.

Media was purchased from Oxoid (Basingstoke, UK), or prepared according to the manufacturer's instructions and sterilised at 121°C for 15 minutes.

2.2.8 Plankonic growth of *P. aeruginosa* strains.

Overnight cultures of *P. aeruginosa* clinical and reference strains were prepared in nutrient broth and incubated at 35°C. Bacterial growth was analysed by plating a range of serial dilutions on TSA and determining the CFU/ml at regular intervals of 0, 1, 2, 4, 6, 24, 48, and 72 hours prior to incubation.

2.2.9 Epsilometer test (E-test).

A 0.5 McFarland (1×10^8 CFU/ml) standard was prepared for each *P. aeruginosa* isolate in sterile saline solution. A lawn was created on Muller-Hinton agar (MHA) plates by

swabbing the culture in two directions and left to dry for several minutes. Using a sterile forceps E-test strips (AB BioDisc, Sweden) containing tobramycin (TM), ceftazidime (TZ), meropenem (MP) and ciprofloxacin (CIP) were placed on the surface of the agar with the lowest concentration at the centre of the plate. The strip was pressed gently onto the agar plate so that no air bubbles were formed. Plates were incubated at 35°C for 24 hours. E-test MIC (μg/ml) values were read where the edge of the inhibition ellipse intersects the strip. Antibiotic susceptibility testing of colistin was carried out using the Vitek 2 system, microbiology laboratory at AMNCH.

2.2.10 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination.

MIC values of antibiotics were determined by the microtitre method as described in the Clinical Laboratory Standards Institute (CLSI) guidelines. A quantity of 100 μl of sterile nutrient broth was added to the wells of a 96-well round-bottom microtitre plate (Nunc Inc., Roskilde, Denmark). Stock solutions of antibiotics were prepared in nutrient broth starting at a final concentration of 256 μg/ml. A quantity of 100 μl of this solution was added to first well of the 96-well plate and serially diluted. A quantity of 100 μl of the suspension of the organism was added to each well at a final concentration of 5×10^5 CFU/ml. The microtitre plates were sealed and incubated at 35°C for 24 hours. After 24 hours incubation, the MIC was determined as the lowest concentration of antibiotic showing no visible bacterial growth.

2.2.11 Optimisation of the biofilm quantification assay.

Biofilm quantification was assessed using four different disruption methods to select a suitable assay for testing biofilm biomass following antimicrobial treatment. An overnight culture of *P. aeruginosa* PA01 was suspended in nutrient broth at a final concentration of 5 x 10^5 CFU/ml. The wells of a round-bottom polystyrene 96-well plate were inoculated with 100 µl of this inoculum. A transferable solid phase (TSP Screening, Nunc, Inc., Roskilde, Denmark) pin lid containing pegs was added to the plate for biofilms to develop and incubated at 35°C for 24 hours. The first assay involved sonication (UW, Ultrawave) of the plate for 5 minutes to dislodge the cells from the pegs, followed by incubation at 35°C for an additional 6 hours. The second assay involved no sonication and immediate incubation of the plate at 35°C for 6 hours. The third assay involved crystal violet staining of the pegs for 30 minutes followed by 1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich) exposure for an additional 15 minutes. The final assay involved crystal violet staining of the pegs for 30 minutes followed by exposure to a 1:1 mixture of 1% Triton X-100 (Sigma-Aldrich) and 95% ethanol (Sigma-Aldrich) for 15 minutes. The OD_{630} was measured in a microplate reader (ELx800, Universal Microplate Reader) after each procedure. All assays were carried out in triplicate on three separate occasions.

2.2.12 Biofilm growth determination.

To assess the ability of *P. aeruginosa* isolates used in this study to form biofilms, strains were grown on TSP pin lids as described in **Section 2.2.11** and incubated at 35°C for 24 and 72 hours. The TSP pin lids containing pegs were washed three times in sterile water

and allowed to dry at room temperature. The pegs were stained with 0.3% crystal violet, CV (Bener Limited, Ireland) for 30 minutes. Lids containing pegs were washed three times again to remove any dye that did not bind to the biofilm cells. Biofilms that were stained with crystal violet were removed with a (1:1 ratio) solution of 95% ethanol and 1% Triton X-100. The OD_{630} was measured in a microplate reader. Biofilm growth was recorded at an OD_{630} of ≥ 0.05 (Moskowitz *et al.*, 2004). All experiments were carried out in triplicate on three separate occasions.

2.2.13 Determination of virulence of selected *P. aeruginosa* isolates in the *in vivo G. mellonella* model.

Sixth instar larvae of *G. mellonella* (Greater wax moth) were obtained from Live Foods Direct (Sheffield, England) and stored in wood shavings in the dark at 15°C. Larvae weighing between 0.2 and 0.4g were selected for all experiments. An overnight culture of *P. aeruginosa* was prepared in nutrient broth at 35°C. *P. aeruginosa* was grown to late exponential phase, diluted to a final concentration of 1×10^8 CFU/ml in nutrient broth and a series of dilutions were prepared Ten larvae were inoculated with 20 µl of each dilution through the last right pro-leg into the haemocoel using a Hamilton syringe (Sigma-Aldrich). Larvae were placed on 9 cm petri dishes containing Whatman filter paper and incubated at 35°C for 48 hours. All experiments included a control which consisted of larvae injected with 20 µl sterile saline. Larval survival was determined at 24 hour intervals by movement in response to touch. All experiments were conducted in triplicate.

2.2.14 Biofilm Inhibitory Concentration (BIC) determination.

Isolates were grown as biofilms as described by Moskowitz *et al.*, (2004), adapted from the Calgary biofilm device to determine the susceptibility of *P. aeruginosa* strains to antibiotics singly and in combination. An overnight culture of *P. aeruginosa* was prepared in nutrient broth. The isolate was suspended in 3 mls of nutrient broth to a 0.5 McFarland standard and adjusted to a concentration of 5×10^5 CFU/ml. A quantity of 100 µl of this suspension was added to the wells of a 96-well round-bottom plate. A TSP pin lid was placed into the microtitre plate and incubated overnight at 35°C. The TSP pin lid was removed and rinsed three times in sterile water to remove any planktonic cells. Serial two-fold dilutions of antibiotics were prepared and added to a new 96-well plate to which the peg lid was transferred and incubated for 24 hours at 35°C. A quantity of 100 µl of crystal violet was added to each well of a 96-well plate. The lid containing pegs was rinsed three times again, dried at room temperature and added to the crystal violet for a further 30 minutes. To remove the stained biofilm, the lid was placed into a new 96-well round-bottom microtitre plate containing a (1:1 ratio) solution of 95% ethanol and 1% Triton X-100. The OD_{630} was measured in a microplate reader. The biofilm inhibitory concentration was determined as the last well that had an $OD_{630} \geq 0.05$. All experiments were conducted in triplicate on three separate occasions.

2.2.15 Fractional Inhibitory Concentration (FIC).

To determine the interactions that occurred between antibiotic combinations the Fractional Inhibitory Concentration (FIC) was calculated: (≤ 0.5 is synergistic, > 0.5 to ≤ 1 is additive, > 1 to ≤ 4 is indifferent and > 4 is antagonistic). This method is frequently

used to determine the activity of interactions between antimicrobial interactions.

2.2.16 Effect of antibiotic combinations on viability of *P. aeruginosa* biofilms.

An overnight culture of each isolate was diluted to an OD_{630} = 0.1 in nutrient broth. A quantity of 100 µl of this culture was added to each well of a 6-well plate containing 10 mls nutrient broth and incubated at 35°C for 24 hours, 72 hours or 7 days. After each time point the media was removed and changed to media containing concentrations corresponding to the MIC, 5 x MIC, 10 x MIC and 20 x MIC of antibiotics. Calculations of antibiotic concentrations are listed in Appendix 4. Biofilms were treated for a further 24 hours at 35°C. Following antibiotic treatment, all contents of the wells were removed and transferred to centrifuge tubes. Cells were centrifuged (Sigma, SK-15) at 3,000 *g* for 10 minutes. Cells were washed in 10 ml 0.9% NaCl and centrifuged at 3,000 *g*, 10 minutes again. Cells were re-suspended in 10 ml 0.9% NaCl followed by sonication and vortexing (Stuart Scientific Autovortex, SA5) until the cells were dispersed. Serial dilutions were prepared and spotted onto TSA plates to determine the CFU/ml.

2.2.17 Statistical analysis.

Statistical analysis was carried out using PASW^{R} Statistics 18 - SPSS. One-way ANOVA or Student's t-test was used to determine statistical variations for most experiments. A $P \leq 0.05$ value was considered statistically significant.

2.3 Results

2.3.1 Diversity of *P. aeruginosa* isolates grown planktonically.

To compare the growth of CF isolates that were common clones among CF patients, log CFU/ml counts were determined at specific time points. Both reference and clinical isolates showed similar growth curves as seen in **Figure 2.1**. *P. aeruginosa* ATCC27853 and *P. aeruginosa* PA01 showed a loss of viability at 72 hours compared to the other strains. The other isolates (*P. aeruginosa* 1826 and *P. aeruginosa* 4276.3) had a longer stationary phase and maintaining growth until 72 hours. The level of cell density reached is important when determining the response of these strains to antimicrobial treatment.

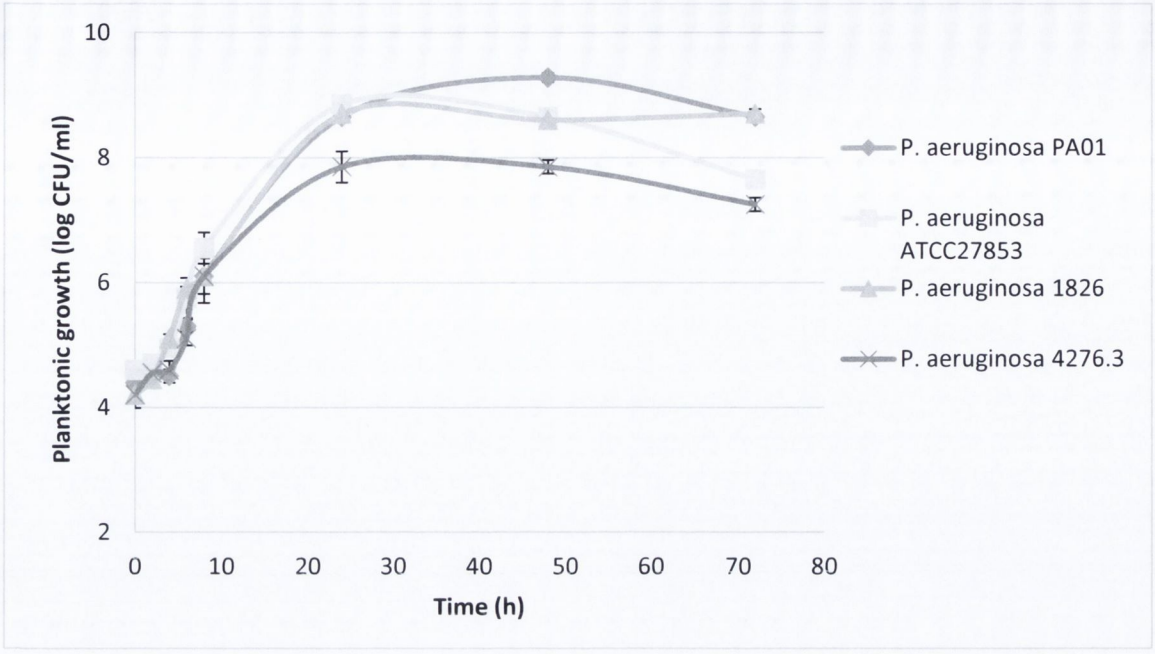


Figure 2.1: Differences in planktonic growth curves of selected *P. aeruginosa* isolates. Total viable counts were measured at regular intervals, every 2 hours for 6 hours with final readings at 24, 48 and 72 hours. Graph represents the mean ± standard deviation of two independent experiments.

2.3.2 Diversity of *P. aeruginosa* isolates grown as biofilms.

Biofilm formation was quantified by (a) the ability to adhere to polystyrene pegs in 96-well plates quantified by crystal violet straining and (b) viable counts following 24 and 72 hours incubation. The majority of *P. aeruginosa* clinical and reference strains showed a progressive increase in biofilm forming capacity from 24 to 72 hours on the pegs. The growth rate of *P. aeruginosa* 4649 was comparable at 24 and 72 hours. Certain strains (*P. aeruginosa* ATCC27853, *P. aeruginosa* 1826, *P. aeruginosa* 4505.2) consistently formed strong biofilm particularly at the 72 hour time point. Biofilm growth of *P. aeruginosa* 4505.2 on polystyrene pegs was significantly higher than the other strains at 24 hours. In

addition, this strain produced significantly more biofilm than ten out of twelve strains at 72 hours (one-way ANOVA), **Figure 2.3 (A)**. Interestingly, the two models showed variation in the amount of biofilm formed by each strain. A total of eight strains were characterised as strong biofilm formers on polystyrene pegs at 24 hours ($OD_{630} \geq 0.05$) with an increase in growth at 72 hours. Four clinical CF strains (*P. aeruginosa* 81599, 1438, 5605 and 6279) which exhibited a low level of adherence to the pegs had high biofilm cell counts at 24 and 72 hours. The results suggest that *P. aeruginosa* strains are capable of producing biofilms of high cell density at 24 and 72 hours even if the level of adherence to the pegs may be weak. Overall, biofilm formation is a common characteristic of CF *P. aeruginosa* strains which may be influenced by quorum sensing systems in the biofilm matrix (Davies *et al.*, 1998).

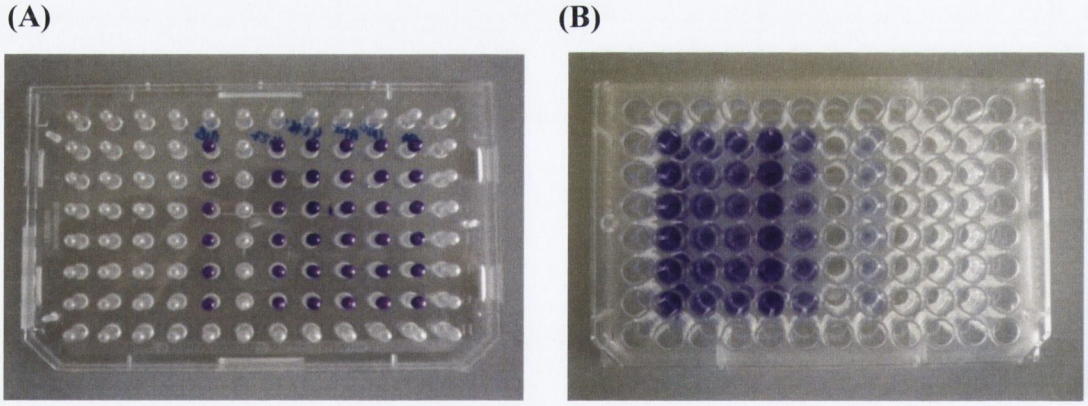
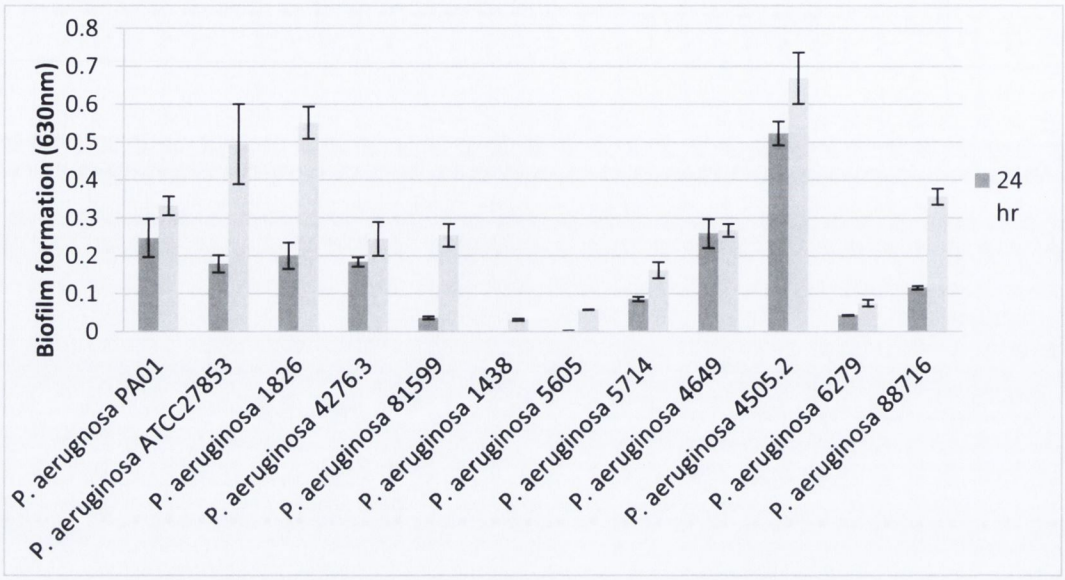


Figure 2.2: 24 hour biofilms of *P. aeruginosa* clinical CF strains grown on polystyrene pegs. Reference strains (*P. aeruginosa* PA01 and *P. aeruginosa* ATCC27853) are in columns 2 and 3, representing positive controls. Columns 4-6 represent clinical CF isolates. **(A)** Biofilm cells that adhered to the pegs were stained with crystal violet. **(B)** Crystal violet was removed from the pegs with 95% ethanol and 1% Triton X-100.

Table 2.1: Phenotypic characteristics of *P. aeruginosa* common clones, uncommon clones and reference strains.

Strain	Pigment	Mucoid/ Non-mucoid	Biofilm former
Reference strains			
***P. aeruginosa* PA01**	pigment	non-mucoid	biofilm former
***P. aeruginosa* ATCC27853**	pigment	non-mucoid	biofilm former
***P. aeruginosa* common clones**			
***P. aeruginosa* 1826**	pigment	non-mucoid	biofilm former
***P. aeruginosa* 4276.3**	no pigment	mucoid	biofilm former
***P. aeruginosa* 1438**	no pigment	non-mucoid	biofilm former
***P. aeruginosa* 81599**	no pigment	mucoid	biofilm former
***P. aeruginosa* 5605**	pigment	non-mucoid	biofilm former
***P. aeruginosa* uncommon clones**			
***P. aeruginosa* 4505.2**	pigment	mucoid	biofilm former
***P. aeruginosa* 4649**	pigment	mucoid	biofilm former
***P. aeruginosa* 5714**	pigment	non-mucoid	biofilm former
***P. aeruginosa* 88716**	pigment	non-mucoid	biofilm former
***P. aeruginosa* 6279**	no pigment	non-mucoid	biofilm former

(A)



(B)

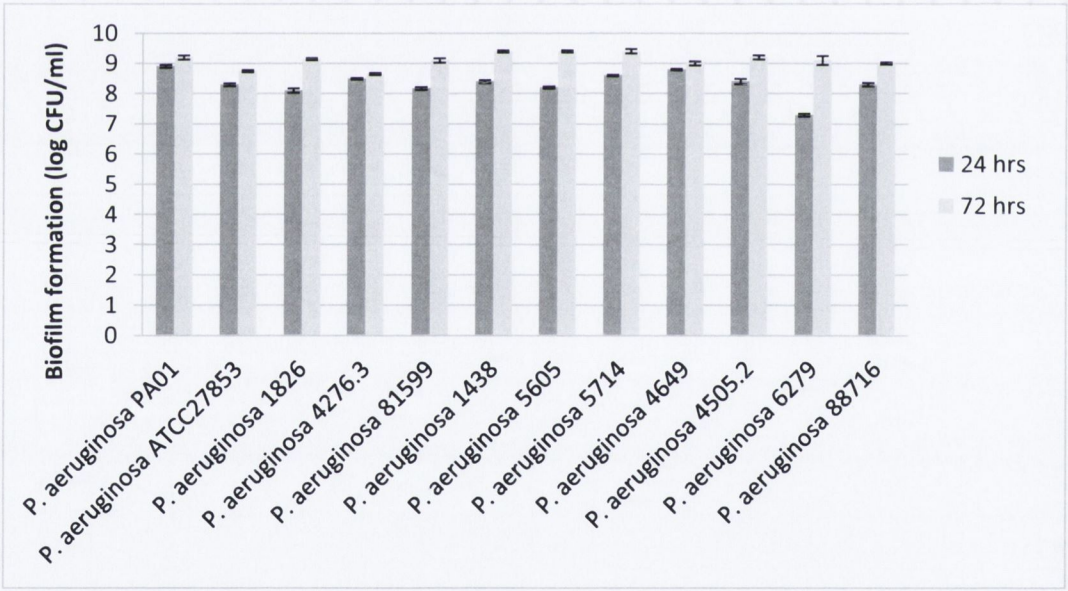
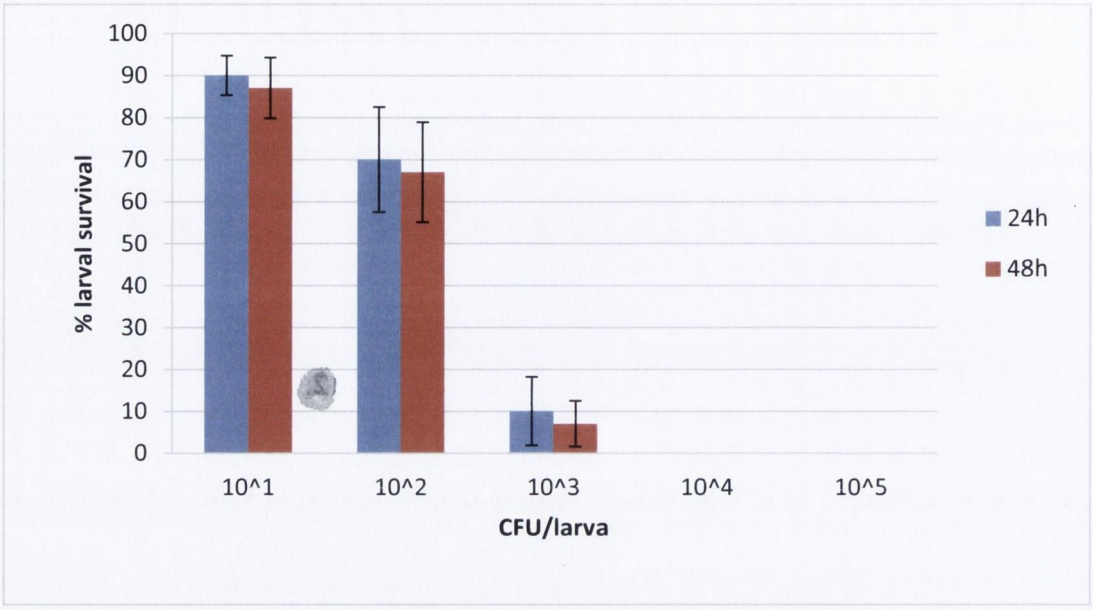


Figure 2.3: Biofilm formation of *P. aeruginosa* strains. Measurements of biomass were based on **(A)** OD_{630} of growth on polystyrene pegs and **(B)** log CFU/ml of viable biofilm cells. At 24 and 72 hours biofilm biomass was quantified for each *P. aeruginosa* strain. Data represent the mean ± standard deviation of three independent experiments.

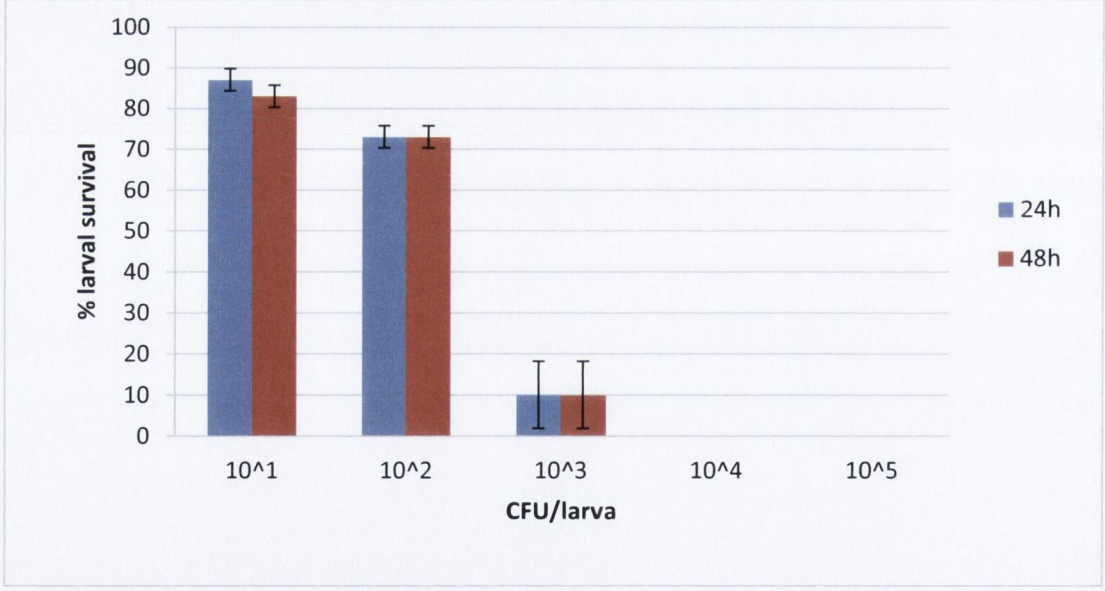
2.3.3 Characterisation of virulence of *P. aeruginosa* isolates in the *in vivo G. mellonella* model.

It was interesting to determine if *P. aeruginosa* strains that form biofilms are more pathogenic in an *in vivo* environment. **Figure 2.4** illustrates *in vivo* virulence of larvae inoculated with the individual *P. aeruginosa* strains used in this study. Larvae were injected with increasing concentrations of *P. aeruginosa* ($1 \times 10^1 - 1 \times 10^5$) and assessed for viability at 24 and 48 hours as described in **Section 2.2.13.** Control larvae were injected with 20 µl sterile saline. Results indicate that the degree of virulence was higher when larvae were inoculated with pigment producing isolates *P. aeruginosa* PA01, *P. aeruginosa* ATCC27853 and *P. aeruginosa* 1826 at 24 and 48 hours. Percentage larval survival following inoculation with clinical isolate *P. aeruginosa* 4276.3 was higher than the other strains. This strain does not secrete any redox-active pigments but does form biofilm of high cell density at 24 hours. Larvae inoculated with higher concentrations of $1 \times 10^3 - 1 \times 10^5$ CFU/ml of clinical CF strains *P. aeruginosa* 5605 and 81599 were more viable than the other inoculated larvae even though a pigment was secreted by *P. aeruginosa* 5605. In addition, the degree of virulence appeared to be less severe during infection with clinical isolate *P. aeruginosa* 1438 which forms a weak biofilm within 48 hours and secretes no pigment, 100% larval survival was recorded at 24 and 48 hours post inoculation. Pigments which are virulence factors produced by *P. aeruginosa* may be important virulence factors influencing larval death.

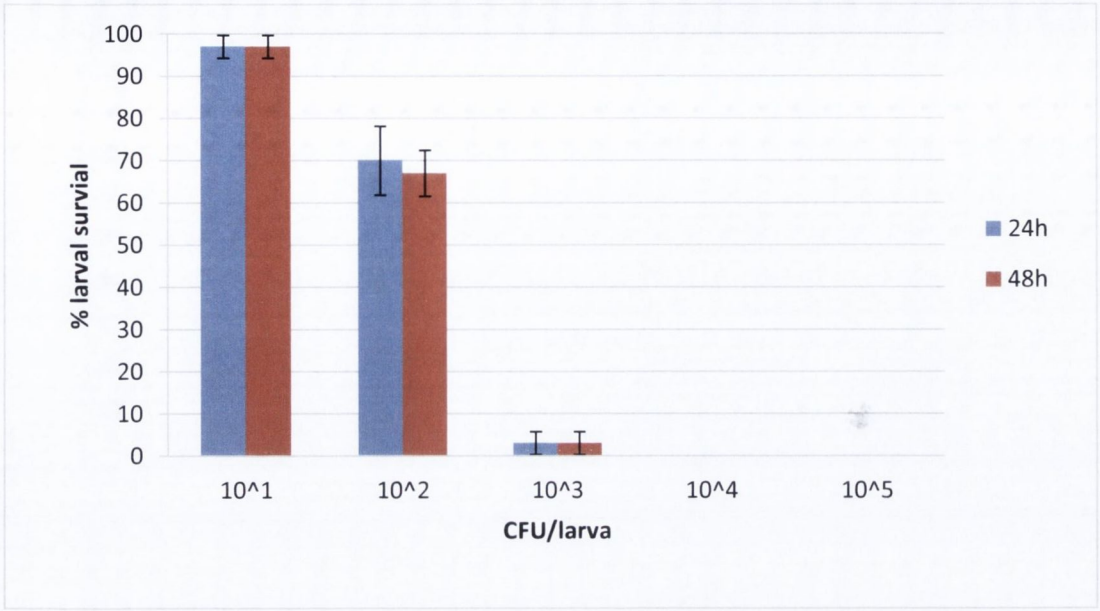
(A) *P. aeruginosa* PA01



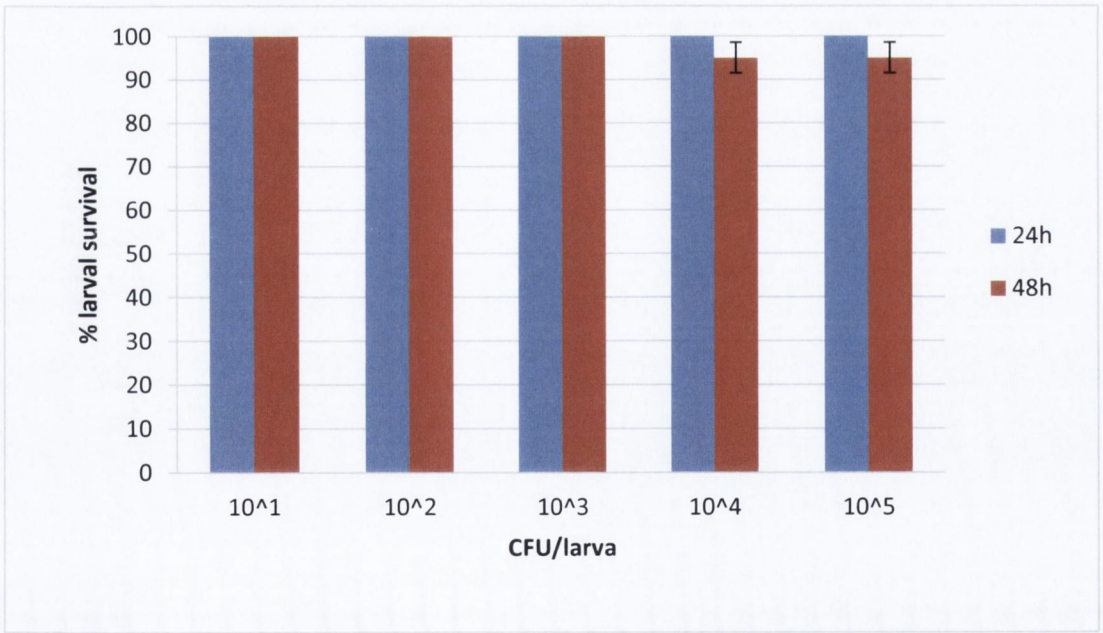
(B) *P. aeruginosa* ATCC27853



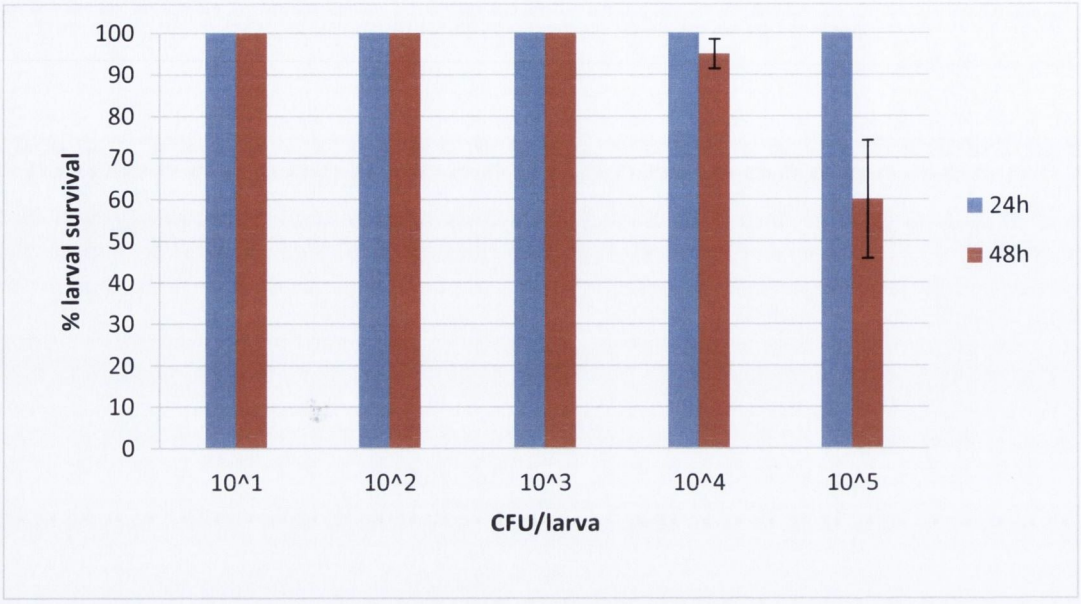
(C) *P. aeruginosa* 1826



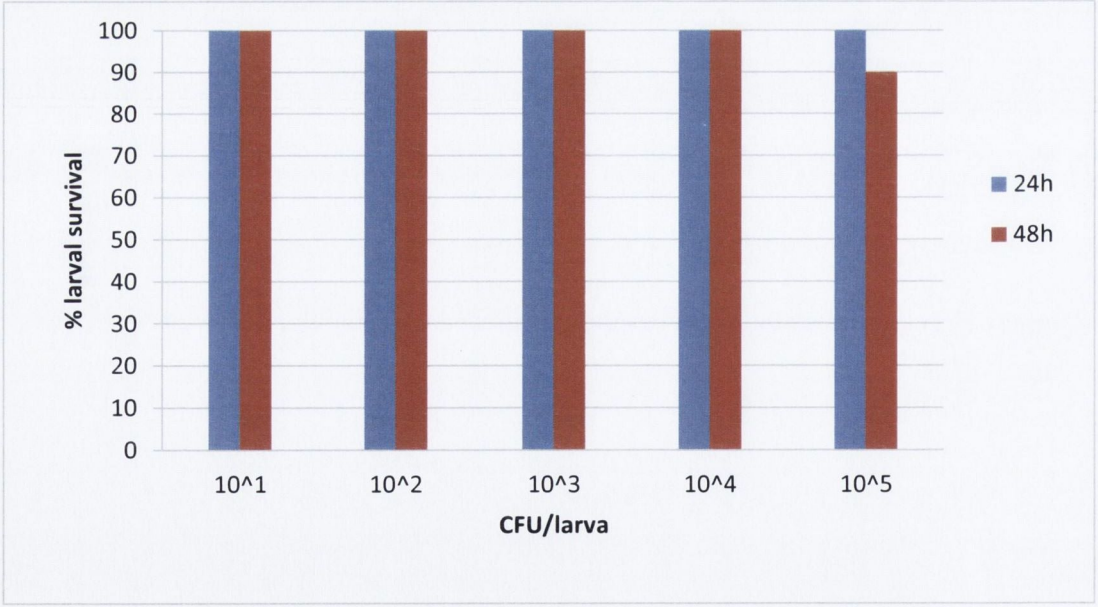
(D) *P. aeruginosa* 4276.3



(E) *P. aeruginosa* 81599



(F) *P. aeruginosa* 5605



(G) *P. aeruginosa* 1438

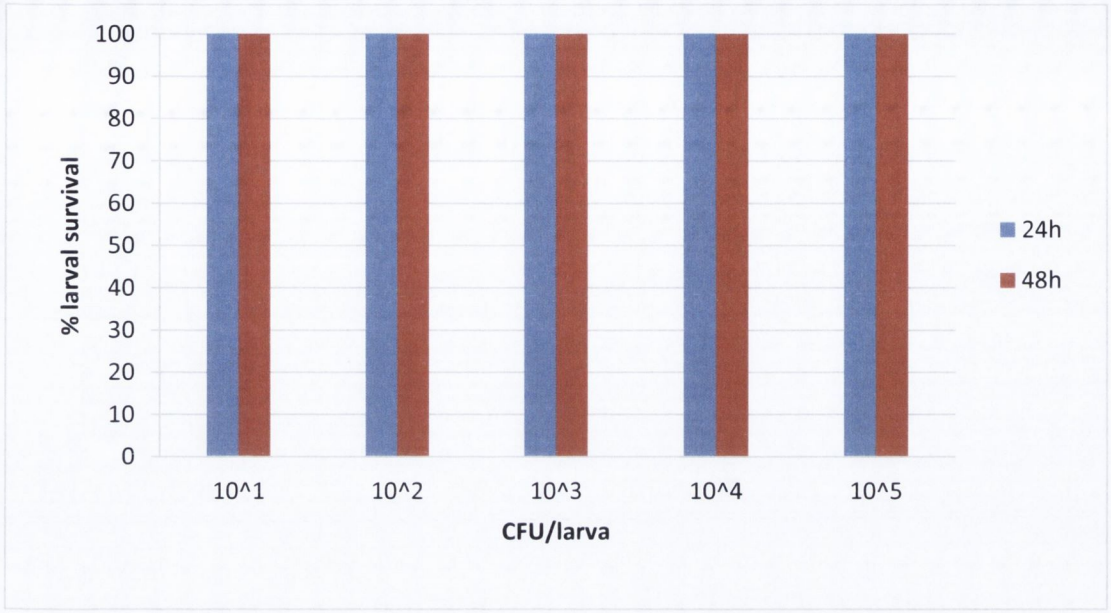


Figure 2.4: Evaluation of virulence of selected reference and CF *P. aeruginosa* strains at different concentrations. Percentage larval survival was recorded at 24 and 48 hours post-infection. Control larvae injected with sterile saline demonstrated 100% survival at 24 and 48 hrs. Larvae were injected with **(A)** *P. aeruginosa* PA01, **(B)** *P. aeruginosa* ATCC27853, **(C)** *P. aeruginosa* 1826, **(D)** *P. aeruginosa* 4276.3, **(E)** *P. aeruginosa* 81599, **(F)** *P. aeruginosa* 5605, **(G)** *P. aeruginosa* 1438 and incubated at 35°C. Larvae infected with concentrations $\geq 10^3$ CFU/ml of *P. aeruginosa* strains (PA01, ATCC27853, 1826) demonstrated significantly reduced viability compared to the other strains (one-way ANOVA). Data represent the mean ± standard deviation of three experiments.



Figure 2.5: *P. aeruginosa* pathogenesis in *G. mellonella* larvae relative to the control. **(A)** Control larvae were injected with PBS which did not affect larval survival. **(B)** Larvae inoculated with *P. aeruginosa* demonstrated melanisation 24 hours, 35°C post-infection.

2.3.4 Optimisation of biofilm growth quantification.

An initial assay was carried out as demonstrated in **Figure 2.6** which illustrates the effect of four different disruption assays against biofilms grown on polystyrene pegs. *P. aeruginosa* PA01 was used as a control organism that forms a biofilm. Findings showed that exposure of the polystyrene pegs to a mixture of 1% Triton X-100 and 95% ethanol at a 1:1 ratio gave the highest absorbance readings indicating that greatest amount of cells were dislodged by this method. This increase in the rate of biomass recovered was found to be significant compared to the other disruption methods ($P \leq 0.001$). Average absorbance readings showed that this assay has good reproducible values. Similarly, sonication for 5 minutes was effective in dislodging cells from the pegs. A recent study by Moskowitz *et al.*, (2004), found sonication for a shorter time as more effective than

centrifuging for biofilm cells. A 1:1 ratio of 1% sodium dodecyl sulfate (SDS) and 95% ethanol gave the weakest absorbance range indicating the two agents are not acting effectively as a combination in altering the subsequent attachment of these cells. Absorbance readings of the assay involving no sonication after biofilm growth was higher than that of 1% SDS and 95% ethanol treatment, suggesting this biofilm model has many limitations.

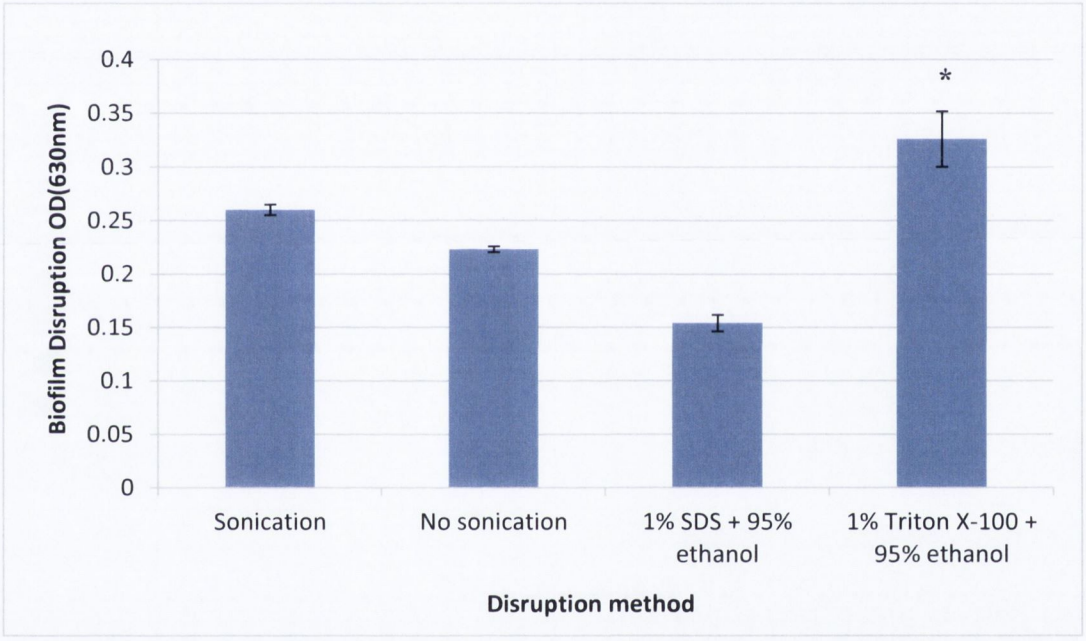


Figure 2.6: The effect of four different disruption methods against biofilms grown on polystyrene pegs. A solution of 1% Triton X-100 and 95% ethanol was the most effective disruption method against pre-grown biofilms. Fewer biofilm cells were dislodged with the solution of 1% SDS and 95% ethanol. Data represents the mean ± standard deviation of three independent experiments.

Table 2.2: E-test (MIC mg/L) results of anti-pseudomonal antibiotics against *P. aeruginosa*.

Results of antibiotic susceptibility determined by E-test and Vitek 2 (colistin only) against *P. aeruginosa* are summarised below in **Table 2.2.**

***P. aeruginosa* strain**	**Tobramycin**	**Ceftazidime**	**Meropenem**	**Ciprofloxacin**	**Colistin**
***P.aeruginosa* PA01**	2	1.5	0.5	0.125	0.5
***P.aeruginosa* ATCC27853**	1.5	1.5	0.5	0.19	1
***P.aeruginosa* 1826**	2	2	0.5	0.125	0.5
***P.aeruginosa* 4276.3**	16	1.5	0.095	1	0.5
***P.aeruginosa* 81599**	8	>32	1	0.25	0.5
***P.aeruginosa* 1438**	16	>32	>32	2	0.5
***P.aeruginosa* 5605**	2	>32	>32	1.5	0.5
***P.aeruginosa* 88716**	1	12	0.38	0.19	1
***P.aeruginosa* 5714**	1	12	0.25	0.25	0.5
***P.aeruginosa* 4649**	1	>32	0.125	0.125	0.5
***P.aeruginosa* 4505.2**	4	0.5	0.38	0.38	2
***P.aeruginosa* 6279**	16	>32	>32	4	0.5

2.3.5 Summary of the number of *P. aeruginosa* strains displaying sensitivity to each antibiotic according to the CLSI standards.

E-test results were defined as sensitive or resistant according to the Clinical Laboratory and Standards Institute (CLSI) as shown in **Table 2.3** with the exception of colistin in which the susceptibility profiles were analysed with the Vitek 2 system. All *P. aeruginosa* strains displayed sensitivity to colistin. A total of 9/12 strains were sensitive to ciprofloxacin and 1/12 was resistant. The susceptibility profile of meropenem was also high with 9/12 strains displaying sensitivity and 3/12 resistance. Tobramycin was also effective against a large (8/12) number of strains. Finally, an equal number of strains (5/12) displayed susceptibility and resistance to ceftazidime.

Table 2.3: Number of antibiotics for which *P. aeruginosa* strains were sensitive (S), intermediate (I) and resistant (R) ($n = 12$).

Antibiotic	**CLSI Breakpoints (MIC µg/ml)**			**Susceptible *P. aeruginosa* strains**	**Intermediate *P. aeruginosa* strains**	**Resistant *P. aeruginosa* strains**
	S	**I**	**R**			
Tobramycin	≤ 4	8	≥ 16	8/12	1/12	3/12
Ceftazidime	≤ 8	16	≥ 32	5/12	2/12	5/12
Meropenem	≤ 4	8	≥ 16	9/12	0/12	3/12
Ciprofloxacin	≤ 1	2	≥ 4	9/12	2/12	1/12
Colistin	≤ 2	4	≥ 8	12/12	0/12	0/12

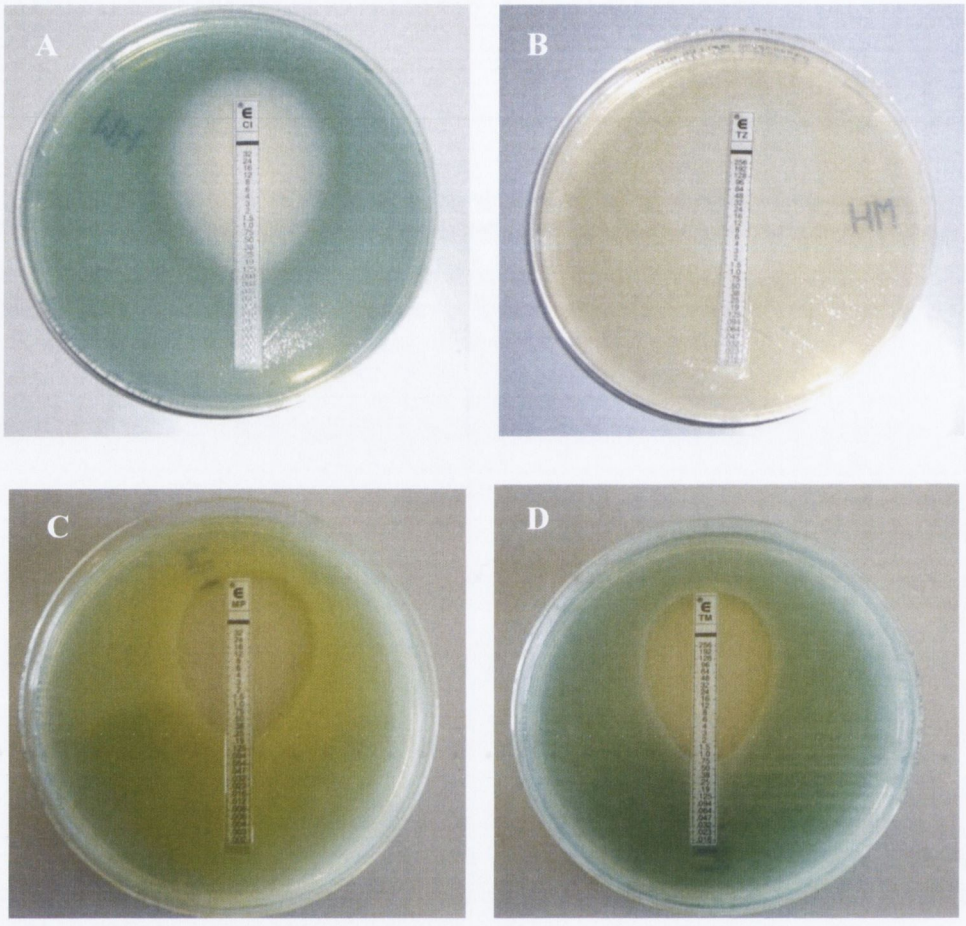


Figure 2.7: The susceptibilities of *P. aeruginosa* isolates to antibiotics by E-test on Mueller-Hinton Agar. **(A)** *P. aeruginosa* 1826, **(B)** *P. aeruginosa* 4276.3, **(C)** *P. aeruginosa* PA01 and **(D)** *P. aeruginosa* ATCC27853. Green agar signifies pigment production by three strains.

2.3.6 Antibiotic susceptibility testing of single antibiotics against *P. aeruginosa.*

Several *P. aeruginosa* isolates representing CF common clones (*P. aeruginosa* 1826, *P. aeruginosa* 4276.3), CF uncommon clones (*P. aeruginosa* 4505.2, *P. aeruginosa* 4649) and reference strains (*P. aeruginosa* PA01, *P. aeruginosa* ATCC27853) were selected for antibiotic susceptibility testing. **Table 2.4** illustrates the susceptibilities of antibiotics tested singly against planktonic and biofilm grown isolates. Among the antibiotics selected ciprofloxacin was the most effective single agent against five out of the six *P. aeruginosa* isolates tested. Clinical isolate, *P. aeruginosa* 4276.3 displayed the greatest sensitivity to meropenem indicating antibiotics may be displaying unique effects against the different strains. Polymyxin antibiotic, colistin displayed the weakest activity against all isolates grown planktonically. For the majority of strains, concentrations required to inhibit biofilm-grown isolates were similar to those of planktonic cells. The MIC and BIC values of *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3 were similar following exposure to ceftazidime. Ceftazidime treated cells exhibited lower MIC values than BIC values against the other *P. aeruginosa* strains indicating this agent can have reduced potency against certain biofilm-forming *P. aeruginosa* isolates. Previous studies have shown that less mature biofilms can have the same antibiotic susceptibility values as planktonic cells (Peeters *et al.*, 2009). Meropenem exhibited weak antibiofilm activity against three isolates, *P. aeruginosa* PA01, *P. aeruginosa* ATCC27853 and *P. aeruginosa* 4505.2 in comparison to planktonic cells. Similarly, tobramycin displayed weaker activity against two isolates, *P. aeruginosa* 4276.3 and *P. aeruginosa* 4505.2, grown as biofilms than planktonic cells.

Table 2.4: Minimum inhibitory concentration (MIC µg/ml) and Biofilm inhibitory concentration (BIC µg/ml) of single antibiotics against *P. aeruginosa*.

Antibiotic	*P. aeruginosa* PA01		*P. aeruginosa* ATCC27853		*P. aeruginosa* 1826		*P. aeruginosa* 4276.3		*P. aeruginosa* 4505.2		*P. aeruginosa* 4649	
	MIC	**BIC**	**MIC**	**BIC**	**MIC**	**BIC**	**MIC**	**BIC**	**MIC**	**BIC**	**MIC**	**BIC**
Tobramycin	0.25	1	0.5	0.5	0.5	2	4	32	2	>256	0.25	0.25
Ceftazidime	4	128	2	2	1	128	1	128	8	>256	4	8
Meropenem	1	8	2	8	2	2	0.0625	0.0625	1	>256	1	1
Ciprofloxacin	0.0625	0.0625	0.0625	0.25	0.125	0.125	1	1	0.5	1	0.25	0.25
Colistin	64	64	16	128	32	128	16	16	64	64	8	64

2.3.7 Susceptibility testing of antibiotic combinations.

All *P. aeruginosa* isolates were grown planktonically and as biofilms and treated with a selection of frequently administered antibiotics, alone and in combination. The chosen antibiotics such as ceftazidime, meropenem and colistin were examined in combination with tobramycin as it is one of the main antibiotics of choice for CF patients infected with *P. aeruginosa.* Ciprofloxacin was also tested in combination with these antibiotics as it displayed the greatest anti-pseudomonal activity against the majority of CF isolates as a single agent.

Table 2.6 shows the fractional inhibitory concentration (FIC) indices which determined the interactions that occurred between antibiotic combinations. Results revealed that combinations tobramycin-ceftazidime and tobramycin-meropenem displayed synergistic activity at an FIC $\leq$ 0.5 against the biofilm of *P. aeruginosa* 1826. Tobramycin-ceftazidime also exhibited synergistic interactions against *P. aeruginosa* 4505.2 at an FIC $\leq$ 0.25. Interestingly, these antibiotic combinations did not have a similar affect against the same strains grown as planktonic isolates, indicating that these interactions may be specifically targeting the development of biofilms at certain stages. The antibiotic combinations tobramycin-ceftazidime and tobramycin-meropenem did not display synergistic interactions against the reference strains in planktonic or biofilm form. Another interesting finding was observed with ciprofloxacin-colistin which displayed synergistic activity against *P. aeruginosa* 1826 grown planktonically but not against the biofilm. The mechanisms of these findings are currently unknown and warrant further study.

Four antibiotic combinations including tobramycin-meropenem, tobramycin-colistin, ciprofloxacin-ceftazidime and ciprofloxacin-meropenem, displayed antagonistic interactions against the biofilm of clinical isolate *P. aeruginosa* 4505.2 at FIC values of 275, 17, 257, and 8.5, respectively. In addition, two combinations, tobramycin-colistin (FIC $\geq$ 64.25) and ciprofloxacin-colistin (FIC $\geq$ 257) were antagonistic against the biofilm of *P. aeruginosa* 4649. These two isolates possess the mucoid phenotype which may be reducing the efficacy of these antibiotics. The remaining results showed that the majority of combinations tested were in the indifferent range. Ciprofloxacin which was an effective single agent displayed the same inhibitory activity in combination with other antipseudomonal antibiotics as ciprofloxacin monotherapy. The combination involving tobramycin-colistin displayed antagonistic interactions (FIC $\geq$ 4) against biofilm and planktonic cells of two reference strains *P. aeruginosa* PA01 and *P. aeruginosa* ATCC27853. Antagonistic interactions of this combination were also observed against biofilm formation of three clinical strains, *P. aeruginosa* 1826, *P. aeruginosa* 4505.2, *P. aeruginosa* 4649 and planktonic growth of *P. aeruginosa* 4276.3.

Table 2.5: Susceptibility of *P. aeruginosa* planktonic (MIC µg/ml) and 24 hour biofilm-grown isolates (BIC µg/ml) to antibiotic combinations.

Antibiotic Combinations	*P. aeruginosa* PA01		*P. aeruginosa* ATCC27853		*P. aeruginosa* 1826		*P. aeruginosa* 4276.3		*P. aeruginosa* 4505.2		*P. aeruginosa* 4649	
	MIC	BIC	MIC	BIC	MIC	BIC	MIC	BIC	MIC	BIC	MIC	BIC
Tobramycin-Ceftazidime	0.5	1	0.25	1	1	1	4	32	1	32	0.25	0.25
Tobramycin-Ciprofloxacin	0.25	0.25	0.25	0.25	0.25	0.25	2	4	1	>256	0.25	0.25
Tobramycin-Meropenem	1	2	0.25	0.5	0.25	0.5	0.25	0.25	0.5	>256	0.25	0.25
Tobramycin-Colistin	2	64	2	16	1	2	16	16	2	>256	2	16
Ciprofloxacin-Ceftazidime	0.125	0.125	0.0625	0.5	0.125	0.125	2	2	1	>256	0.25	0.25
Ciprofloxacin-Meropenem	0.125	0.125	0.25	0.25	0.125	0.125	0.5	1	1	>256	0.25	0.25
Ciprofloxacin-Colistin	0.125	0.125	0.0625	0.25	0.0625	0.125	2	2	1	256	0.25	64

Table 2.6: Fractional Inhibitory Concentration's (FIC) of antibiotic combinations according to FIC Index (FICI).

FIC of Antibiotic Combinations	*P. aeruginosa* PA01		*P. aeruginosa* ATCC27853		*P. aeruginosa* 1826		*P. aeruginosa* 4276.3		*P. aeruginosa* 4505.2		*P. aeruginosa* 4649	
	MIC	**BIC**	**MIC**	**BIC**	**MIC**	**BIC**	**MIC**	**BIC**	**MIC**	**BIC**	**MIC**	**BIC**
Tobramycin-Ceftazidime	2	1	0.6	2.5	3	**0.5***	5	1.5	0.652	**0.25***	1	1
Tobramycin-Ciprofloxacin	5	4	1.5	1.5	2.5	2	2.5	4	2.5	2	2	2
Tobramycin-Meropenem	1.4	2	0.6	1	0.6	**0.5***	4	4	0.75	275	1.25	1.25
Tobramycin-Colistin	8	65	4	32	2	1	5	1.5	1	17	1	64.25
Ciprofloxacin-Ceftazidime	2	2	1	2.25	1.1	1.1	4	2	2	257	1	1
Ciprofloxacin-Meropenem	2	2	1	1	1.06	1.06	8.5	17	3	8.5	1.25	1.25
Ciprofloxacin-Colistin	2	1	1	1	**0.5***	1	2.1	2	2	2	1	257

*** FIC value for synergy ≤ 0.5**

2.3.8 Activity of effective antibiotic combinations against mature (72 hour) *P. aeruginosa* biofilms.

Mature *P. aeruginosa* biofilms were grown for 72 hours and exposed to effective antibiotic combinations, tobramycin-ceftazidime and tobramycin-meropenem. These combinations displayed synergistic interactions against particular biofilms grown for 24 hours. Two reference strains (*P. aeruginosa* PA01, *P. aeruginosa* ATCC27853) and two clinical CF strains (*P. aeruginosa* 1826, *P. aeruginosa* 4505.2) were selected for further study. Findings revealed that *P. aeruginosa* 1826 was synergistically inhibited with tobramycin-ceftazidime at an FIC $\leq$ 0.5. Tobramycin-meropenem showed indifference under the same experimental conditions, indicating antibiotic interactions can be influenced by the age of the biofilm. Similarly, tobramycin-ceftazidime which acted synergistically against clinical isolate *P. aeruginosa* 4505.2 after 24 hours growth did not display the same inhibitory effect against the older biofilm (FIC $\geq$ 2). Findings suggest that the activity of antibiotic combinations may be reduced against mature *P. aeruginosa* biofilms in comparison to the same strains at 24 hours.

Table 2.7: Susceptibility of mature *P. aeruginosa* biofilms (BIC µg/ml) grown for 72 hours and treated with effective single and double antibiotic combinations.

Antibiotic	*P. aeruginosa* PA01	*P. aeruginosa* ATCC27853	*P. aeruginosa* 1826	*P. aeruginosa* 4505.2
Tobramycin	4	1	>256	>256
Ceftazidime	>256	>256	>256	>256
Meropenem	>256	>256	>256	>256
Tobramycin-Ceftazidime	4	1	64	>256
Tobramycin-Meropenem	4	2	>256	>256

Table 2.8: Fractional Inhibitory Concentration (FIC) of the most effective antibiotic combinations against mature (72 hour) biofilms.

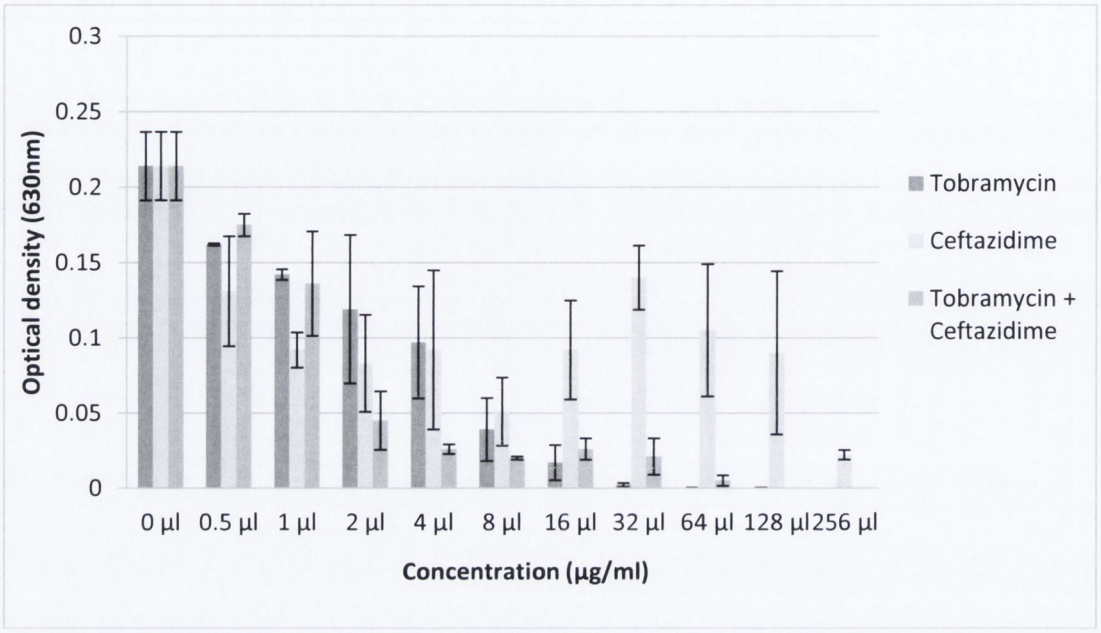
Antibiotic Combination	*P. aeruginosa* PA01	*P. aeruginosa* ATCC27853	*P. aeruginosa* 1826	*P. aeruginosa* 4505.2
Tobramycin-Ceftazidime	1.03	1	**0.5***	2
Tobramycin-Meropenem	1	2	2	2

*** FIC value for synergy ≤ 0.5**

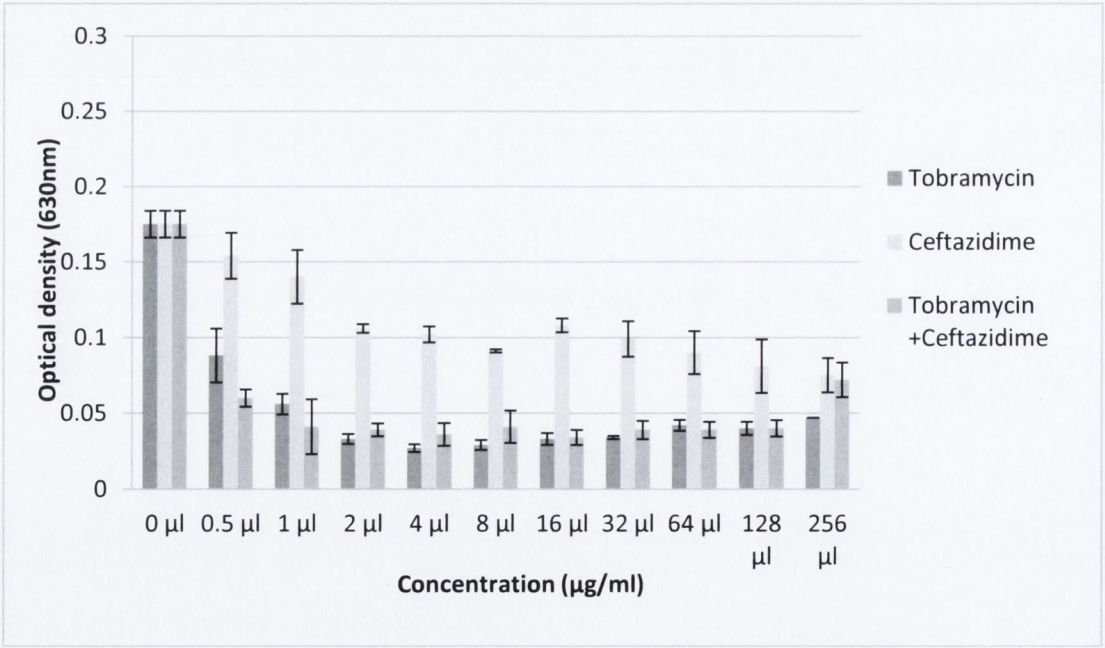
2.3.9 The effect of tobramycin, ceftazidime, tobramycin-ceftazidime against *P. aeruginosa* biofilms.

Figure 2.8 shows survival of 24 hour *P. aeruginosa* biofilms grown on polystyrene pegs following treatment with effective single (tobramycin, ceftazidime) and combination antibiotics (tobramycin-ceftazidime) at varying concentrations. At concentrations > 64 μg/ml the activities of tobramycin and tobramycin-ceftazidime against *P. aeruginosa* PA01 were similar indicating that tobramycin is the most effective single agent. In contrast, this strain displayed resistance to concentrations > 8 μg/ml of ceftazidime. The activity of tobramycin-ceftazidime matched the activity of tobramycin against *P. aeruginosa* ATCC27853. Clinical isolates *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3 were also more sensitive to concentrations > 64 μg/ml of tobramycin and tobramycin-ceftazidime than ceftazidime. The activity of single antibiotics showed *P. aeruginosa* 1826 was more resistant to concentrations > 0.5 μg/ml of ceftazidime whereas *P. aeruginosa* 4276.3 was more resistant to concentrations ranging between 8 - 32 μg/ml of tobramycin.

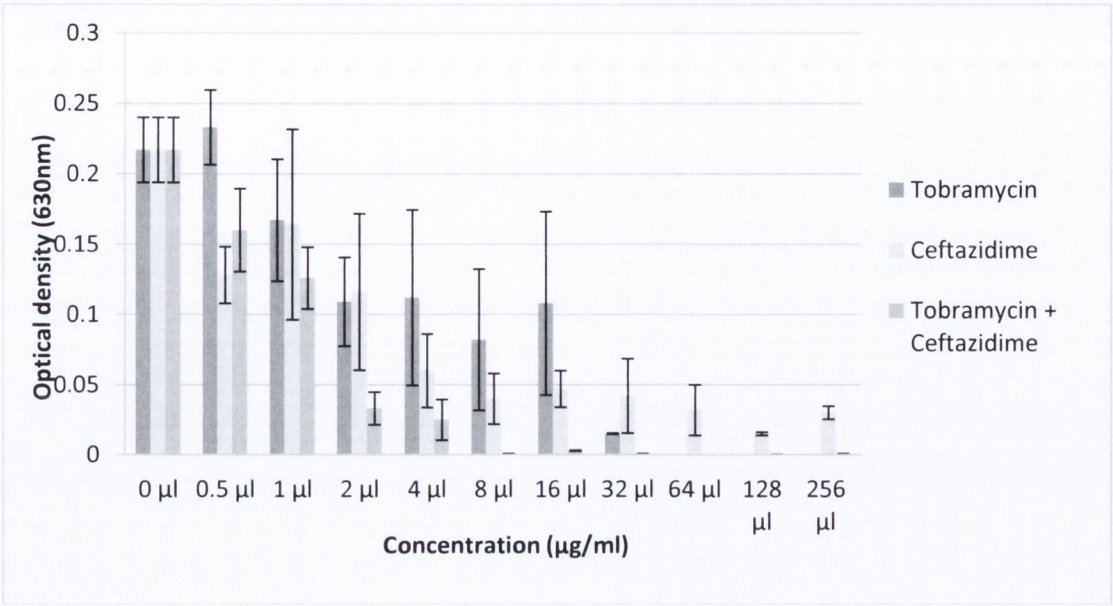
(A)


0.3
0.25
0.2
0.15
0.1
0.05
0
Optical density (630nm)
0 µl
0.5 µl
1 µl
2 µl
4 µl
8 µl
16 µl
32 µl
64 µl
128 µl
256 µl
Concentration (µg/ml)
Tobramycin
Ceftazidime
Tobramycin + Ceftazidime

(B)


0.3
0.25
0.2
0.15
0.1
0.05
0
Optical density (630nm)
0 µl
0.5 µl
1 µl
2 µl
4 µl
8 µl
16 µl
32 µl
64 µl
128 µl
256 µl
Concentration (µg/ml)
Tobramycin
Ceftazidime
Tobramycin +Ceftazidime

(C)



(D)

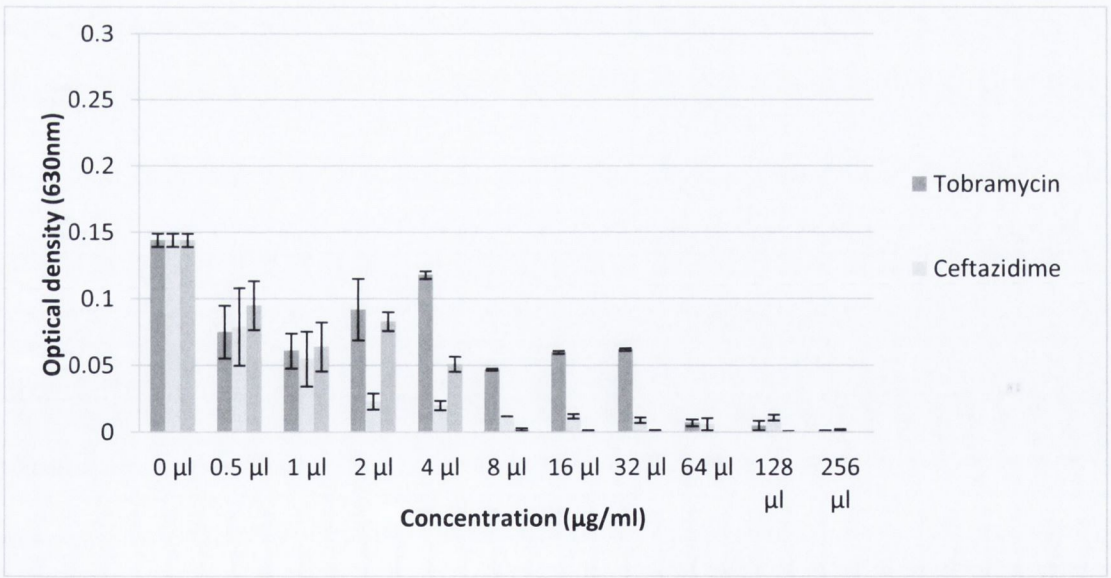


Figure 2.8: Optical density (630nm) of *P. aeruginosa* biofilms **(A)** *P. aeruginosa* PA01, **(B)** *P. aeruginosa* ATCC27853, **(C)** *P. aeruginosa* 1826, **(D)** *P. aeruginosa* 4276.3 treated with tobramycin, ceftazidime and tobramycin-ceftazidime at varying

concentrations (μg/ml). Results represent the mean ± standard deviation of three independent experiments.

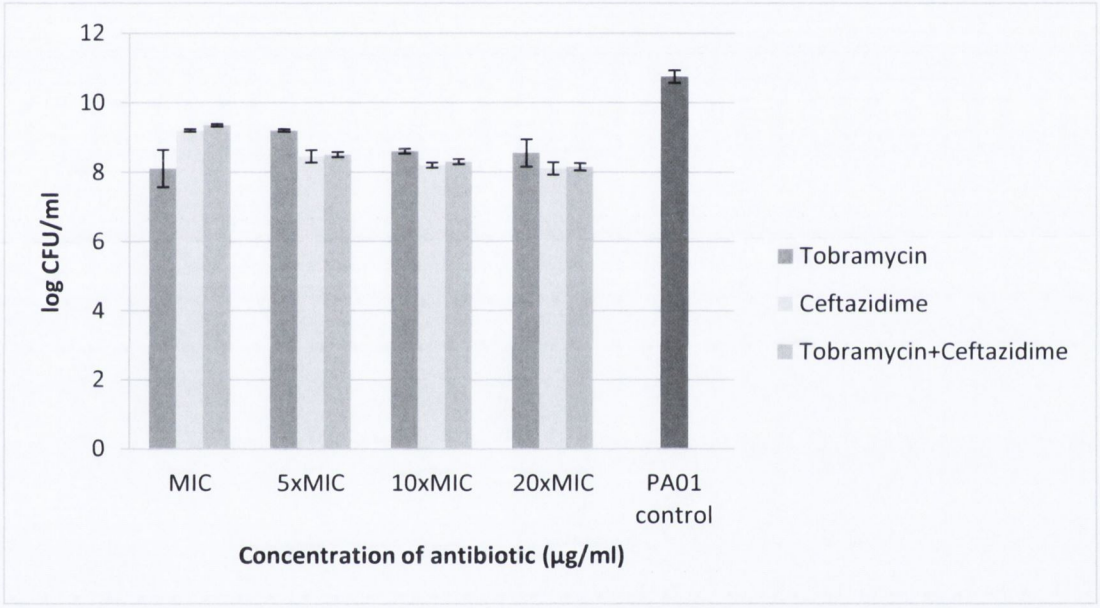
2.3.10 The effect of biofilm maturation on the efficacy of antibiotic treatment.

The activity of combined antibiotic treatment of tobramycin and ceftazidime was evaluated against one reference strain (*P. aeruginosa* PA01) and one clinical strain (*P. aeruginosa* 1826). The concentrations selected were the MIC, 5 x MIC, 10 x MIC, 20 x MIC based on results obtained by the broth microdilution method. The activity of antibiotic combinations was determined based on age of the biofilm and concentration of each agent. The interaction of tobramycin-ceftazidime against *P. aeruginosa* PA01 did not show any significant difference compared to each antibiotic administered alone. On the other hand, at a concentration of 10 x MIC biofilms growth for 72 hours and treated with tobramycin-ceftazidime was significantly reduced (P = 0.008) in comparison to each single agent. The activity of the antibiotic combination against seven day old biofilms of *P. aeruginosa* PA01 showed no significant difference to each single agent as shown in **Figure 2.9**.

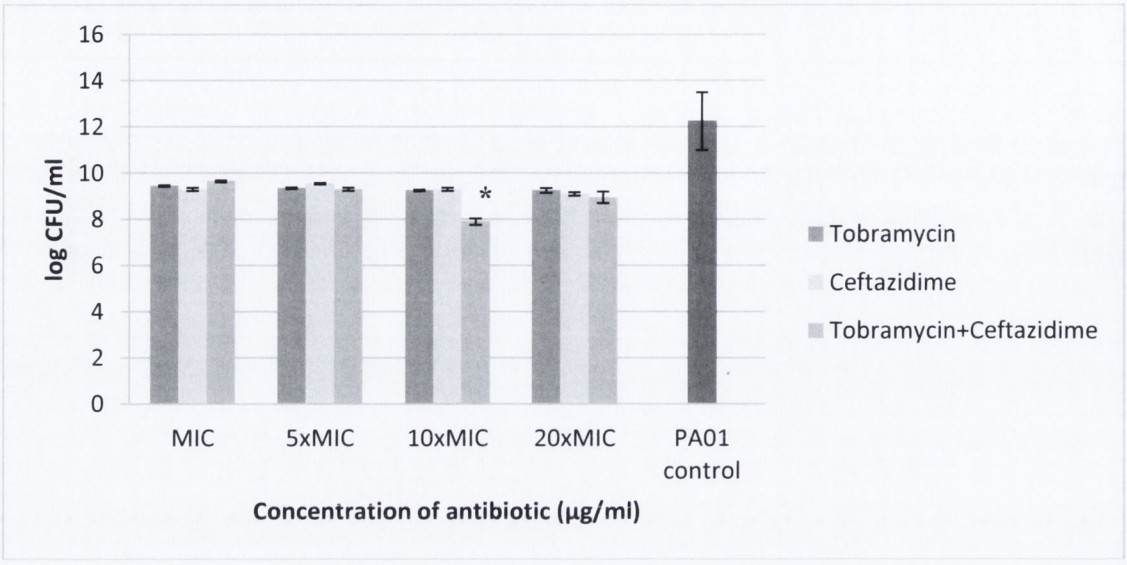
Figure 2.10shows greater survival of clinical strain *P. aeruginosa* 1826 at 72 hours in comparison to 24 hours following treatment with the same antibiotics as shown in **Figure 2.9**. *P. aeruginosa* 1826 grown for 24 hours was eradicated with 20 x MIC of tobramycin, indicating high concentrations of particular antibiotics may effectively inhibit less mature biofilm structures. The activity of the tobramycin-ceftazidime revealed a resemblance to the activity of either tobramycin or ceftazidime used singly. The comparisons of each

single and double antibiotic treatment showed no significant difference at any particular time or concentration. The results also revealed that 7 day old biofilm biomass of *P. aeruginosa* PA01 and *P. aeruginosa* 1826 was similar to the less mature biofilm structures suggesting optimum growth may be reached before 7 days with this *in vitro* model.

(A)



(B)



(C)

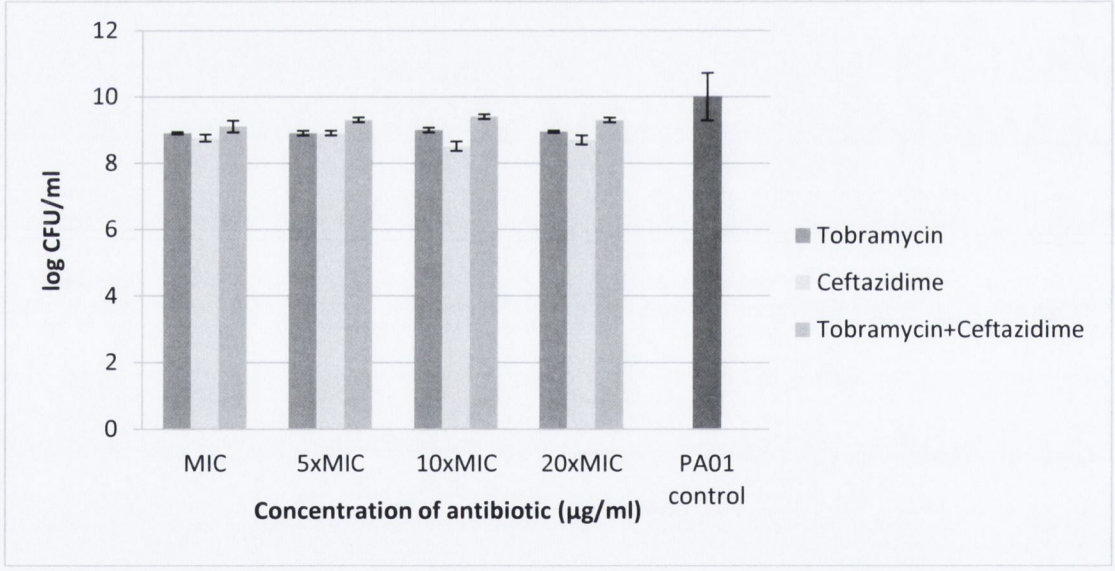
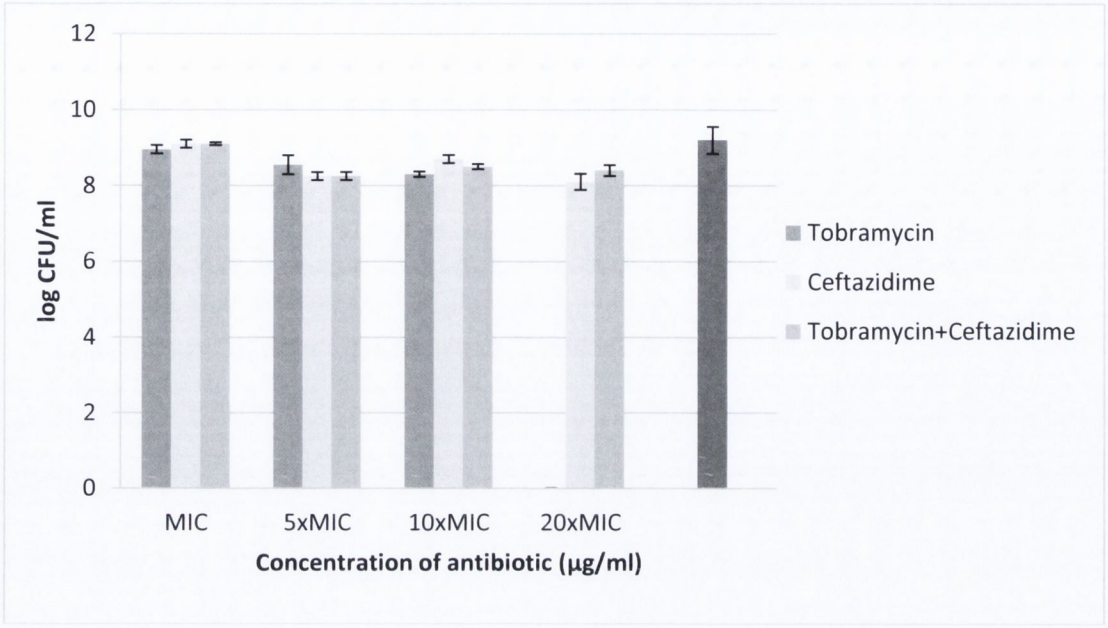
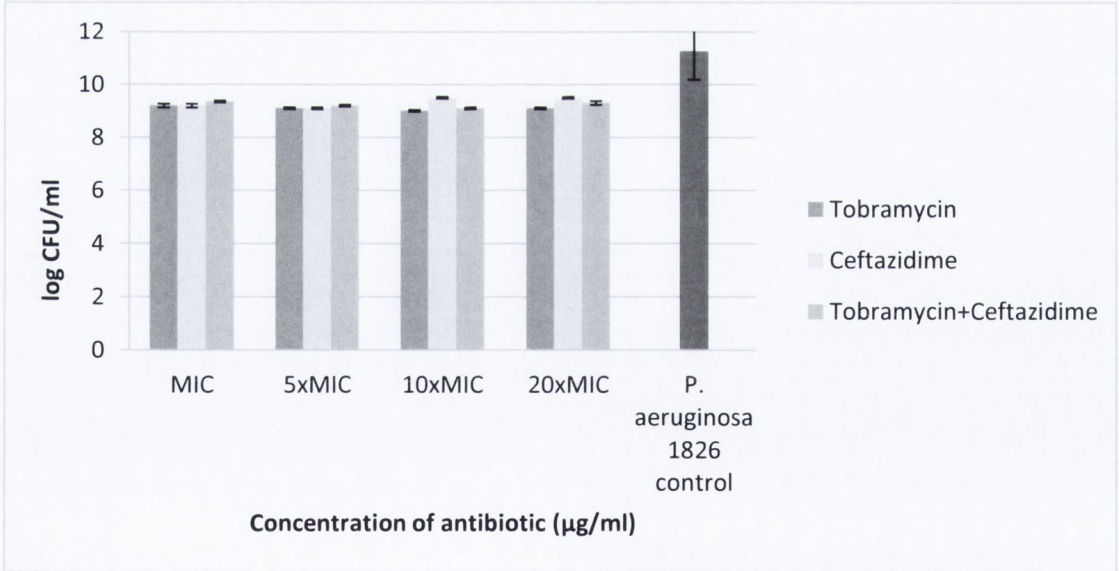


Figure 2.9: Reduction in biofilm biomass (*P. aeruginosa* PA01) following antibiotic treatment. *P. aeruginosa* PA01 grown for **(A)** 24 hours, **(B)** 72 hours and **(C)** 7 days and challenged with antibiotics at final concentrations of MIC, 5 x MIC, 10 x MIC and 20 x MIC. Viability of the biofilm was recorded following 24 hours treatment.

(A)


12
10
8
6
4
2
0
log CFU/ml
MIC
5xMIC
10xMIC
20xMIC
Concentration of antibiotic (µg/ml)
Tobramycin
Ceftazidime
Tobramycin+Ceftazidime

(B)


12
10
8
6
4
2
0
log CFU/ml
MIC
5xMIC
10xMIC
20xMIC
P. aeruginosa 1826 control
Concentration of antibiotic (µg/ml)
Tobramycin
Ceftazidime
Tobramycin+Ceftazidime

(C)

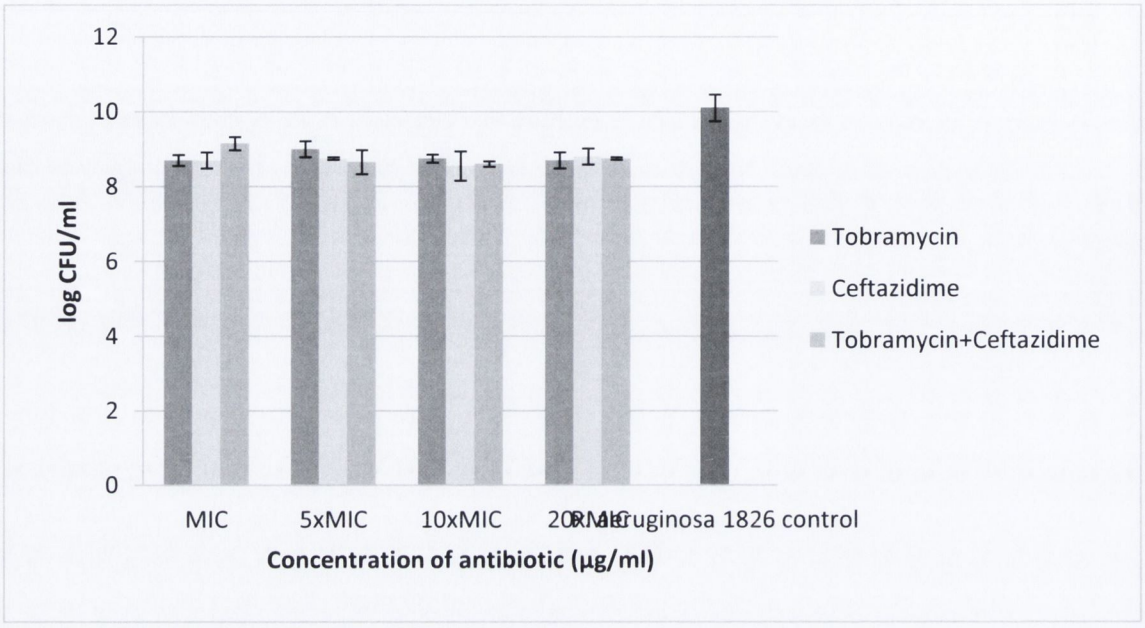


Figure 2.10: Reduction in biofilm biomass (*P. aeruginosa* 1826) following antibiotic treatment. *P. aeruginosa* 1826 was grown for **(A)** 24 hours, **(B)** 72 hours and **(C)** 7 days and treated with effective antibiotics at final concentrations of MIC, 5 x MIC, 10 x MIC and 20 x MIC. Viability of the biofilm was recorded following 24 hours treatment.

2.4 Discussion

The analysis of clinical isolates described in this chapter was achieved by recovering *P. aeruginosa* from CF sputum samples. A selection of *P. aeruginosa* isolates from the different Irish CF clinics displayed diversity in terms of phenotypic characteristics and biofilm formation. All clinical CF *P. aeruginosa* isolates which included the 5 identified common clones and the 5 uncommon clones had different phenotypic characteristics. Four out of twelve isolates grew as mucoid colonies. The mucoid phenotype identified generally relates to a more rapid impairment in lung function (Parad *et al.*, 1999). Several of the CF strains such as *P. aeruginosa* strains 81599, 5605 and 1438 grew as small white colonies on TSA agar plates. Lam *et al.*, (1980), found *P. aeruginosa* small colony variants (SCV's) were highly adherent and commonly associated with biofilm formation in the CF lung. SCV's are often associated with greater biofilm biomass than other strains and increased resistance to antibiotics (Drenkard *et al.*, 2002). The morphological diversity of CF *P. aeruginosa* strains has been documented extensively and may be due to mutations that occur when strains are adapting to a heterogeneous environment (Rainey *et al.*, 1998). Another difference observed was the blue-green pigment produced by eight out of twelve *P. aeruginosa* strains on agar plates. These minor phenotypic differences may be accounting for survival and adaptation by each individual strain.

Biofilm growth of reference and clinical CF strains was examined using two different *in vitro* models. The data demonstrated a progressive increase in growth from 24 to 72

hours for the majority of *P. aeruginosa* isolates. The study also found the biofilm density reached by several *P. aeruginosa* strains differed with each model. Four clinical CF isolates *P. aeruginosa* 81599, *P. aeruginosa* 1438, *P. aeruginosa* 5605 and *P. aeruginosa* 6279 which appeared as weakly adherent on polystyrene pegs grew biofilms of high cell density as determined by viable counts following 72 hours incubation. Results are in accordance with previous studies that have suggested certain species may not colonise the surfaces of pegs but may be depositing on the bottom of the microtitre wells (Smith *et al.*, 2008). Other studies have suggested microcolonies may not be attaching to any surface but may display similar characteristics to adherent bacteria (Alhede *et al.*, 2011). According to Oliver *et al.*, (2000), environmental factors may alter adaptation and survival and influence phenotypic properties of CF isolates. Houry *et al.*, (2009), indicated motility may play a role in biofilm formation through which cells need to find suitable places for biofilms to settle. Heidelberg *et al.*, (2002), suggested the type IV-pili of *P. aeruginosa* may be involved in the attachment of bacteria to various abiotic surfaces. O' Toole and Kohter, (1998), suggested flagella may also promote attachment and that mutant strains may exhibit limited biofilm formation compared to wild-type strains. Findings indicate that several *in vitro* models should be used to assess biofilm development of different *P. aeruginosa* strains.

P. aeruginosa mutants that produce a reduced level of pyocyanin may be less virulent in various animal models than wild-type strains (Hendrickson *et al.*, 2001). Rambaugh *et al.*, (1999), linked quorum sensing to the production of virulence factors and increased levels of mortality and morbidity under *in vivo* conditions. The study described the

inactivation of the *las* QS system caused a reduction in pathogenicity of CF bacteria in the *G. mellonella* model. Results from the present study demonstrated *G. mellonella* infected with *P. aeruginosa* expressed different rates of survival depending on concentration and each individual strain. A number of strains, *P. aeruginosa* PA01, *P. aeruginosa* ATCC27853 and *P. aeruginosa* 1826, which secreted pigments, lead to a greater killing effect than the other strains. Recently, Jander *et al.*, (2000), described pyocyanin as a main virulence factor in *G. mellonella* infected with *P. aeruginosa*. The toxic effects of pyocyanin have been characterised of which contribute to inactivation of vacuolar ATPase of lung epithelial cells and apoptosis of neutrophils (Hasset *et al.*, 2003; Usher *et al.*, 2002). Although *P. aeruginosa* 5605 secretes a pigment, this strain was less virulent in the *in vivo* model suggesting other exoproducts produced by *P. aeruginosa* may be influencing larval mortality. The exact mechanisms that are influencing differences in larval survival with these strains remain inconclusive.

Virulence factor production which is controlled by quorum sensing systems of *P. aeruginosa* strains may also influence the rate at which antimicrobial agents affect the biofilm structure (Pearson *et al.*, 1994). Antibiotic susceptibility testing enables the selection of suitable agents for treatment of CF bacterial infections (Aaron *et al.*, 2002). Findings from the current study indicate the clinical CF isolates develop a unique response to antibiotic treatment. *P. aeruginosa* biofilms were more resistant to antibiotics than planktonic cells. These results correspond to the study by Aaron *et al.*, (2002), which found antibiotics singly or in combination were less active against the strains grown as biofilms than planktonic cells. Recently, Yu *et al.,* (2012), found all *P. aeruginosa*

isolates that were tolerant to antibiotics such as aztreonam were non-mucoid. Mutants defective in the *psl* gene which is responsible for EPS component synthesis exhibited an increase in resistance to the antibiotic. Findings from the current study indicate that synergistic interactions of antibiotics against *P. aeruginosa* do not rely on whether the isolate is mucoid or non-mucoid.

Ciprofloxacin was identified as a more effective single agent than tobramycin against planktonic and biofilm cells. Previously, susceptibility testing against biofilms found fluoroquinolones such as ofloxacin and ciprofloxacin penetrate biofilm structures more effectively. Aminoglycosides such as tobramycin penetrate the biofilm structure at a slower rate than ciprofloxacin which may be allowing bacteria to acquire adaptive stress responses (Shigeta *et al.*, 1997; Yasuda *et al.*, 1993; Whiteley *et al.*, 2001). The exopolysacchairde matrix can reduce the rate of antibiotic diffusion and aminoglycosides have been reported to stimulate alginate production and increase *P. aeruginosa* biofilm biomass (Hogdes and Gordon, 1991; Bagge *et al.*, 2004). In addition, Stewart *et al.*, (1996), suggested the efficacy of antimicrobial agents may be reduced once adsorbed through the exopolysaccharide matrix. The multi-drug resistance pump MexAB-OprM expressed in *P. aeruginosa* is accountable for much of the intrinsic antimicrobial resistance of this organism (Li *et al.*, 1995).

This section found the MIC and BIC values of colistin were higher in planktonic and biofilm form in comparison to the other anti-pseudomonal antibiotics. Resistance to colistin treatment has been linked to the two signal transduction systems, *phoPQ* and

pmrAB which regulate the expression of the OprH gene. It has been suggested that the OprH gene increases membrane permeability of biofilms thereby generating a higher rate of resistance (MacFarlane *et al.*, 1999; Bell *et al.*, 1991). Haagensen *et al.*, (2006), observed *P. aeruginosa* was more resistant to colistin treatment during the early stages of biofilm formation. The study showed the effect of colistin against *P. aeruginosa* biofilms was not influenced by the age or maturation stage of the biofilm and the mechanisms are incompletely understood.

The effect of antibiotic combination therapy was investigated against *P. aeruginosa* planktonic cells and biofilms. Single and combination antibiotic therapy observed higher BIC values for 72 hour *P. aeruginosa* biofilms treated with ceftazidime and meropenem, indicating reduced activity of these agents against mature biofilms. Increased production of exopolysaccharide matrix may slow the penetration of antibiotics through the biofilm structure (Stewart *et al.*, 1996). Another observation involving the antibiotic combinations examined, found tobramycin-ceftazidime and tobramycin-meropenem displayed synergistic interactions against biofilm growth of clinical isolate *P. aeruginosa* 1826 and tobramycin-ceftazidime acted synergistically against clinical isolate *P. aeruginosa* 4505.2. Additionally, tobramycin-ceftazidime displayed synergistic interactions against the 72 hour biofilm of clinical isolate *P. aeruginosa* 1826 but not *P. aeruginosa* 4505.2 while tobramycin-meropenem had reduced activity against all isolates at this time point. Tobramycin belongs to the aminoglycoside group of antibiotics and ceftazidime belongs to the cephalosporin antibiotics. Antibiotics that have different modes of action have been shown to be more effective at eliminating CF pathogens and

synergy of antibiotics is not a novel finding. The exact mechanisms that are causing synergistic interactions warrant further investigation. Recently, the study by Chaudhary *et al.*, (2009), found a patient group with lower respiratory tract infection treated with tobramycin-ceftazidime experienced a better clinical outcome that the group receiving ceftazidime monotherapy. Findings from the present study indicate this combination of antibiotics may be effective for inhibiting *P. aeruginosa* biofilms, however, changes that occur during biofilm maturation may reduce the efficacy of this treatment. Kuman *et al.*, (1995), described certain antibiotics may display synergistic interactions against biofilms during the different stages of the biofilm developmental cycle. Hermann *et al.*, (2010), revealed the combination of colistin and tobramycin was more effective *in vitro* than each single agent in which different areas of the biofilm structure were targeted by each agent. Combined antibiotic treatment of colistin and ciprofloxacin allows biofilm cells to be treated during the different stages of metabolic activity (Pamp *et al.*, 2008). In addition, Skindersoe *et al.*, (2008), found biofilms treated with azithromycin and ceftazidime caused alterations in membrane permeability and signal molecule uptake by interfering with quorum sensing.

Total viable counts were obtained for biofilms treated with tobramycin, ceftazidime and tobrmaycin-ceftazidime as this combination of antibiotics displayed synergistic activity against biofilms grown on polystyrene pegs. *P. aeruginosa* biofilms were grown for different lengths of time to determine the response of young and mature biofilms to this treatment. Recently, Tre-Hardy *et al.*, (2009), described a biofilm grown for 12 days as more resistant to antibiotic treatment than a biofilm grown for 24 hours. In addition,

Anwar *et al.*, (1989), reported a reduced effect of tobramycin against a 7 day biofilm in comparison to a 2 day biofilm. Consistent with these previous findings, the current study found antibiotic combinations were less effective against 72 hour biofilms than 24 hour biofilms. Synergistic activity was not reported in most cases with tobramycin-ceftazidime as was previously identified against biofilms formed on polystyrene pegs. These differences suggest that therapeutic approaches seem to rely on the age of the biofilm and concentration of antimicrobial agent administered. Inefficacy of antibiotics may be due to slow growth of biofilms rendering the strain more resistant to treatment (Dunne *et al.*, 1993; Evans *et al.*, 1987). Another possible explanation for the difference between the results obtained with different assays may be due to a less developed biofilm structure forming on polystyrene pegs making such strains more susceptible to treatment (Aaron *et al.*, 2002). Recently, Gillis *et al.*, (2009), observed azithromycin displays inhibitory activity against *P. aeruginosa* PA01 during early biofilm development. Cells within the deeper layers of biofilms may be more resistant to certain agents that can effectively inhibit the outer areas (Costerton *et al.*, 1999). Finally, phenotypic and genotypic differences have been identified during the biofilm development process which may be a major factor in the biofilm response to treatment (Ito *et al.*, 2009).

Chapter 3: Antimicrobial activity of metal-based compounds against *P. aeruginosa* biofilms.

3.1 Introduction

The phenotypic and genotypic diversity among bacteria of the same species may influence the ability of bacterial communities to withstand environmental stresses (Tilman *et al.*, 1994). Since many bacteria found in the CF lungs are able to form biofilms and develop resistance mechanisms to antibiotics (Cos *et al.*, 2010), many alternative agents have been explored in the literature to suppress biofilm formation.

The use of metals as antimicrobial agents began in ancient times to treat a variety of infectious diseases. Many recent studies have described the use of silver based compounds for many medical applications. Silver is frequently used as a polymer or coating agent for indwelling medical devices such as heart valves and catheters to prevent the formation of biofilms (Dasgupta *et al.*, 1999). Bjarnsholt *et al.*, (2007), found *P. aeruginosa* biofilms were inhibited by silver sulfazidime at wound infection sites. The study also found that concentrations required to kill biofilms can be 10-100 times the concentrations required to kill planktonic cells. It is reported that silver uptake by bacteria can cause damage to the cell membrane (Kim *et al.*, 2007). One of the disadvantages of using silver as a treatment is argyia. This is caused by precipitates of silver within the dermis and leads to discolouration of the skin (Bouts *et al.*, 1999).

Other metals such as copper and zinc which are required in trace amounts for bacterial

growth can be toxic to many species at high concentrations (Balsalobre *et al.*, 2003). Studies have found bacterial species may develop resistance following treatment with certain metals. Parsek *et al.*, (2003), and his group reported that biofilms exhibit resistance to heavy metals such and copper and zinc. The *cus*A or *cus*CFBA systems of *E. coli* enhance the rate of resistance to silver and copper agents (Rensing *et al.*, 2003). Resistance of bacteria to silver has rarely been reported in the literature. The study by Pervical *et al.*, (2008), found wound isolates of *P. aeruginosa* and *S. aureus* did not display any resistance to silver.

Recent studies have found coumarin agents possess potent antimicrobial activity. Coumarins are benzopyrene derivatives which are found naturally in many plants and microorganisms and have many uses in pharmaceutical preparations such as antibacterials, antivirals, antioxidant, antiinflammatory and anticoagulants (Hoult *et al.*, 1996; Kostova *et al.*, 2007). The study by Rosselli *et al.*, (2007), showed that coumarins exhibit antimicrobial activity by targeting bacterial nucleic acid synthesis. Coumarin antibiotics such as novobiocin and clorobiocin have potent activity against bacterial infections by inhibiting DNA gyrase (Maxwell *et al.*, 1993). Flavonoids which contain the properties of coumarin agents are able to interact with many drugs through the inhibition of P-glycoprotein (Wang *et al.*, 2007). Warfarin, a derivative of dicoumarol, can prevent tumour formation by reducing the ability of tumor cells to be retained in the pulmonary microvasculature (Mousa *et al.*, 2002).

The aim of this chapter was to determine the effect of metal-based agents against *P.*

aeruginosa. The antibacterial activity of synthesised copper-benzotriazole and silver-coumarin compounds was explored. This section also studied the effect of gallium nitrate in combination with antibiotics against *P. aeruginosa* planktonic and biofilm cultures.

3.2 Materials and Methods

3.2.1. Bacterial strains and media.

Two reference strains *P. aeruginosa* PA01 and *P. aeruginosa* ATCC27853 were used as controls. Two clinical CF isolates, *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3 were also used for this study. An overnight culture of each isolate was diluted to $OD_{630} = 0.1$ in nutrient broth. *P. aeruginosa* biofilms were grown for 24 and 72 hours at 35°C.

3.2.2 Media for flow cell biofilm growth.

BTrace medium

BTrace media was prepared by adding 10 ml 1M magnesium chloride ($MgCl_2$), 1 ml calcium chloride ($CaCl_2$) and 1 ml Trace metals solution which are required for *P. aeruginosa* biofilm growth in 9 L of sterile Milli-Q-water (Millipore Corporation).

A-10

A-10 containing 30g sodium chloride (NaCl), 20g ammonium sulphate, $(NH_4)2SO_4$, 60g disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) and 30g monopotassium phosphate (KH_2PO_4) in 1 L sterile water adjusted to a pH 6.4 ± 0.1, acts as a buffer for biofilm cultivation.

BTrace media and A-10 were added together mixed for one minute before supplementing the medium with 10% glucose as a carbon source and mixing again. Media was

autoclaved at 121°C for 15 minutes before use. All chemicals used to prepare this media were purchased from Sigma-Aldrich.

3.2.3 Culture preparation for flow cell experiments.

For flow cell experiments bacterial cultures were streaked on Luria agar (LA) (Sigma-Aldrich) plates and incubated overnight. A single colony was incubated in 5 ml Luria broth (LB) (Sigma-Aldrich) at 35°C on an orbital shaker (200 rpm). The OD_{630} = 0.1 was standardised in sterile 0.9% (w/v) sodium chloride solution.

3.2.4 Synthesis of silver-coumarin compounds.

Silver-coumarin compounds and metal-free coumarin ligands were synthesised by Dr. Bernie Creaven, Department of Chemistry, Institute of Technology, Tallaght, Dublin 24. All compounds and molecular weights are listed in the tables below.

Table 3.1: Molecular weights of synthesised metal-free coumarin ligands.

Coumarin compound	**Molecular weight (g/mol)**
Coumarin 2	224.60
Coumarin 3	220.18
Coumarin 4	269.05
Coumarin 5	220.18
Coumarin 6	254.62
Coumarin 7	254.62
Coumarin 8	220.18
CCa (coumarin-3-carboxylic acid)	190.15

Table 3.2: Molecular weights of synthesised silver-coumarin compounds.

Silver-coumarin compound	**Molecular weight (g/mol)**
Ag(6-OHCCa)	313.01
Ag(7-OHCCa)	313.01
Ag(8-OHCCa)	313.01
AgCCa	296.01
Compound 2	331
Compound 3	327.04
Compound 4	375.91
Compound 5	327.04
Compound 6	361.48
Compound 7	361.48
Compound 8	327.04
Compound 10	366
Silver nitrate ($AgNO_3$)	169.87

3.2.5 Synthesis of copper-benzotriazole compounds.

Copper-benzotriazole compounds were synthesised by Dr. Denis O'Shea, School of Food Science and Environmental Health, Dublin Institute of Technology (DIT), Cathal Brugha Street, Dublin 1. Copper sulphate (Sigma-Aldrich) and a benzotriazole (Sigma-Aldrich) were added to 50 cm^3 ethanol and the resulting clear solutions were refluxed for 3 hours. The reaction mixtures were filtered off, washed in ethanol and air-dried.

Table 3.3: Molecular weights of synthesised copper-benzotriazole compounds and metal-free benzotriazole ligands.

Copper-benzotriazole compound	**Molecular weight (g/mol)**
$Cu(C_7H_7N_3)_2(SO_4)$	425.62
$Cu(C_6H_5N_3)_2(SO_4)$	397.62
$Cu(C_7H_7N_3O)_2(SO_4)$	457.62
Copper sulphate ($CuSO_4$)	159.62
Benzotriazole	119.12
1H-Benzotriazole-1-methanol	149.15
5-Methyl-1H-benzotriazole	133.15

3.2.6 Gallium nitrate.

Gallium hydrate nitrate, $Ga(NO_3)_3.xH_2O$, was purchased from Sigma-Aldrich. The molecular weight is 255.73 g/mol. All stock solutions were prepared in sterile water.

3.2.7 Solubility testing and preparation of metal-based compounds for experimental procedures.

Stock solutions of 1%, 2.5%, 5% and 10% (v/v) DMSO (99% GC, Sigma-Aldrich) in 1 ml sterile water were prepared. Pre-grown *P. aeruginosa* biofilms were treated with each solution in order to select a concentration to dilute the compounds that would not affect bacterial growth. A 5% (v/v) DMSO solution was selected to dilute the compounds as it was the highest concentration that did not display any inhibitory activity against *P. aeruginosa*.

Stock solutions of silver-coumarin compounds, metal-free coumarin ligands, copper-benzotriazole compounds and metal-free benzotriazole ligands were prepared in a 5% DMSO solution with sterile water to form a uniform solution. Compounds 5 and 10 required heating and vigorous stirring to dissolve the solute. The metal-free coumarin ligands also required heating and vigorous stirring in 5% DMSO to form a uniform solution. Stock solutions of gallium nitrate and silver nitrate were prepared in sterile water as these agents were more readily soluble.

3.2.8 Effect of synthesised compounds against planktonic cultures of *P. aeruginosa*.

Serial dilutions of gallium nitrate and synthesised compounds were prepared and minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) were carried out as described in **Chapter 1**, **Section 2.2.12**. To determine the MBC, the contents of non-turbid wells were sub-cultured by streaking 10 µl of suspension onto 5% Columbia sheep blood agar plates which were incubated overnight at

35°C and examined for complete inhibition the following day.

3.2.9 Effect of synthesised compounds against biofilms grown on polystyrene pegs.

An overnight culture of *P. aeruginosa* strains was prepared in nutrient broth. The isolates were suspended in 3 mls of nutrient broth to a 0.5 McFarland standard and adjusted to a concentration of 5 x 10^5 CFU/ml. A quantity of 100 µl of this suspension was added to the wells of a 96-well round-bottom plate. A transferable solid phase (TSP) pin lid was placed into the microtitre plate and incubated overnight at 35°C. The TSP pin lid was removed and rinsed three times in sterile water to remove planktonic cells. The lid was placed into another plate containing serial dilutions of all compounds starting at a final concentration of 512 µg/ml. After a further 24 hours at 35°C the peg lids were removed and rinsed three times with sterile water and dried at room temperature. The TSP lid was added to a 96-well plate containing 100 µl 0.3% crystal violet for a further 30 minutes. The TSP lid was rinsed again three times in sterile water to remove any crystal violet that did not bind to the biofilm cells. To remove crystal violet stained biofilms attached to the pegs the lid was placed into a new 96-well round-bottom microtitre plate containing a solution (1:1 ratio) of 95% ethanol and 1% Triton X-100. The OD_{630} was measured in a microplate reader. The biofilm inhibitory concentration was determined as the last well that had an $OD_{630} \geq 0.05$ (Moskowitz *et al.*, 2004). All experiments were conducted in triplicate on three separate occasions

3.2.10 Effect of synthesised compounds on viability of *P. aeruginosa* biofilms.

An overnight culture of *P. aeruginosa* was diluted to $OD_{630} = 0.1$ in LB. A quantity of 10

mls of BTrace minimal medium containing A-10 and 0.5% glucose was added to 6-well microtitre plates (Tissue culture testplate 6, Techno Plastic Products (TPP), Switzerland). A quantity of 100 µl of the culture was added to each well and incubated at 35°C for 72 hours. All of the media was removed from the wells using a 10 ml syringe without destroying clumps at the bottom of the wells. An additional 10 mls of BTrace (A-10 and 0.5% glucose) media containing 100 µl of the appropriate concentrations of silver-coumarin compounds were added to the wells and incubated for a further 24 hours at 35°C. All contents from the wells were transferred to centrifuge tubes. Cells were centrifuged for 3,000 *g*, 10 minutes. Cells were washed in 10 ml 0.9% NaCl and centrifuged at 3,000 *g*, 10 minutes again. Cells were re-suspended in 10 ml 0.9% NaCl followed by sonication and vortexing until the cells were dispersed. Serial dilutions were prepared and spotted onto TSA plates to determine the CFU/ml.

3.2.11 Establishing the flow cell system.

3.2.12 Assembly of the flow cell system channels.

Assembly of the flow cell began by adding a thin layer of silicone (Bluestar Silicones) with a syringe between the flow cell channels, without touching the plexiglass. A glass coverslip was placed over the silicone and pressed firmly in order to cover the whole area of plexiglass. Any coverslips that cracked were removed with a scalpel and replaced again. Flow cells were left for 24 hours to allow the silicone to dry. Tubing (Marpene Tubing, Ole Dich Instrumentmakers Aps.) was connected to each end of the flow channels. Bubble traps were also connected to the flow channels. The 0.5 ml syringes

were mounted on the bubble traps with the inlet and outlet inside the bubble traps and closed with a stopper. Flasks were placed at each end of the system to collect the media. Flasks that were to collect effluent were placed at or above the level of the flow channels so that media will not run out and air will not enter into the system. The flow system was started by removing the stoppers from the bubble traps. The stoppers were replaced when the bubble traps began to overflow.

3.2.13 Sterilisation of the flow system.

A 13% sodium hypochloride (AppliChem) solution in 1L Milli-Q-water was prepared and allowed to flow through the system for 3-4 hours. When this had completed, 2L Milli-Q-water was added to the system for 2 hours to get rid of any remaining sodium hypochloride. Sterile tubes were connected to the flask containing BTrace minimal media which was allowed to flow through the system. When the bubble traps began to overflow they were capped so the media was able to flow through the flow cells. Flow rate was set with the 16-channel Watson Marlow pump (Watson-Marlow Flexicon A/S, Denmark) at 0.5 rpm at 35°C.

3.2.14 Cultivation of biofilms in the flow cell system.

A-10 and 10% glucose were added to BTrace minimal medium and mixed for 1 minute. Sterile tubes were placed into each flask containing media. Before inoculation, flow was stopped and all tubing between the flow cells and bubble traps was clamped. The flow channels were inoculated with *P. aeruginosa* by injecting 250 µl of the overnight culture diluted to OD_{630} = 0.1 into each flow channel with a 0.5 ml syringe (Thermo Fisher

Scientific, Ireland). The syringe was inserted into the tubing and the hole was sealed with silicone. After inoculation, flow channels were inverted and left without flow for 1 hour, after which medium flow was resumed. Flow velocity was set at 1.75 rpm for all experiments. All bubbles were removed from the flow cell. Biofilms were allowed to develop for 72 hours prior treatment.

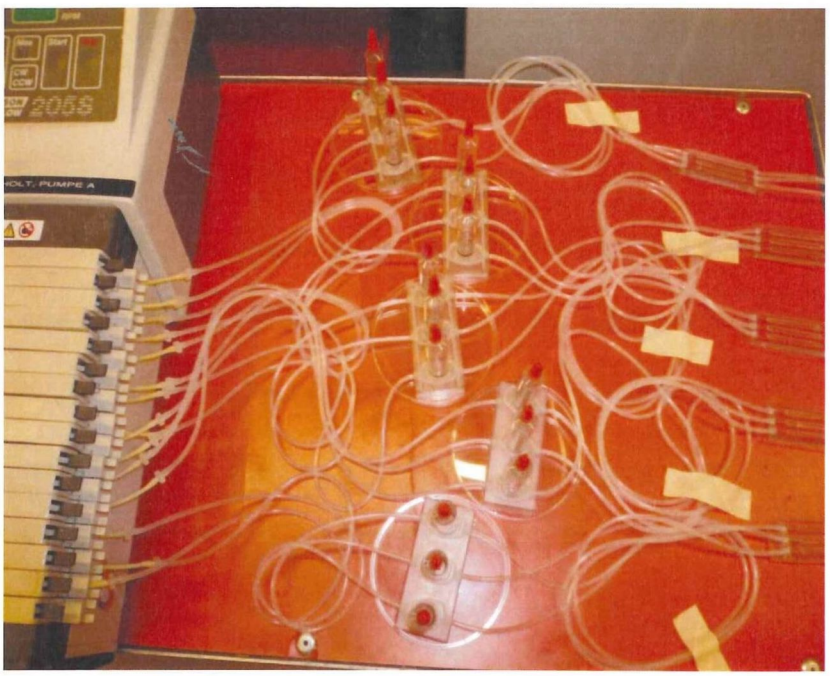


Figure 3.1: Fully assembled flow cell system showing connecting flow chambers, effluent vessels and connecting tubes. Media was circulated through the bubble traps and flow chambers by a 16-channel periplasmic pump. Waste was removed through a series of tubes into flasks.

3.2.15 Exposure of biofilms to silver nitrate and silver-coumarin compounds.

After 72 hours incubation the flow was stopped and all tubing between the flow cells and bubble traps was clamped. Lids were removed from the bubble traps and flow was resumed at 90 rpm to empty all the bubble traps. The media was changed to medium containing 10% glucose and the appropriate concentrations corresponding to the MIC and

10 x MIC values of silver nitrate and selected silver-coumarin compounds. Concentrations of the agents used are listed in Appendix 5. All clamps were removed and flow was started again at 1.75 rpm. Biofilms were continuously exposed to the compounds for a further 24 hours.

3.2.16 Fluorescent staining.

Baclight Live-Dead stain (Invitrogen, Molecular Probes, 5 mM solution in DMSO) was added to the biofilm after 24 hours. An equal amount of Syto 9 and propidium iodide (PI), (1 µl) was added to 1 litre of sterile Milli-Q-water and covered with foil to avoid contact with light. A quantity of 250 µl was injected the opposite end of the flow cell, covered with foil and left for 15 minutes before viewing under the microscope. The red-fluorescing stain displayed non-viable cells and the green-fluorescing stain displayed cells that were viable.

3.2.17 Microscopy and image processing.

All imaging of *P. aeruginosa* treated and untreated biofilms was conducted using a Zeiss LSM510 confocal scanning laser microscopy (CSLM; Carl Zeiss, Germany), equipped with an argon-NeHe laser and detectors. The filters were set for simultaneous monitoring of Syto 9 and propidium iodide (PI). Images were obtained using a 63X/1.3 Plan-Neofluar oil immersion objective. Cross sectional analysis through the biofilm was generated by IMARIS (Bitplane AG) software.

3.2.18 Statistical analysis.

Statistical analysis was carried out using PASW^{R} Statistics 18 - SPSS. One-way ANOVA was used to determine statistical variations for most experiments. A $P \leq 0.05$ value was considered statistically significant.

3.3 Results

3.3.1 Effect of copper sulphate/benzotriazole compounds on *P. aeruginosa* planktonic and biofilm formation.

The sensitivity of *P. aeruginosa* to synthesised copper-benzotriazole compounds was assessed by carrying out MIC, MBC and BIC assays. Interestingly, the synthesised compounds did not exhibit any inhibitory effects against *P. aeruginosa* grown planktonically. Copper sulphate displayed antibacterial activity at a high concentration of 3.2 mM against three out of four strains which included two reference strains and one clinical strain. Similarly, compounds displayed weak antimicrobial activity against *P. aeruginosa* biofilm-grown isolates in which results were comparable to the copper sulphate control. The metal-free benzotriazole ligand 5-methyl-1H-benzotriazole exhibited antibiofilm activity against two clinical isolates *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3 at a concentration of 1.9 mM. The other ligands benzotriazole and benzotriazole-1H methanol displayed similar biofilm inhibitory activity against *P. aeruginosa* 4276.3 at high concentrations of 2.1 mM and 1.7 mM, respectively. Findings from this study suggest that the metal-free ligands are disrupting *P. aeruginosa* biofilms established on polystyrene pegs and warrant further study.

Table 3.4: Effect of synthesised copper-benzotriazole compounds against planktonic *P. aeruginosa* strains. Results are expressed as Minimum Inhibitory Concentration (MIC μM).

Copper sulphate-benzotriazole compound	***P. aeruginosa* ATCC27853**	***P. aeruginosa* PA01**	***P. aeruginosa* 1826**	***P. aeruginosa* 4276.3**
Cu (BZT)	>1.28	>1.28	>1.28	>1.28
Cu (MBZT)	>2.4	>2.4	>2.4	>2.4
Cu (BZTM)	>2.2	>2.2	>2.2	>2.2
Copper sulphate	3.2	3.2	>3.2	3.2
Benzotriazole	>4.3	>4.3	>4.3	>4.3
5-Methyl-1H Benzotriazole	>3.8	>3.8	>3.8	>3.8
1H Benzotriazole 1-methanol	1.7	1.7	0.85	0.85

Table 3.5: Effect of synthesised copper-benzotriazole compounds against *P. aeruginosa* biofilms. Results are expressed as Biofilm Inhibitory Concentrations (BIC μM).

Copper sulphate-benzotriazole compound	***P. aeruginosa* ATCC27853**	***P. aeruginosa* PA01**	***P. aeruginosa* 1826**	***P. aeruginosa* 4276.3**
Cu (BZT)	>1.2	>1.2	>1.2	>1.2
Cu (MBZT)	>2.4	>2.4	>2.4	>2.4
Cu (BZTM)	>2.2	>2.2	>2.2	>2.2
Copper sulphate	>6.4	>6.4	>6.4	6.4
Benzotriazole	>4.3	>4.3	>4.3	2.1
5-Methyl-1H Benzotriazole	>7.6	3.8	1.9	1.9
1H Benzotriazole 1-methanol	3.4	6.8	3.4	1.7

3.3.2 Effect of transition metal gallium nitrate ($Ga(NO_3)_3$) on growth of *P. aeruginosa* planktonic and biofilm bacteria.

The potential antimicrobial action of gallium nitrate was assessed against *P. aeruginosa* isolates grown planktonically and as biofilms on polystyrene pegs. Gallium nitrate displayed no obvious antibacterial effect as a single agent against planktonic and biofilm formation at concentrations ranging between 1 – 500 μM. Optical density readings of cells treated with gallium nitrate had similar absorbance values to the untreated control. Previous studies have shown gallium to inhibit biofilm bacteria by targeting cells located within the interior of the structure (Singh *et al.*, 2007). The inhibitory activity of gallium in combination with aminoglycoside antibiotic tobramycin was also assessed against biofilms grown on polystyrene pegs. Concentrations of tobramycin ranging between 0.5 - 512 μg/ml were prepared. In contrast to the results obtained from the study by Singh *et al.*, (2007), tobramycin appeared as the only agent inhibiting *P. aeruginosa* biofilm formation in this model. Findings revealed gallium nitrate is not enhancing the anti-pseudomonal activity of tobramycin.

Table 3.6: Effect of gallium nitrate ($Ga(NO_3)_3$) on antibiotic efficacy of tobramycin.

Results are expressed as MIC and BIC (μg/ml).

Gallium nitrate (μM) + Antibiotic	***P. aeruginosa* PA01**		***P. aeruginosa* ATCC27853**		***P. aeruginosa* 1826**		***P. aeruginosa* 4276.3**	
	MIC	**BIC**	**MIC**	**BIC**	**MIC**	**BIC**	**MIC**	**BIC**
2 μM Ga + Tobramycin	1	2	0.5	1	2	4	8	32
5 μM Ga + Tobramycin	1	1	0.5	1	2	4	8	32
20 μM Ga + Tobramycin	1	1	0.5	1	1	4	4	32
50 μM Ga + Tobramycin	1	2	0.25	2	1	4	8	16
200 μM Ga + Tobramycin	1	2	0.5	1	1	4	8	16
500 μM Ga + Tobramycin	0.5	2	0.5	1	2	4	8	32
Tobramycin control	**0.5**	**1**	**0.5**	**1**	**0.5**	**0.5**	**4**	**32**

Gallium nitrate displayed no effect against *P. aeruginosa.* Results are shown in Appendix 3.

3.3.3 Effect of silver-coumarin compounds against planktonic and biofilm formation of *P. aeruginosa*.

The metal-free coumarin ligands did not display any antimicrobial effect against *P. aeruginosa* strains grown planktonically or as biofilms. Results are shown in Appendix 2.

Results presented in **Tables 3.7** and **3.8** shows the sensitivity of *P. aeruginosa* strains to silver-coumarin compounds in comparison to the silver nitrate control. Silver-coumarin compounds used in this study were designed to enhance this effect by complexing with coumarins. Results revealed compounds 6, 7, 8 and 10 were active against all four strains grown planktonically. Compounds 5 and 6 showed the most potent inhibitory effect against planktonic cultures of two *P. aeruginosa* isolates at 6.1 and 5.5 μM, respectively. Several compounds exhibited a moderate decrease in biofilm formation in comparison to the silver nitrate control when exposed to various concentrations of the different agents. Compounds 3 and 7 resulted in biofilm inhibition greater than silver nitrate against three strains, as quantified by staining with crystal violet. Another three compounds 4, 5 and 6 reduced biofilms in two out of the four strains tested. Overall, the compounds displayed enhanced activity to silver nitrate against biofilm formation in at least one of the isolates examined. In addition, compounds 3 and 4 exhibited greater activity against biofilms than planktonic cells. The interactions of these compounds with *P. aeruginosa* appear to be specific to each strain.

Table 3.7: Effect of silver-coumarin compounds against planktonic *P. aeruginosa* strains. Results are expressed as MIC (μM) and MBC (μM).

Silver-coumarin compound	***P. aeruginosa* PA01**		***P. aeruginosa* ATCC27853**		***P. aeruginosa* 1826**		***P. aeruginosa* 4276.3**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Ag60H	12.7	25.5	25.5	51.1	12.7	102.2	25.5	51.1
Ag70H	25.5	25.5	25.5	102.2	25.5	51.1	25.5	102.2
Ag80H	12.7	25.5	25.5	102.2	12.7	102.2	25.5	51.1
AgCCa	13.46	26.9	26.9	107.7	26.9	107.7	26.9	53.87
Compound 2	24.1	193.3	48.3	96.6	48.3	96.6	24.1	48.3
Compound 3	24.4	195.6	24.4	48.9	48.9	195.6	48.9	195.6
Compound 4	21.3	85.1	42.5	170.2	42.5	170.2	21.3	170.2
Compound 5	6.1	12.2	24.46	195.6	12.2	24.46	12.2	12.2
Compound 6	11	44.2	22.1	44.2	5.5	88.5	11	22.1
Compound 7	22.1	44.26	22.1	44.26	44.26	88.5	11	22.1
Compound 8	12.2	97.8	24.46	48.9	12.2	97.8	12.2	24.46
Compound 10	10.9	21.8	21.8	43.7	10.9	87.4	10.9	21.8
Silver nitrate ($AgNO_3$) control	**23.5**	**94.18**	**23.5**	**94.18**	**23.5**	**162.5**	**11.7**	**94.18**

Table 3.8: Effect of synthesised silver-coumarin compounds against *P. aeruginosa* biofilms. Results are expressed as BIC (μM).

Silver-coumarin compound	***P. aeruginosa* PA01**	***P. aeruginosa* ATCC27853**	***P. aeruginosa* 1826**	***P. aeruginosa* 4276.3**
Ag60H	215.48	1635.7	107.7	107.7
Ag70H	408.9	1635.7	51.1	204.46
Ag80H	817.8	1635.7	51.1	204.46
AgCCa	204.46	408.9	204.46	51.1
Compound 2	24.1	3093	3093	24.16
Compound 3	48.9	782.7	48.92	97.84
Compound 4	21.3	2724	85.1	340.5
Compound 5	24.46	1565.5	97.84	48.92
Compound 6	22.13	2832.7	354.09	22.13
Compound 7	44.26	1416.39	44.26	177
Compound 8	24.46	1565.5	195.69	1565.5
Compound 10	174.8	2797.8	43.71	174.86
Silver nitrate ($AgNO_3$) control	**94.18**	**1506.9**	**188.36**	**47.09**

3.3.4 Summary of selected clinical CF *P. aeruginosa* strains.

A selection of *P. aeruginosa* CF strains were recovered from sputum samples from different Irish CF clinics and typed using pulsed-field gel electrophoresis (PFGE) at the CF reference laboratory, AMNCH. Samples were plated onto selective agar as described in **Section 2.2.5** and phenotypic characteristics are listed below in **Table 3.9**. Four out of the sixteen strains grew as mucoid colonies while twelve were non-mucoid. Approximately half of the isolates produced a green pigment and the remaining grew as small white colonies on MHA after 24 hours.

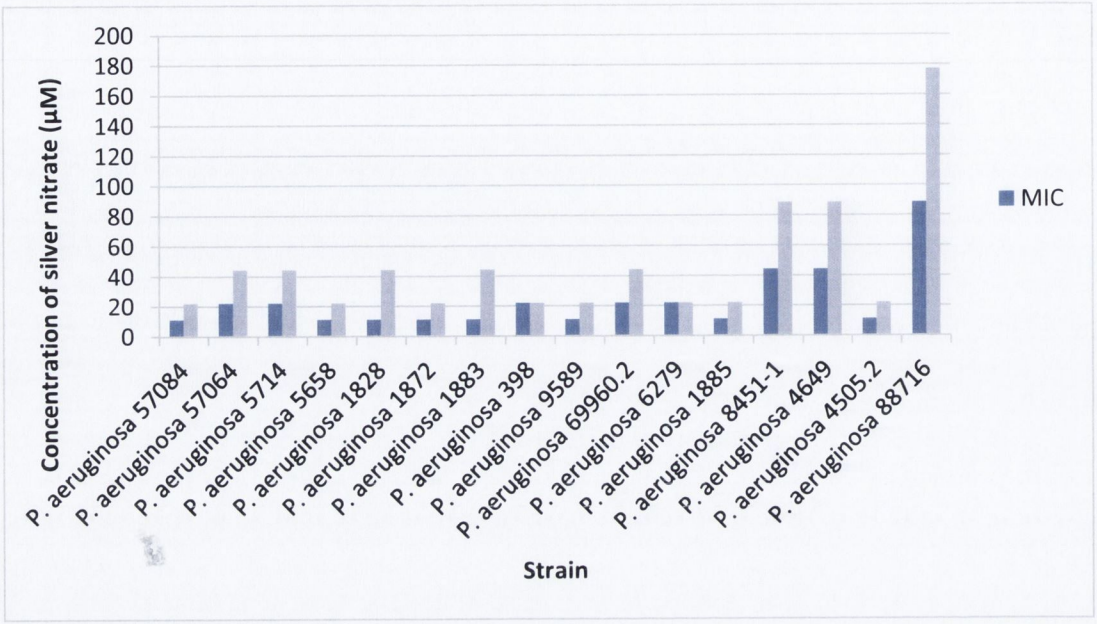
Table 3.9: Phenotypic characteristics of CF *P. aeruginosa* strains.

***P. aeruginosa* strain**	**pigment/ no pigment**	**mucoid/ non-mucoid**
P. aeruginosa 57084	no pigment	non-mucoid
P. aeruginosa 57064	no pigment	non-mucoid
P. aeruginosa 5714	pigment	non-mucoid
P. aeruginosa 5658	no pigment	non-mucoid
P. aeruginosa 1828	no pigment	non-mucoid
P. aeruginosa 1872	no pigment	non-mucoid
P. aeruginosa 1883	pigment	non-mucoid
P. aeruginosa 398	no pigment	non-mucoid
P. aeruginosa 9589	no pigment	non-mucoid
P. aeruginosa 69960.2	no pigment	non-mucoid
P. aeruginosa 6279	no pigment	non-mucoid
P. aeruginosa 1885	pigment	non-mucoid
P. aeruginosa 8456-1	pigment	mucoid
P. aeruginosa 4649	pigment	mucoid
P. aeruginosa 4505.2	pigment	mucoid
P. aeruginosa 88716	pigment	mucoid

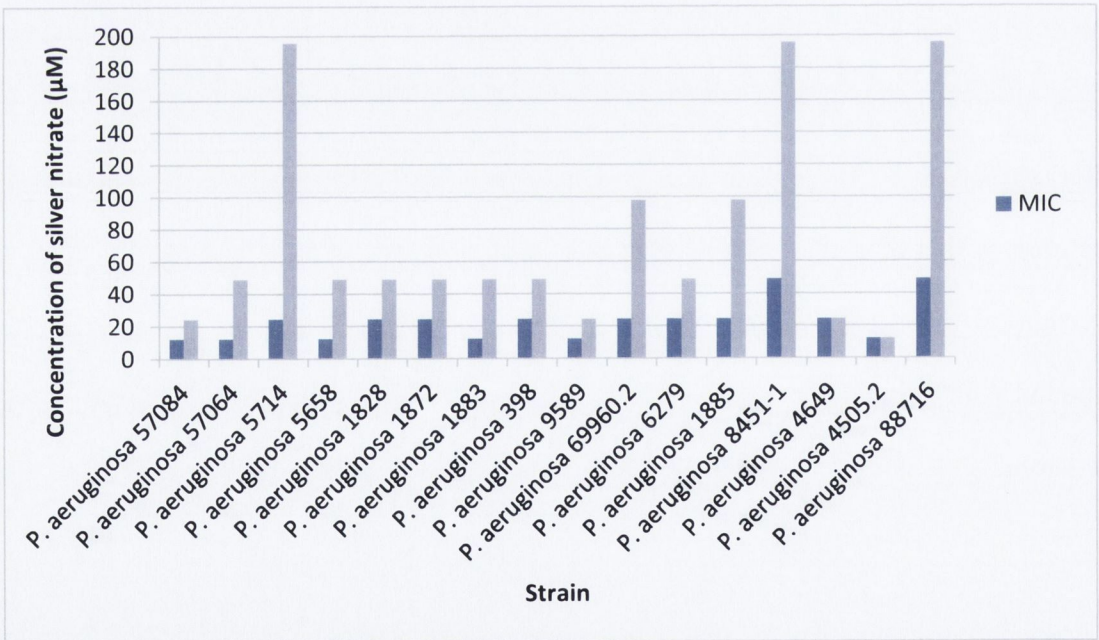
3.3.5 Investigation of the effect of silver-coumarin agents on planktonic growth of clinical CF *P. aeruginosa* strains.

The antimicrobial activity of effective silver-coumarin compounds was assessed against a range of CF *P. aeruginosa* strains of different phenotypic characteristics. Virulence factors including pyocyanin production secreted by *P. aeruginosa* are known to target epithelial cell function in the CF lung resulting in chronic lung infection (Gardner *et al.*, 1996). The amount of exopolysaccharide has been shown to be an important component in biofilm production and function (Matsukawa *et al.*, 2004). Strains appeared sensitive to these agents irrespective of their phenotypic traits as seen in **Figure 3.2**. Following treatment with synthesised compounds 6 and 8, a greater reduction in MIC and MBC values in comparison to silver nitrate was not observed for most strains. Differences between MIC and MBC values were generally not more than one or two-fold greater in concentration for each agent. *P. aeruginosa* 57064 and *P. aeruginosa* 4505.2 displayed greater sensitivity to the compounds in comparison to silver nitrate with a one-fold reduction in either the MIC or MBC values. Three additional strains (*P. aeruginosa* 5714, *P. aeruginosa* 69960.2, *P. aeruginosa* 6279) showed greater bactericidal sensitivity to compound 6 in comparison to silver nitrate in which MBC values were reduced by at least 2-fold.

(A) Compound 6



(B) Compound 8



(C) Silver nitrate ($AgNO_3$)

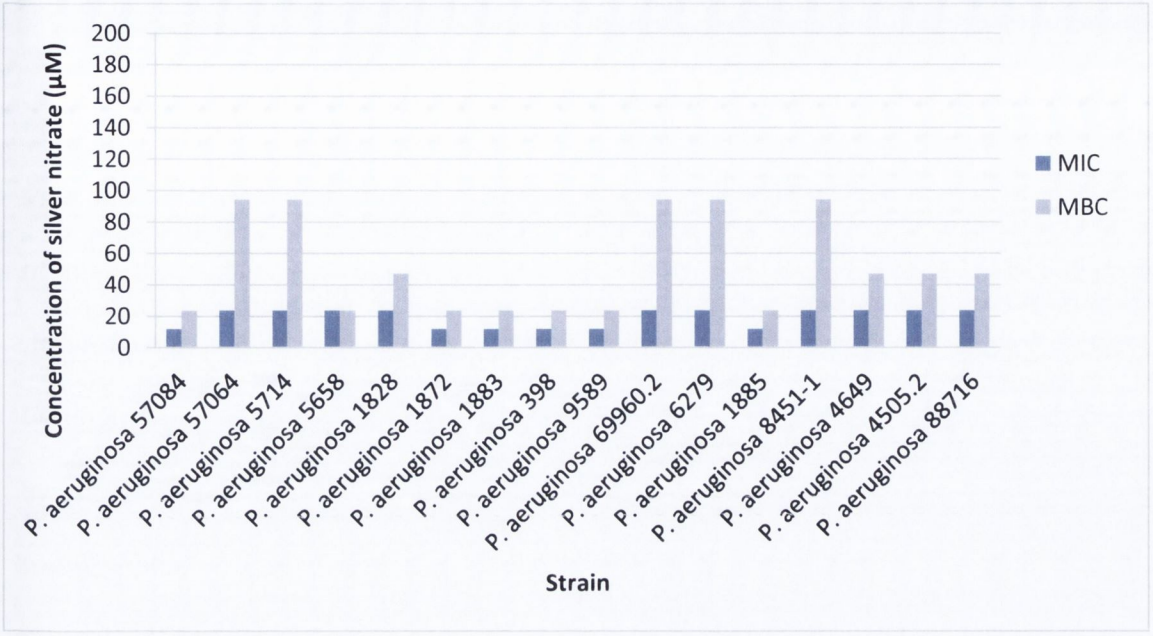
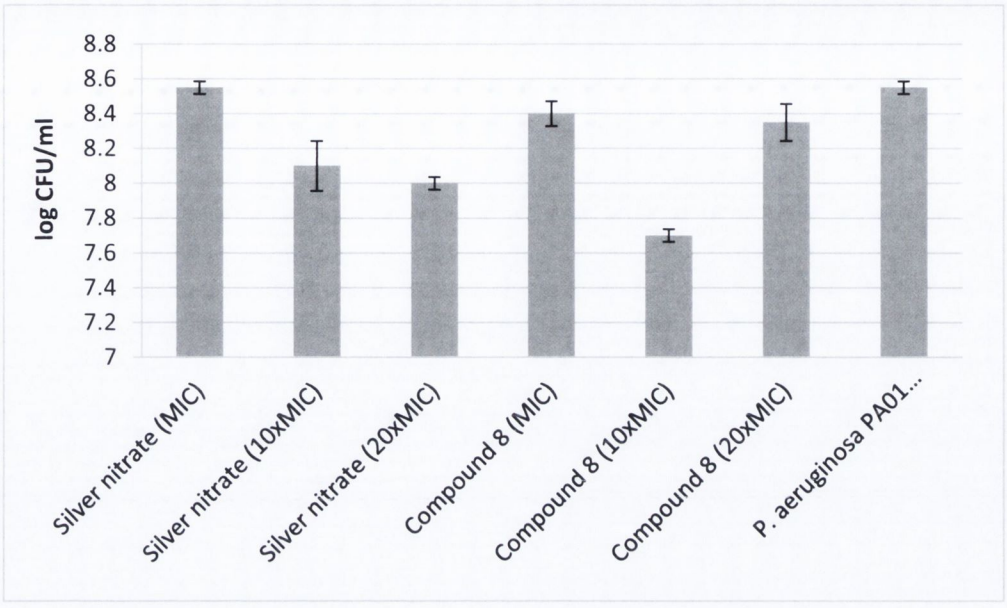


Figure 3.2: MIC and MBC (μM) values of **(A)** compound 6 (mw 361.48), **(B)** compound 8 (mw 327.04) and **(C)** silver nitate control against clinical CF *P. aeruginosa* strains. Results represent the mean ± standard deviation of three independent experiments.

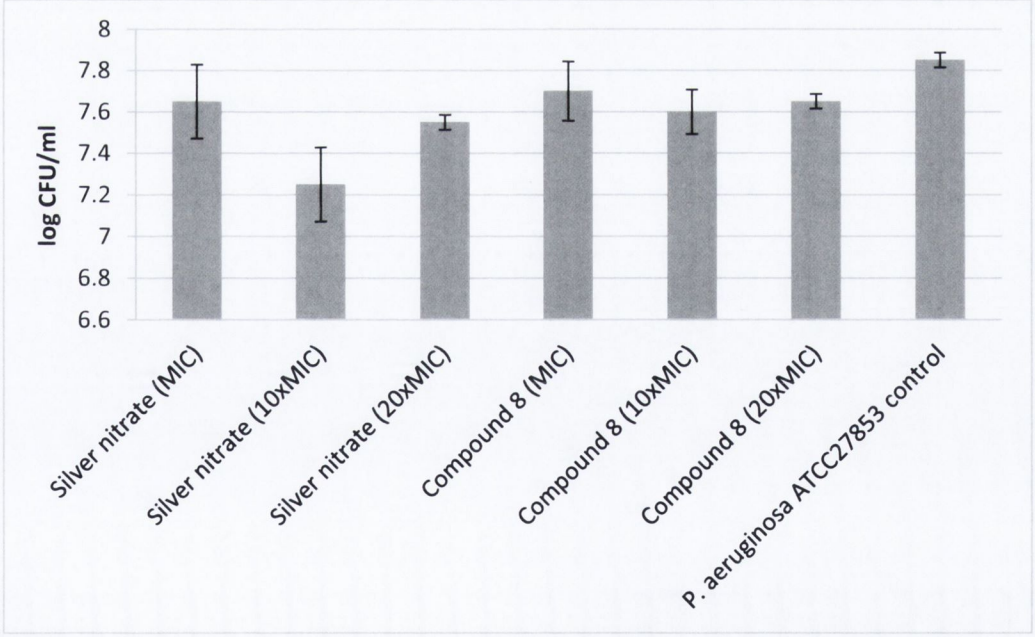
3.3.6 Inhibition of mature *P. aeruginosa* biofilms by silver-coumarin compounds using two different static models.

To investigate the influence of effective silver-coumarin compounds on mature biofilm structures, analysis was carried out using two different static models. Biofilms were grown for 72 hours and viability was assessed following a further 24 hours exposure to silver nitrate and compound 8. Biofilm biomass was compared to the untreated control. Less mature biofilms have been described as generally more susceptible to antimicrobial treatment than cells in more older structures (Tre-Hardy *et al.*, 2009). Comparative studies of biofilms grown for 72 hours revealed that reference strains (*P. aeruginosa* PA01 and *P. aeruginosa* ATCC27853) were more resistant to treatment since the activity of silver nitrate and compound 8 did not significantly reduce growth in comparison to the untreated control. As shown in **Figure 3.3** biomass of clinical isolate *P. aeruginosa* 1826 was significantly reduced with concentrations of MIC of silver nitrate ($P = 0.029$), 10 x MIC of silver nitrate ($P = 0.011$) and MIC of compound 8 ($P = 0.043$) in comparison to the control. In addition, the agents did affect bacterial viability of clinical isolate *P. aeruginosa* 4276.3, suggesting that the concentrations sufficient to suppress mature biofilm formation are strain dependant. Taken together the data indicate that biofilm suppression by these agents may be influenced by the age of the biofilm.

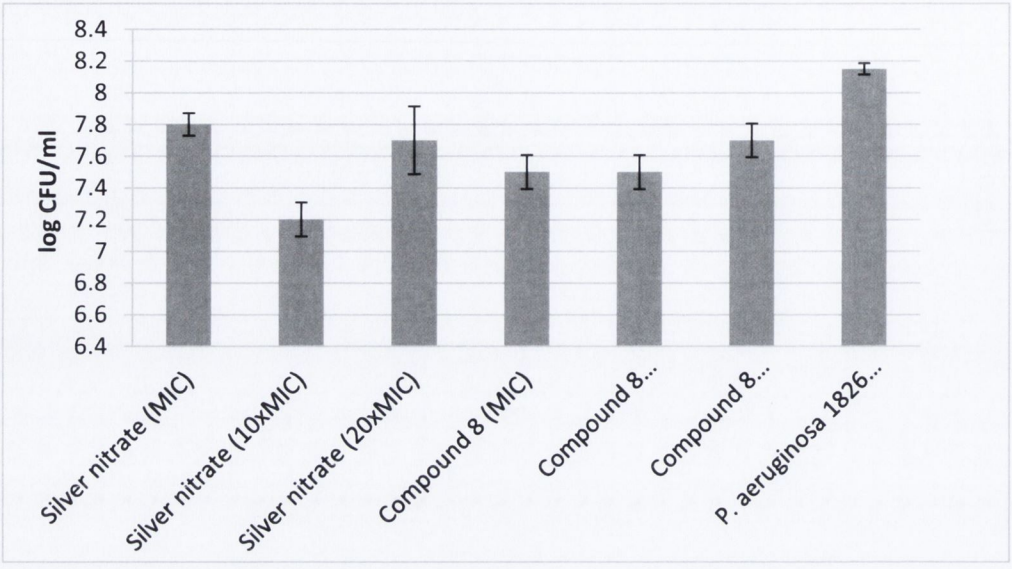
(A) *P. aeruginosa* PA01



(B) *P. aeruginosa* ATCC27853



(C) *P. aeruginosa* 1826



(D) *P. aeruginosa* 4276.3

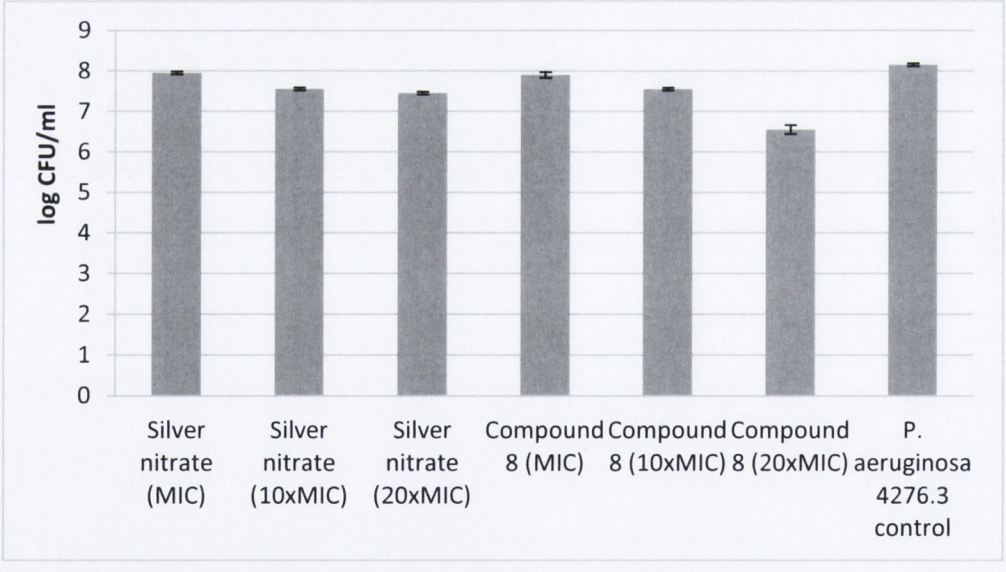
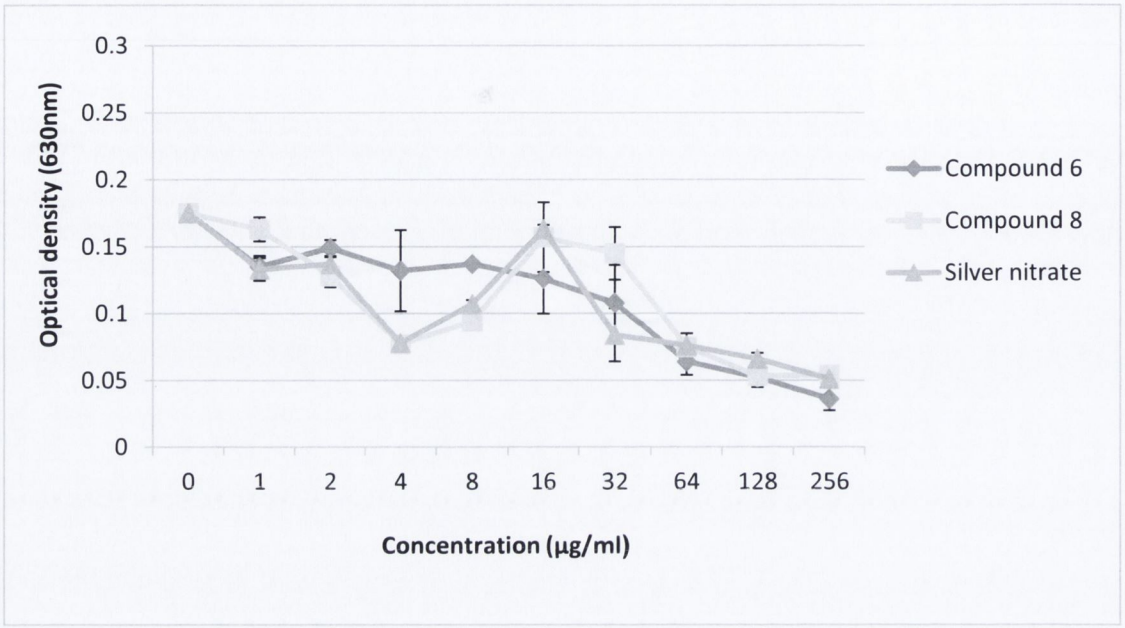


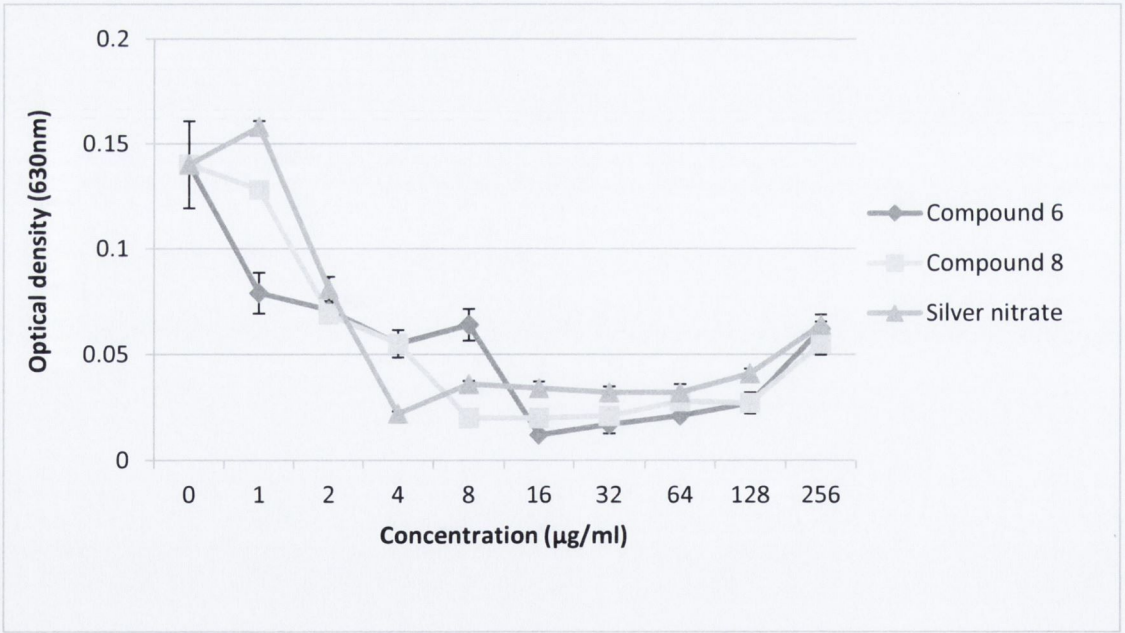
Figure 3.3: The effect of silver nitrate and compound 8 against 72 hour *P. aeruginosa* biofilms grown in BTrace medium. The activity of all agents was compared to the untreated control. Results are shown as total log counts and represent the average of three experiments.

The results obtained from 72 hour biofilms formed on polystyrene pegs, treated with effective compounds showed that the activity of the silver-coumarin compounds was comparable to silver nitrate as shown in **Figure 3.4**. Results obtained with this static model are in keeping with the previous model, in which the optical densities of 72 hour biofilms treated with the compounds were similar to the silver nitrate control. Increasing concentrations did not necessarily increase the rate of biofilm inhibition against *P. aeruginosa* PA01. The results indicate that resistance of 72 hour *P. aeruginosa* biofilms may be greater than at 24 hours. The two clinical isolates, *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3, were more sensitive strain to all agents at higher concentrations. The efflux system has been described as one of the main resistance mechanisms of most microorganisms exposed to high concentrations of heavy metals for a lengthy period of time (Nies *et al.*, 1999).

(A) *P. aeruginosa* PA01



(B) *P. aeruginosa* 1826



(C) *P. aeruginosa* 4276.3

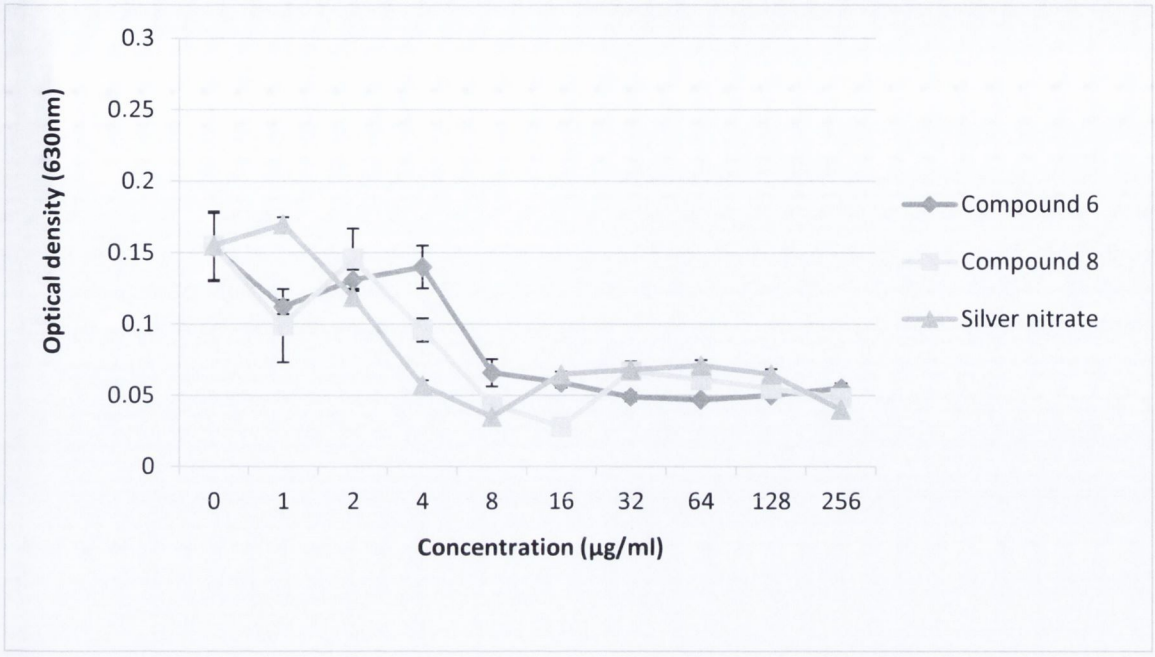


Figure 3.4: The effect of varying concentrations of compound 6, compound 8 and silver nitrate against mature *P. aeruginosa* biofilms. Biofilms were grown on polystyrene pegs for 72 hours prior treatment. Results represent the mean ± standard deviation of two experiments.

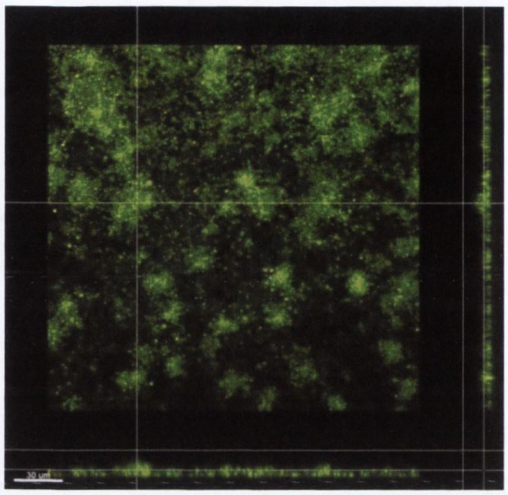
3.3.7 Investigation of *P. aeruginosa* biofilm formation using the flow cell system.

Biofilm forming ability of the reference and CF clinical isolates was investigated in the flow cell system. This model allows the structural development of biofilms to be monitored with confocal laser scanning microscope (CLSM). The results show that clinical CF and reference strains develop different structural biofilms as depicted in **Figure 3.5**. The microcolonies formed by clinical isolates, *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3 were larger than *P. aeruginosa* PA01. Both of these CF isolates developed large, thick structures while the overall structure of *P. aeruginosa* 1826 was more uniform. In addition, an uneven distribution of microcolonies was observed with the clinical strains. The microcolonies of *P. aeruginosa* PA01 appeared smaller and less defined while *P. aeruginosa* ATCC27853 formed a flat structure on the surface of the flow-cell chamber. It is interesting that *P. aeruginosa* PA01 which produced a strong biofilm with the crystal violet staining biofilm assay showed reduced thickness in the flow cell system. The results suggest that the environmental conditions of the static model may be favouring biofilm growth of *P. aeruginosa* PA01.

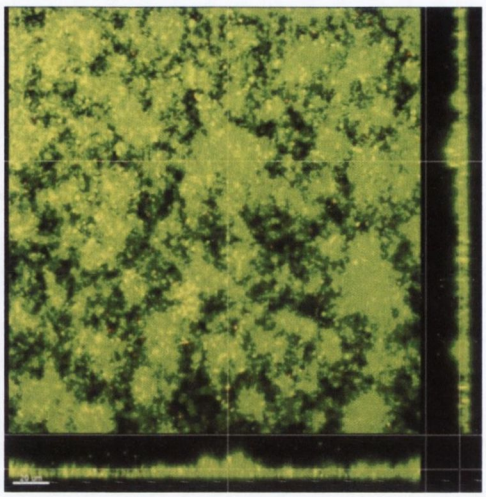
Table 3.10: Phenotypic characteristics of isolates selected for examination and treatment in the flow cell system.

Strain	Pigment/ No pigment	Mucoid/ Non-mucoid
P. aeruginosa PA01	pigment	non-mucoid
P. aeruginosa ATCC27853	pigment	non-mucoid
P. aeruginosa 1826	pigment	non-mucoid
P. aeruginosa 4276.3	no pigment	mucoid

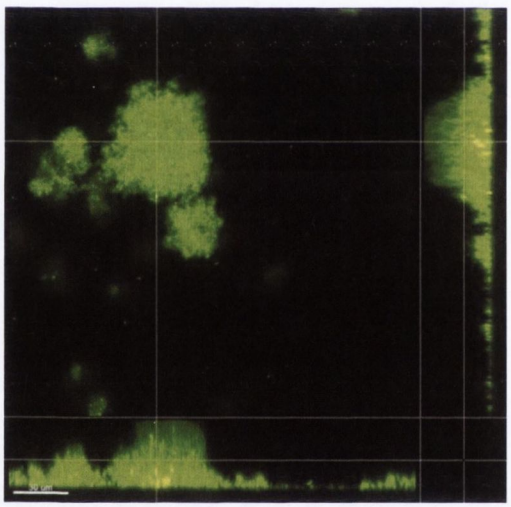
(A) *P. aeruginosa* PA01



(B) *P. aeruginosa* ATCC27853



(C) *P. aeruginosa* 1826



(D) *P. aeruginosa* 4276.3

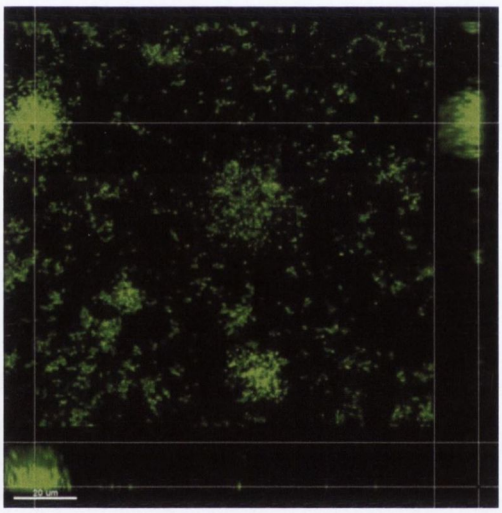
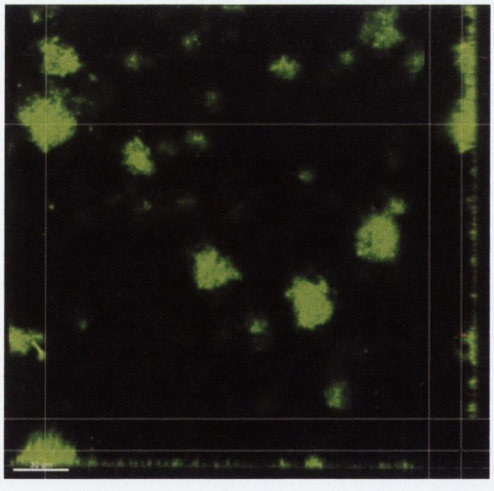


Figure 3.5: CLSM images of 72 hour *P. aeruginosa* biofilms in 10% glucose supplemented minimal media showing different biofilm structures. Images show sections through the biofilm depicting microcolony profiles of the structures.

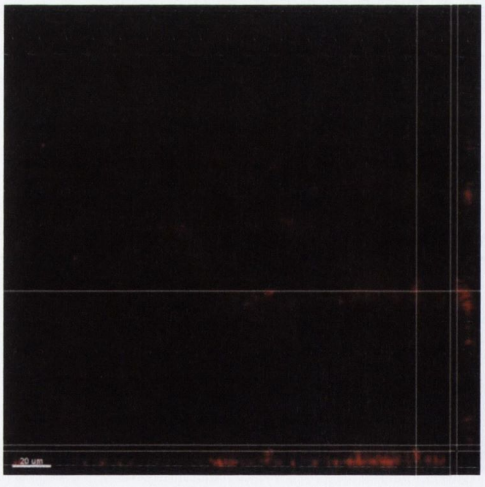
3.3.8 The effect of silver-coumarin compounds on biofilm development in the flow cell system.

The study progressed to a continuous flow cell model to further determine the mechanisms by which synthesised compounds reduce the viability of established *P. aeruginosa* biofilms in comparison to the silver nitrate control. *P. aeruginosa* 1826 biofilms were grown for 72 hours in separate channels followed by 24 hours treatment with 10 x MIC compound 8 and 10 x MIC of silver nitrate **(Figure 3.6).** Eradication of the biofilm was observed in comparison to the untreated control in the presence of these agents. The results show no clear differences in the activities of each compound. Findings are consistent with the results obtained of 72 hour *P. aeruginosa* 1826 biofilm formed in the static biofilm models.

(A) *P. aeruginosa* 1826 control



(B) *P. aeruginosa* 1826 + $AgNO_3$



(C) *P. aeruginosa* 1826 + compound 8

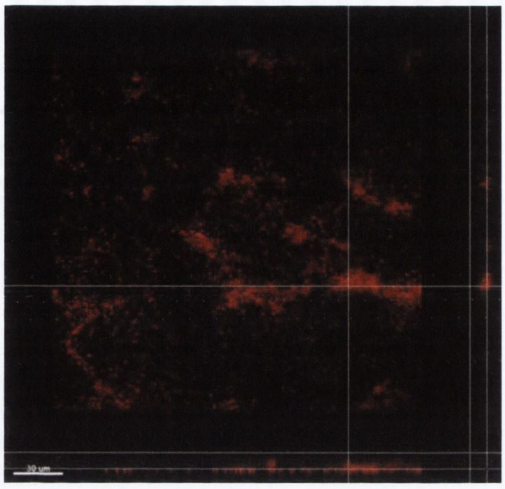
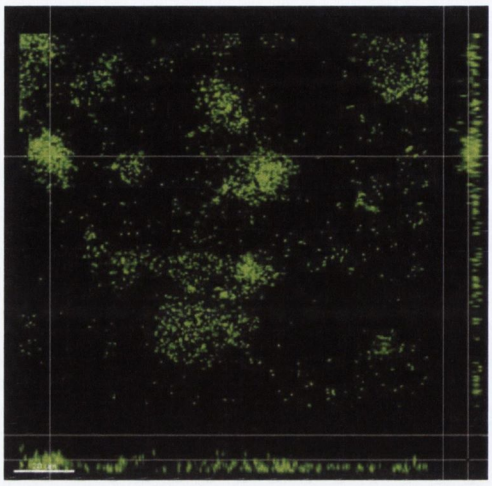


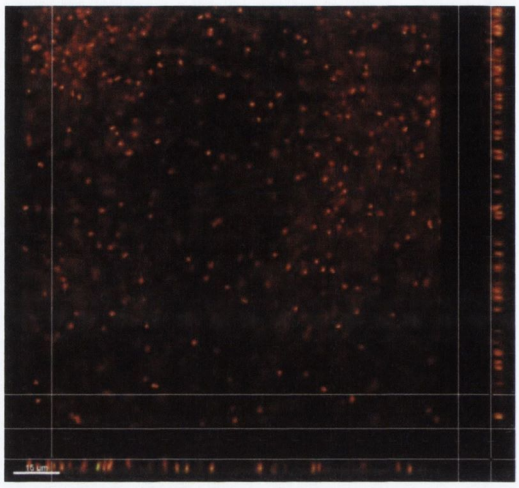
Figure 3.6: Live-dead stained *P. aeruginosa* 1826 grown for 72 hours in minimal medium and treated with 10 x MIC of silver nitrate and 10 x MIC of compound 8. Images were obtained by CLSM, live cells appear green and dead cells appear red as a result of staining.

The effect of compound 8 and silver nitrate at the same concentration of 10 x MIC was also investigated against *P. aeruginosa* PA01 biofilms in the flow cell system **(Figure 3.7)**. Prior to treatment *P. aeruginosa* PA01 formed small, flat microcolonies. Previously, this strain displayed greater sensitivity to the synthesised silver-coumarin compounds in comparison to the silver-nitrate control in the static biofilm model. This strain was not able to form biofilm of such high surface coverage in the flow cell system. In addition, there was complete inhibition of this strain in the presence of all agents following 24 hours exposure. The exact stages at which the agents affect *P. aeruginosa* biofilm development remain unknown in the present study and warrant further investigation.

(A) PA01 control



(B) PA01 + $AgNO_3$



(C) PA01 + compound 8

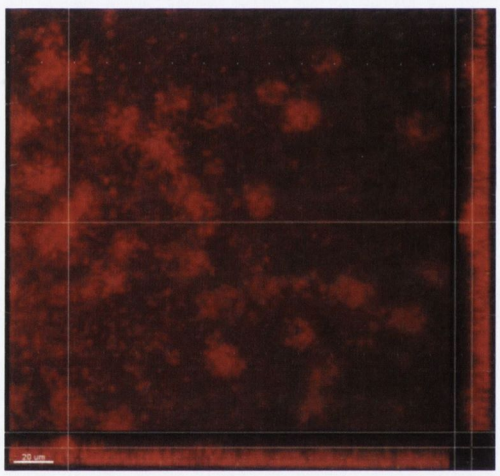


Figure 3.7: Live-dead stained images of 72 hour *P. aeruginosa* PA01 biofilm treated with 10 x MIC of silver nitrate and 10 x MIC of compound 8. The results show *P. aeruginosa* PA01 biofilms following 24 hours treatment. Images were obtained by CLSM.

3.4 Discussion

The results so far have demonstrated that CF *P. aeruginosa* strains have different phenotypic characteristics which may influence the response to treatment. This section of the study was aimed at determining the ability of novel metal-based compounds to disrupt *P. aeruginosa* biofilms. Findings were based on crystal violet staining which is a standard method for biofilm quantification. A more advanced study of biofilms using the flow cell system was also used as part of this study.

Results of the antimicrobial action of copper-benzotriazole agents showed that these agents exhibit low antimicrobial activity against *P. aeruginosa*. As mentioned in this Chapter, Section 3.1, reduced concentrations of copper and various other metals may facilitate the growth of certain bacterial species. The current study found *P. aeruginosa* growth was unaffected with all synthesised copper-benzotriazole agents. In addition, copper sulphate exhibited antimicrobial activity against three *P. aeruginosa* strains at a concentration of 3.2 μM indicating that this metal may be toxic to *P. aeruginosa* cells at this concentration. Previously, Visca *et al.*, (1992), described *P. aeruginosa* genes involved in the synthesis of pyochelin were repressed by copper activity. Copper is also known to produce reactive oxygen species which can lead to damage of cellular components (Damerson and Morrison, 1998). Teitzel *et al.*, (2006), described oxidative stress as an important factor for copper toxicity in *P. aeruginosa*. As well as damage to the cell membrane, oxidative stress can increase membrane permeability leading to death of the bacterial species. High amounts of copper may also enhance protein misfolding,

repression of virulence genes, *agr* and *sae* in *S. aureus* (Baker *et al.*, 2010). In other studies, copper has shown to display greater activity through synergistic interactions with other agents. Harrison *et al.*, (2008), found combinations of copper and certain quaternary ammonium compounds enhance toxicity by affecting the nitrate reduction of *P. aeruginosa* biofilms.

Another agent examined against *P. aeruginosa* biofilms grown on polystyrene pegs was gallium nitrate. This agent has been studied by many researchers since it has been FDA approved for intravenous treatment of hypercalcemia of malignancy (Bernstrin *et al.*, 1998). Other studies have shown gallium can enter bacterial cells and act as a substitute for iron in many biological processes including DNA replication (Hubbard *et al.*, 1986; Chitambar *et al.*, 1988). Olakanmi *et al.*, (2000), showed that gallium is effective against *Mycobacterium tuberculosis* under conditions in which the concentration of iron are high. In contrast, Singh *et al.*, (2007), showed gallium was taken up by *P. aeruginosa* biofilms when the concentration of iron was low. Results from the current study found gallium nitrate did not display antibacterial activity against *P. aeruginosa* planktonic and biofilm cultures. This study also found that gallium nitrate did not enhance the activity of the aminoglycoside antibiotic tobramycin. The study by Singh *et al.*, (2007) described biofilms formed under conditions in which concentrations of iron are reduced are more susceptible to antimicrobial treatment. Synergistic interactions were observed with gallium and tobramycin. Tobramycin acts on the cell membrane allowing gallium to inhibit the interior of the biofilm structure. Unlike the study by Singh *et al.*, (2007), *P. aeruginosa* biofilms developed in this study were not grown under conditions in which

the concentration of iron was low.

The synthesised metal-free coumarin compounds used in this study were ineffective against all *P. aeruginosa* strains. In contrast, many of the synthesised silver-coumarin compounds displayed enhanced antibacterial activity in comparison to the silver nitrate control against *P. aeruginosa*. Previous studies have shown coumarins have the potential to enhance the antimicrobial effect of other agents. The study by Thati *et al.,* (2007), showed that these silver-coumarin compounds reduced the rate of respiration of *C. albicans* by disrupting the synthesis of cytochromes in the mitochondrion. Reeves *et al.,* (2004), found coumarin agents increased leakage of the components of the cell membrane. Another study by Smyth *et al.*, (2009), found antibiotic oxicillin displayed greater activity against MRSA in combination with 8-Iodo-5, 7-dihydroxycoumarin. In addition, Zeng *et al.*, (2007), found coumarin agent, esculin enhanced the susceptibility of *P. aeruginosa* to tobramycin by interfering with quorum sensing signalling.

This study adds to the current knowledge about the antimicrobial activity of silver-based agents. The results revealed the effect of silver nitrate and silver-coumarin compounds are effective against *P. aeruginosa* planktonic cells and biofilms. The majority of the compounds showed greater efficacy against planktonic cells than 24 hour biofilms. In addition, biofilms grown for 72 hours on polystyrene pegs were more resistant to treatment with these compounds suggesting the efficacy of these agents may be reduced against mature biofilms. Silver is effective against a broad range of bacteria by acting against the cell membrane by binding to DNA and thiol groups and inactivating enzymes

affecting bacterial replication (Feng *et al.*, 2000; Slawson *et al.*, 1992; Lansdown and Williams, 2007). The antibacterial properties have also been described by Kalishwaralal *et al.*, (2010), which found silver nanoparticles may enhance the detachment of *P. aeruginosa* and *S. aureus* from the wells of a microtitre plate in a concentration dependent manner. Greenfield *et al.*, (1995), described silver sulfadiazine in combination with chlorhexidine inhibited bacterial adherence on vascular catheters. Other *in vivo* studies found silver iontophoretic catheters reduce the rate of *S. aureus* infection (Raad *et al.*, 1996). Youngs *et al.*, (2009), suggested silver-complexes that are water soluble may be suitable for administration via inhalation.

This study also assessed the activity of the silver-coumarin compounds against a broad range of *P. aeruginosa* strains since many clinical CF strains have different phenotypic characteristics as described in this section. The bacteriostatic and bactericidal activity of silver nitrate was recorded against planktonic cultures of 16 *P. aeruginosa* strains. The differences between the MIC and MBC values of silver-coumarin agents in comparison to silver nitrate were generally not more than two-fold, indicating the phenotypic characteristics of *P. aeruginosa* do not appear to influence the efficacy of these agents.

The activity of these compounds was also tested against *P. aeruginosa* biofilms formed in microtitre wells in minimal BTrace media. The concentrations selected were based on the MIC, 10 x MIC and 20 x MIC as determined by the broth microdilution method. Findings revealed that the activity of silver nitrate and the silver-coumarin compounds was not dependant of the concentration. The results revealed that two strains, *P. aeruginosa* PA01

and *P. aeruginosa* 1826 exhibited an increase in biofilm density at the 20 x MIC in comparison to a lower concentration of 10 x MIC. In addition, *P. aeruginosa* 4276.3 was more resistant to the activity of these agents. Previously, Parson *et al.*, (2006), showed that wound dressings that released a larger amount of silver do not lead to greater degree of antimicrobial activity. Bridges *et al.*, (1979), found a selection of *P. aeruginosa* strains collected from burn patients that were resistant to gentamicin were equally resistant to silver, the reasons of which are currently unknown. Gupta *et al.*, (1999), and his group reported that the ability of silver to bind to proteins and efflux pump mechanisms may reduce the toxicity of silver.

P. aeruginosa biofilms were grown in the flow cell system to determine if results correlated with those obtained with the static models. Results suggested that silver is able to inhibit and detach established biofilms of certain strains. Assays which enable bacteria to be cultured continuously allow phenotypic characteristics of bacteria to be maintained for lengthy periods of time (Herbert *et al.*, 1956). A continuous nutrient supply is essential for bacteria to grow in any system (Nichols *et al.*, 1999). Results from the current study showed distinct differences in the biofilm structures of selected *P. aeruginosa* strains which may be influencing their response to treatment. Clinical strains, *P. aeruginosa* 1826 and *P. aeruginosa* 4276.3 developed large microcolonies. Reference strain *P. aeruginosa* PA01 formed small microcolonies and *P. aeruginosa* ATCC27853 developed a flat biofilm structure. Previous studies have suggested the matrix of biofilms under flow conditions may be thicker than those formed in other models, which may be enhancing resistance to antimicrobial agents (Douglas *et al.*, 2006). Davies *et al.*, (1998),

suggested that quorum sensing signalling molecules, known as autoinducers (AI), of *P. aeruginosa* have an important role in differential biofilm morphology which may be giving biofilms their defining structures in a flow environment. The two QS systems, *las* system and *rhl* system, influence biofilm architecture through different mechanisms (DeKievit *et al.*, 2001). It remains unclear if the *las*, *rhl* or both QS systems are involved in attachment and microcolony formation in the present study.

In this study, biofilms grown in the flow cell system that were exposed to effective silver-coumarin compounds showed biofilms were inhibited following 24 hours treatment. These findings were different to the results obtained with the static biofilm models. One possible explanation for the results obtained in the current study could be the continuous nutrient supply containing a suitable concentration of the silver-based agent sloughing off the different cell layers of the biofilm. Recently, Sillankorva *et al.*, (2008), reported that the rate at which some agents reach their host under dynamic conditions can depend on force. The amount of exopolysaccharide secreted by bacteria can be influenced by the different growth conditions. It remains unclear if a greater amount of exopolysaccharide was produced in the static models, indicating that the efficacy silver-based compounds may not be influenced by the matrix of *P. aeruginosa* biofilms.

Chapter 4: Mixed-species biofilm infection.

4.1 Introduction

The pathogens present in the CF lung may change due to environmental conditions and patient-to-patient transmission (LiPuma *et al.*, 1990; Salunkhe *et al.*, 2005). According to Wahab *et al.*, (2004), patients co-colonised with mucoid strains of *P. aeruginosa* and less common bacterial species can experience more severe lung function complications than patients that have contracted just the mucoid phenotype.

Pathogens within the CF airways change with disease progression and antibiotic exposure (Tunney *et al.*, 2013). *P. aeruginosa*, *S. aureus* and *H. influenzae* have been identified as the primary causes of acute exacerbations and lung deterioration (Saiman *et al.*, 2004). Pathogens less frequently associated with chronic infection include *Streptococcus pneumoniae*, *Stenotrophomonas maltophilia, Burkholderia cepacia* complex (Bcc), *Achromobacter xylesoxidans, Acinetobacter* sp., *Herbaspirillum* sp., *Pandoraea* sp. and *Ralstonia* sp. (LiPuma *et al.*, 2010).

As previously described in Chapter 1, Section 1.5, biofilms are bacterial communities encased within an exopolysaccharide matrix. Biofilms may form as single species in which the cell clusters are separate or in a mixed culture consisting of two or more species (Bjarnsholt *et al.*, 2011). Mixed species biofilms may be classified as synergistic

or antagonistic in which the presence of one organism influences the growth of the other organism (Kuramitsu *et al.*, 2007).

Biofilms consisting of one or more species have been described as more thicker and stable than those formed by a single species (Allison *et al.*, 2000). Rao *et al.*, (2004), suggested that microcolonies of certain species may act as protective layers during biofilm formation, enhancing the presence of one species during colonisation. Skillman *et al.*, (1999), found that interactions between the extracellular polysaccharide of two different species resulted in changes to the polymer matrix. Other studies have described rheological interactions between different species may decrease the rate at which antimicrobial agents penetrate the membrane of the biofilm (Allison *et al.*, 1992).

Many standard culturing techniques have been directed at identifying pathogens that are generally more dominant within the lungs. In order for clinicians to select more accurate treatments, a more detailed analysis of the bacteria that exist within these communities of each sputum sample would be necessary (Malic *et al.*, 2009; Gu *et al.*, 2005). The use of molecular methods such as phylogenetic assays has increased the understanding of CF airway pathogens. The 16S rRNA phylochip has detected more than 2,015 bacterial taxa from CF sputum samples (Sibley *et al.*, 2011). Fluorescent *in situ* hybridization (FISH) is an emerging technique that allows simultaneous identification and visual analysis of bacteria within mixed communities (Moter *et al.*, 2000). With this technique Fazli *et al.*, (2009), noticed *P. aeruginosa* and *S. aureus* biofilms colonise the wound surface at

different levels. *P. aeruginosa* tends to reside in the deeper layers and virulence factors that are produced lead to polymorphonucleotide (PMN) destruction and inflammation.

The CF reference laboratory in AMNCH has identified a number of unusual pathogens in CF sputum samples. CF pathogens may develop their own biofilm structures or integrate into biofilms formed by more dominant species (Akiyama *et al.*, 2003; Lembke *et al.*, 2006). In this section the characterisation of unusual CF species was carried out. In parallel studies mixed biofilm interactions of these species with *P. aeruginosa* was conducted with the aim of finding pathogens that may be interfering with the biofilm forming capacity of *P. aeruginosa* and any possible mechanisms of these interactions.

4.2 Materials and Methods

4.2.1 Bacterial strains.

Clinical and reference isolates of *P. aeruginosa* and unusual CF strains are listed below in **Table 4.1.** The clinical CF strains were identified in the cystic fibrosis reference laboratory in AMNCH by 16S ribosomal RNA (rRNA) gene sequencing. The *Paracoccus yeeii* strains were obtained from the Centre for Disease Control and Prevention (CDC). All isolates were routinely grown on TSA and inoculated in nutrient broth for 24 hours at 35°C when conducting experimental procedures. A culture of all bacterial strains was stored in glycerol at -80°C.

Table 4.1: Cystic fibrosis species used in this study.

Species:	**Gram-positive/ Gram-negative**	**Aerobic/Anaerobic**
Stenotrophomonas maltophilia	gram-negative	aerobic
Ralstonia sp.	gram-negative	aerobic
Acinetobacter sp.	gram-negative	aerobic
Herbaspirillum sp.	gram-negative	aerobic
Paracoccus yeeii	gram-negative	aerobic

4.2.2 Planktonic growth of unusual CF species.

An overnight culture of each isolate was suspended in nutrient broth to a 0.5 McFarland standard and adjusted to a final concentration of 5 x 10^5 CFU/ml. A quantity of 100 µl of each culture was added the wells of a 96-well microtitre plate and incubated for 24 hours. After 24 hours, the OD_{630} was measured in a microplate reader to determine planktonic growth of the unusual CF species.

4.2.3 Single and mixed-species biofilm formation on polystyrene pegs.

The ability of unusual strains isolated from the CF lung to form biofilms as single species and mixed species with *P. aeruginosa* was examined using the microtitre plate assay as described by Moskowitz *et al.*, (2004). An overnight culture of each isolate was suspended in nutrient broth to a 0.5 McFarland standard. A quantity of 5 µl of this inoculum was added to 1 ml nutrient broth to give a final concentration of 5 x 10^5 CFU/ml. For single species experiments, 100 µl of the prepared inoculums of each isolate was added to the wells of a 96- well round-bottom plate. A TSP lid containing pegs was placed into the microtitre plate for biofilms to develop and incubated at 35°C for 24, 48 and 72 hours.

For mixed species experiments, a 0.5 McFarland standard of *P. aeruginosa* and the unusual CF isolates was prepared and adjusted to a final concentration of 5 x 10^5 CFU/ml as described above. A quantity of 50 µl of two different species was added to the wells of a 96-well plate to give a final volume of 100 µl. A TSP pin lid was added to the microtitre plate and incubated at 35°C for 24, 48 and 72 hours. After each time point the

lids containing pegs were rinsed three times in sterile water, dried at room temperature and added to another plate containing 0.3% crystal violet (CV) for 30 minutes to stain the cells. The plates were rinsed again thoroughly and placed into a new 96-well round-bottom microtitre plate containing an equal volume of 95% ethanol and 1% Triton X-100 to remove the CV stained biofilms. The OD_{630} was measured in a microplate reader. All experimental procedures were carried out in triplicate on three separate occasions.

4.2.4 Cell culture biofilm growth assay of single and mixed species.

To a 6-well microtitre plate, 10 mls of nutrient broth was added. An overnight culture of each isolate was diluted to OD_{630} = 0.1 in nutrient broth. A quantity of 100 µl of the culture was added to each well and incubated at 35°C for 24 and 72 hours to allow biofilms to develop. For mixed species biofilm growth, each mixture was prepared at a 1:1 ratio. Following 24 hours incubation, all contents of the wells were removed and transferred to centrifuge tubes. Cells were centrifuged for 3,000 *g*, 10 minutes. Cells were washed in 10 ml 0.9% NaCl and centrifuged at 3,000 *g*, 10 minutes again. Cells were re-suspended in 10 ml 0.9% NaCl followed by sonication and vortexed until the cells have been dispersed. Serial dilutions were prepared and spotted onto TSA plates to determine the CFU/ml.

For mature biofilm experiments, biofilms were prepared as described above in **Section 4.2.4** and incubated for 72 hours. The media was removed from the wells every 24 hours and replaced with fresh media using a 10 ml syringe. This was dispensed gently down the side of the wells to avoid shear force which can destroy any biofilm that has formed on

the bottom of the wells. After 72 hours incubation, all contents from wells were transferred to centrifuge tubes. Cells were centrifuged for 3,000 *g*, 10 minutes. Cells were washed in 10 ml 0.9% NaCl and centrifuged at 3,000 *g*, 10 minutes again. Cells were re-suspended in 10 ml 0.9% NaCl followed by sonication and vortexed until cells are dispersed. Serial dilutions were prepared and spotted onto TSA plates to determine the CFU/ml.

4.2.5 Effect of *S. maltophilia* whole cells and cell-free supernatants on the biofilm forming ability and pre-formed biofilms of *P. aeruginosa*.

Preparation of cell-free supernatants involved inoculating one or two colonies of *S. maltophilia* strains (668, 747) in 50 mls nutrient broth for 96 hours at 35°C. Cell-free supernatants were collected by centrifuging for 3,000 *g*, 10 minutes and filtering through 0.2 μm pore size filters (Sigma-Aldrich). To assess the interactions between *P. aeruginosa* and *S. maltophilia*, mixed biofilms were prepared by inoculating a cell suspension of 5 x 10^5 CFU/ml of each species at a 1.1 ratio. In addition, a cell suspension of *P. aeruginosa* and an equal volume of cell-free supernatants or concentration of whole cells of *S. maltophilia* were prepared. A quantity of 100 μl of each mixture was added to the microwell plate containing peg lids and incubated for 24 hours at 35ºC to allow biofilms to develop.

To determine the effect of *S. maltophilia* on pre-formed *P. aeruginosa* (PA01, 1826, 4505.2) biofilms, strains were grown on polystyrene pegs for 24 hours at 35°C, as previously described. The lids containing pegs were rinsed three times with sterile water

and added to another plate containing 100 µl of *S. maltophilia* supernatants and whole cells. The untreated *P. aeruginosa* strains and cell-free supernatants were added to the wells as controls. Biofilm quantification was carried out after a further 24 hours, 35°C incubation.

4.2.6 Effect of antibiotics on *P. aeruginosa* biofilm formation and pre-formed biofilms with *S. maltophilia* whole cells and cell-free supernatants.

P. aeruginosa strains were allowed to form mixed biofilms as previously described in **Section 4.2.5**. Two-fold serial dilutions of antibiotics were prepared in nutrient broth at a final concentration of 256 µg/ml. Biofilms were exposed to antibiotics for a further 24 hours, stained with 0.3% crystal violet and the optical density measured at 630nm.

4.2.7 Susceptibility of unusual CF isolates to antibiotic treatment.

Minimum inhibitory concentrations (MIC) of unusual species were carried out by the broth microdilution method as previously described in **Chapter 2, Section 2.2.10**. A quantity of 100 µl sterile nutrient broth was added to the wells of a 96-well round-bottom microtitre plate (Nunc Inc., Roskilde, Denmark). A serial two-fold dilution of tobramycin was prepared in nutrient broth at a final concentration of 256 µg/ml. A 100 µl suspension of each organism was added to the wells at a final concentration of 5 x 10^5 CFU/ml and the microtitre plates were incubated at 35°C for 24 hours. At 24 hour, the contents of non-turbid wells were sub-cultured by streaking 10 µl of suspension onto 5% Columbia sheep blood agar plates, incubated overnight at 35°C and examined for complete inhibition.

4.2.8 Isolation of proteins from *S. maltophilia* isolates.

S. maltophilia isolates were prepared as described in **Section 4.2.5**, centrifuged at 4,000 g, 10 minutes and filter sterilised to collect the cell-free supernatant. A quantity of 15 µl was added to an ultracentrifuge tube (Amicon, Ultra-15 Centrifugal Filter Devices) and centrifuged at 4,000 g for 30 minutes to collect the protein sample. The concentration of each protein sample was determined using the Nanodrop^{TM} 1000.

4.2.9 10% Ammonium persulfate (APS).

APS (10% w/v) was prepared by adding 0.1g APS to 1 ml distilled water. APS (10% w/v) was prepared fresh on the day for use.

4.2.10 10% Sodium dodecyl sulphate (SDS).

SDS (10% w/v) was prepared by adding 10 g to 100 mls distilled water until all the SDS had been solubilised.

4.2.11 10% Running buffer.

The running buffer (10%) was prepared by dissolving 250 mM Tris base, 1.92 M glycine and 1% SDS in 1 litre distilled water. The mixture was adjusted to a pH 8.3 and stored at room temperature. The buffer was adjusted to a 1X concentration in distilled water before use.

4.2.12 SDS-polyacrylamide gel electrophoresis.

Analysis of proteins produced by *S. maltophilia* was carried out using the SDS-page method. The resolving and stacking gels were prepared as described in **Table 4.2**. Following the addition of TEMED and 10% APS the gel was poured immediately as it begins to polymerise rapidly. The prepared resolving gel was poured into the plates, overlaid with water and allowed to set for approximately 30 minutes. Once the gel had polymerised, the water was removed, the stacking gel was added and the combs inserted to allow the wells to form. This was left to set for approximately 45 minutes.

Table 4.2: Materials for preparation of resolving and stacking gel.

Materials:	**Resolving gel**	**Stacking gel**
dH_2O	5.3 ml	3.66 ml
0.3% Bis/Acrylamide	4.3 ml	780 µl
1M Tris/ 2M Tris	3.25 ml 2M Tris	1.25 ml 1M Tris
10% Sodium dodecyl sulfate (SDS)	130 µl	60 µl
N,N,N',N'-Tetramethylethylenediamine (TEMED)	6.5 µl	6 µl
10% (w/v) Ammonium Persulfate (APS)	65 µl	30 µl

Protein samples were prepared by adding 5X Laemmli loading dye to each sample, vortexing and centrifuging. The samples were boiled at 95°C for 5 minutes to denature the proteins and cooled for a further 2 minutes. The electrophoresis rig was prepared and filled with running buffer. The well combs were removed and a quantity of 6 µl of protein

sample was added to each well and 3 µl of Colorburst™ electrophoresis marker (Fermentas, Thermo Scientific). Samples were electrophoresed at 130 volts, 200 mA for approximately 110 minutes. The gel was removed, rinsed in distilled water and stained overnight with 20 ml PageBlue Protein staining solution (Thermo Scientific) followed by rinsing with distilled water until the bands were visible.

4.2.13 Preparation of protein bands for liquid chromatography/mass spectrometry (LC/MS) analysis.

Protein bands of interest were excised from the sds-page gel. The trypsin buffer was prepared by adding ammonium bicarbonate (100 mM) to a solution of 1 ml acetone nitrile and 9 mls water. A quantity of 1.5 ml modified porcine trypsin (Promega) was added to the mixture to give a final concentration of 15 ng/ml trypsin. A quantity of 5 mls acetone nitrile was added to 5 mls 100 mM ammonium bicarbonate and 200 µl was added to the microfuge tubes containing excised gel pieces to be de-stained. Acetone nitrile (200 µl) was then added for 30 minutes. The acetone was removed and 10 µl trypsin buffer was added. The samples were left on ice for 2 hours with frequent checking so that all gel pieces remained submerged in trypsin. The microfuge tubes were then placed in the incubator overnight at 37°C.

To extract the peptide digestion products the contents of the microfuge tubes were centrifuged and transferred into a fresh tube. The extracts were dried in a vaccum centrifuge overnight. Dried peptides were re-suspended in 15 µl of a 0.1% formic acid solution. Analysis of digested peptides was achieved using an Aligent 6340 Ion Trap

Liquid Chromatography/Mass Spectrometry. Data were analysed using MASCOT search engine. Proteins identified from the database are listed.

4.2.14 Statistical analysis.

Statistical analysis was carried out using PASW^{R} Statistics 18 - SPSS. One-way ANOVA or Student's t-test was used to determine statistical variations for most experiments. A $P \leq 0.05$ was considered statistically significant.

4.3 Results

4.3.1 Growth of planktonic cultures of unusual CF species.

The growth rates represented by each bacterial species may influence the response to antimicrobial treatment. Few studies have investigated the growth patterns of the species used in this study. Measurements were based on turbidity following 24 hours, 35°C incubation as shown in **Figure 4.1**. Analysed data showed strong growth capacity of *S. maltophilia* and *Herbaspirillum* species. Growth patterns of seven *Paracoccus* strains were comparable and had the weakest growth rate among the strains tested. Finally, *Ralstonia* sp. and *Acinetobacter* sp. had moderate growth levels. CF strains are showing unique patterns of growth distinguishable from other species.

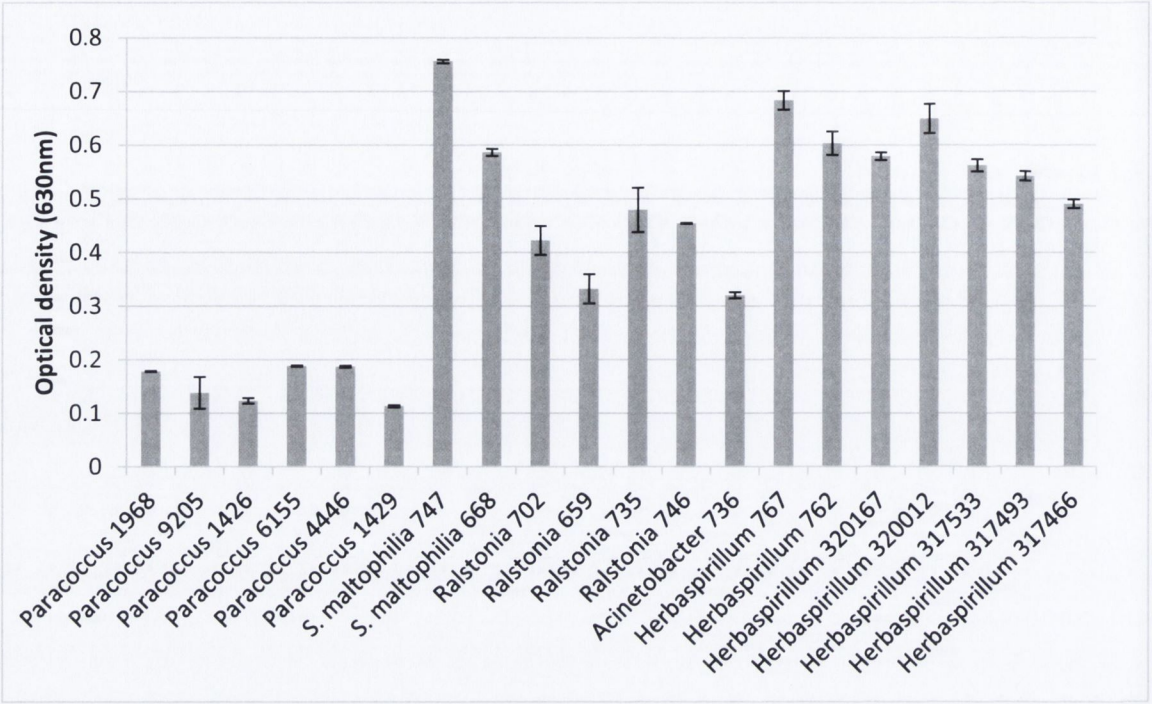
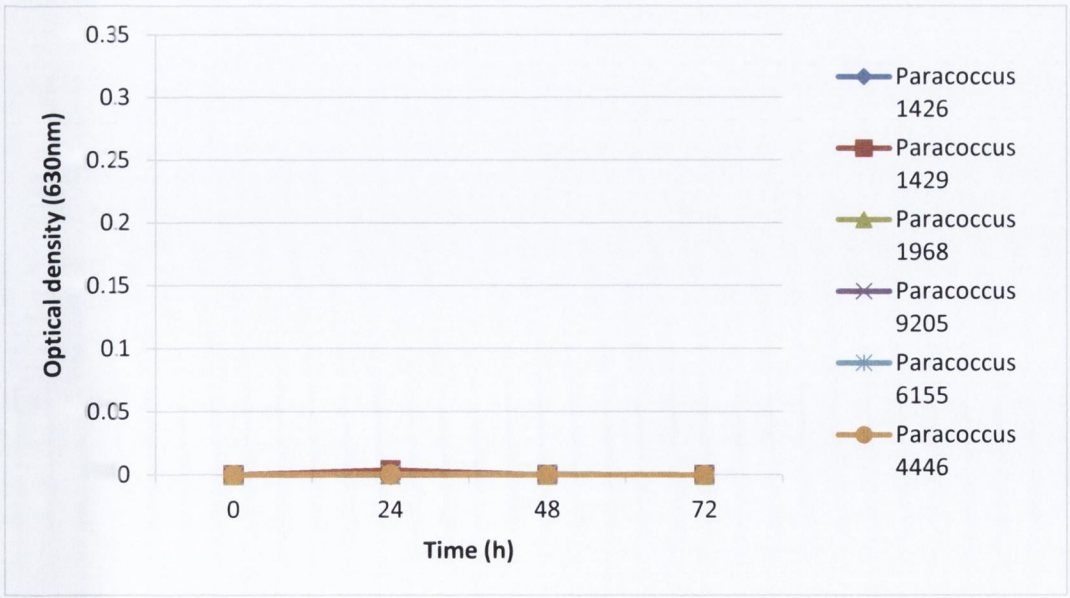


Figure 4.1: Optical density (630nm) of planktonic growth of unusual CF species measured after 24 hours incubation. Results represent the mean ± standard deviation of three independent experiments.

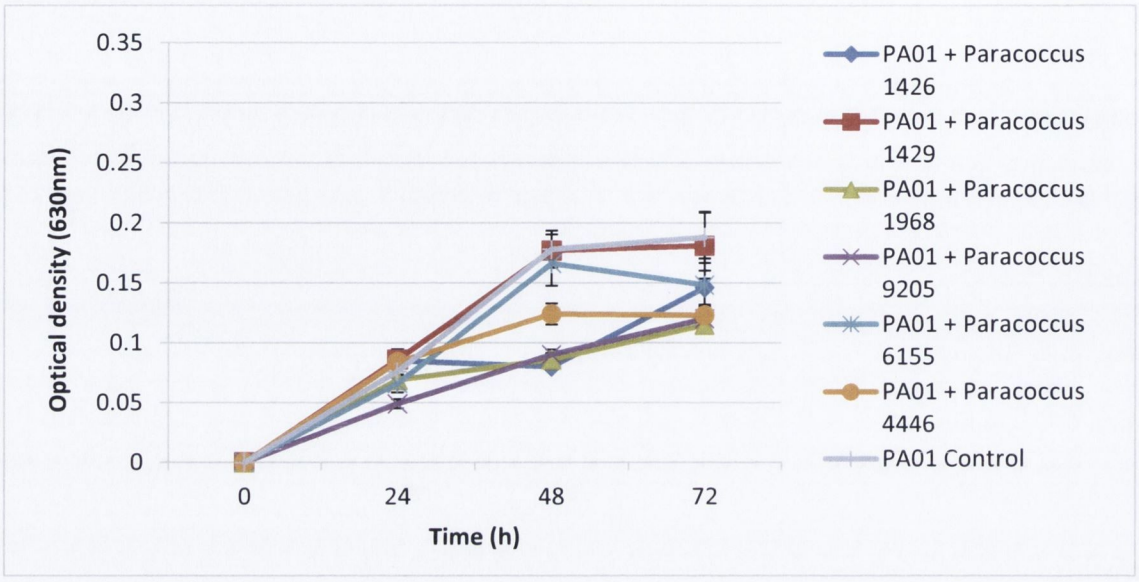
4.3.2 Adherence of single and mixed species of *Paracoccus yeeii* and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.

The sputa of CF patients are frequently assayed for a certain number of bacterial species. There have been reports of unusual bacteria that are rarely isolated from the CF lungs but may be common inhabitants (Rogers *et al.*, 2004). There are limited studies on the virulence factors of *Paracoccus* sp. reported in the literature. The current study found individual species of *P. yeeii* did not form biofilms under the experimental conditions in which OD_{630} values were ≤ 0.05. *P. aeruginosa* isolates which form strong biofilms were inoculated with *Paracoccus* sp. at an equal ratio and found to be comparable to biofilm growth of *P. aeruginosa* PA01. In addition, mixed species *P. aeruginosa* ATCC27853 and *P. yeeii* 1968 exhibited a significant reduction in biofilm growth by 72 hours in comparison to the single strain ($P = 0.003$).

(A)



(B)



(C)

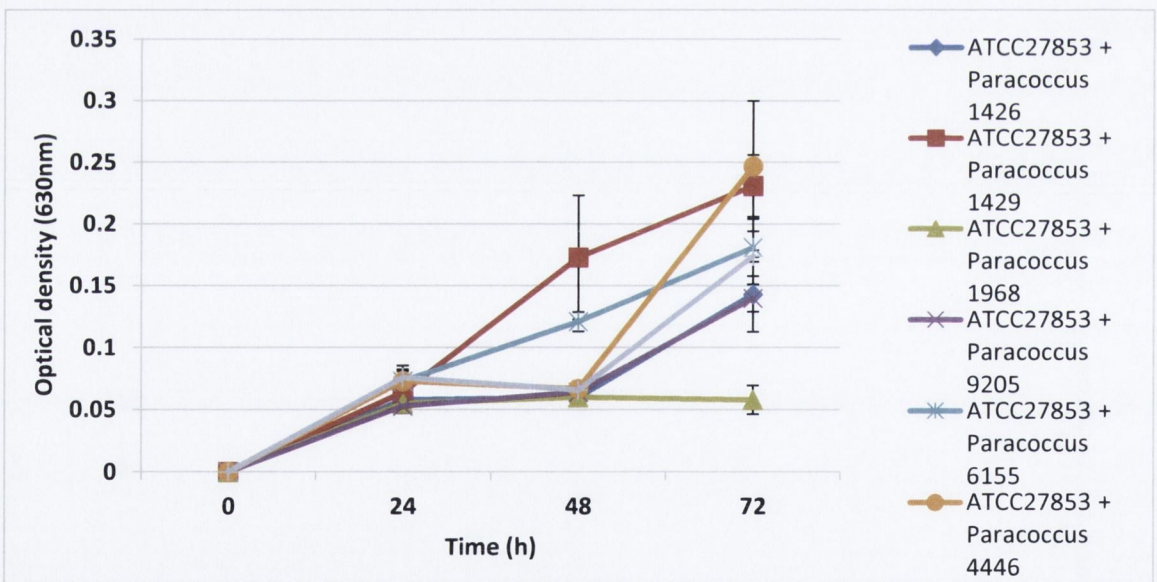


Figure 4.2: Comparison of growth of single and mixed species biofilms with *Paracoccus yeeii*.

(A) *Paracoccus yeeii* controls

(B) *Paracoccus yeeii* + *P. aeruginosa* PA01

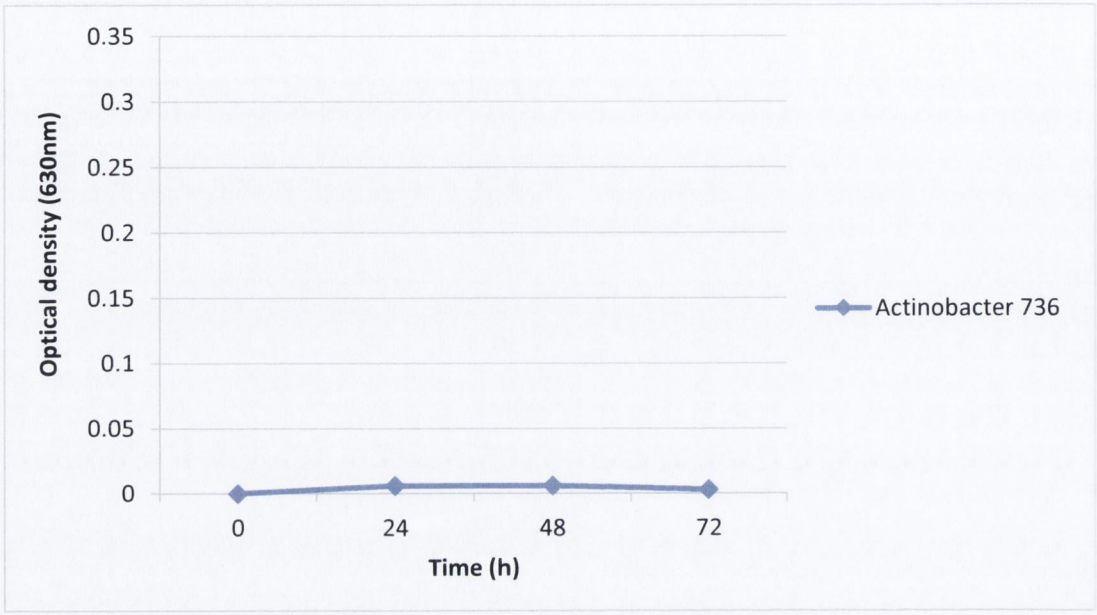
(C) *Paracoccus yeeii* + *P. aeruginosa* ATCC27853

Data represent the mean ± standard deviation of three independent experiments.

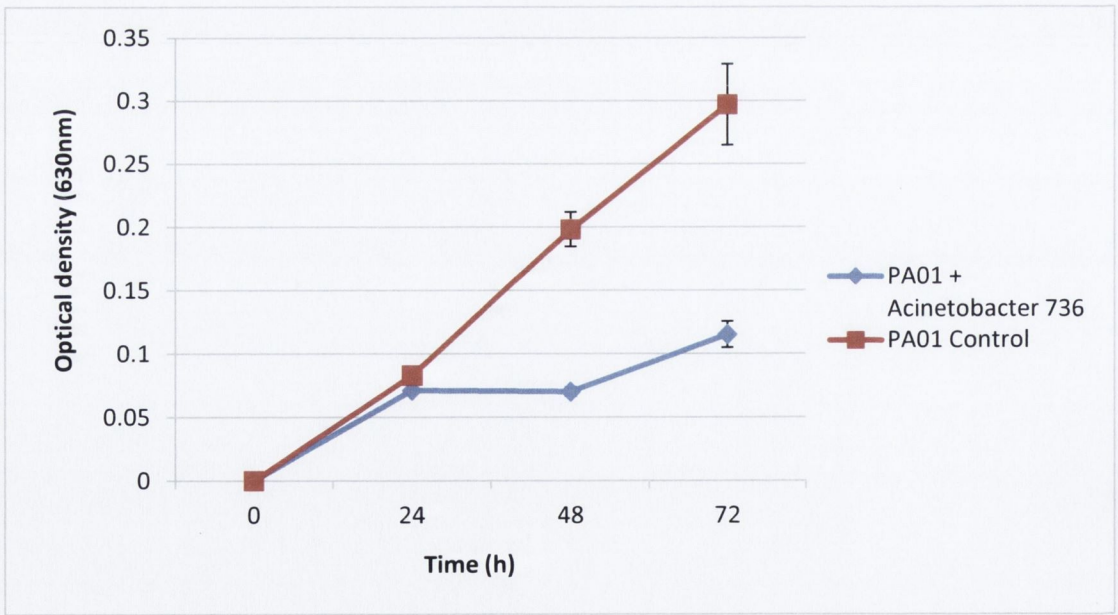
4.3.3 Adherence of single and mixed species of *Acinetobacter* sp. and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.

Acinetobacter sp. has been implicated in a wide variety of nosocomial respiratory infections (Towner *et al.*, 1996). There is evidence to suggest that *Acinetobacter* sp. forms biofilms under static and dynamic conditions and mixed species interactions showed an alteration in the dynamic structure of *P. putida* (Hansen *et al.*, 2007). Quantitative analysis showed that cell attachment of the strain used in the present study was weak with an $OD_{630} \leq 0.003$ following 72 hours incubation. Mixed species biofilms of *P. aeruginosa* and *Acinetobacter* 736 at an equal ratio found the adherence of *P. aeruginosa* PA01 was significantly reduced at 72 hours in combination with this strain ($P = 0.002$). Similarly, *P. aeruginosa* ATCC27853 experienced a significant reduction in biofilm formation by 72 hours when grown in conjunction with the strain. The values shown in **Figure 4.3** are not representative of the quantity of each species in mixed culture.

(A)


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Optical density (630nm)
0
24
48
72
Time (h)
Actinobacter 736

(B)


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Optical density (630nm)
0
24
48
72
Time (h)
PA01 + Acinetobacter 736
PA01 Control

(C)

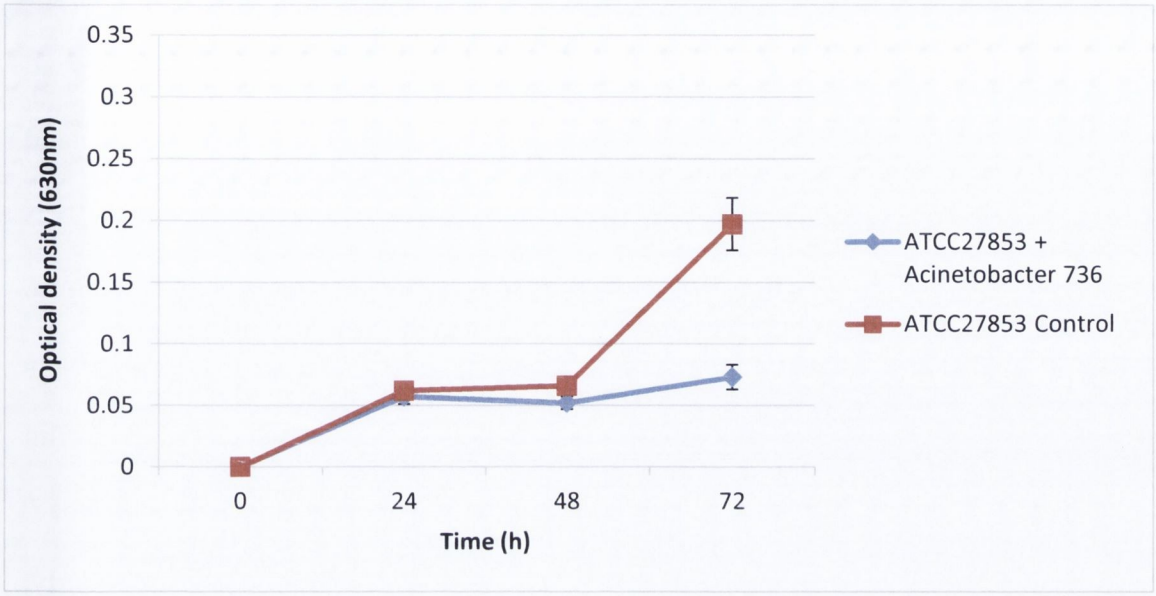


Figure 4.3: Comparison of growth of single and mixed species biofilms with *Acinetobacter* sp.

(A) *Acinetobacter* 736 control

(B) *Acinetobacter* 736 + *P. aeruginosa* PA01

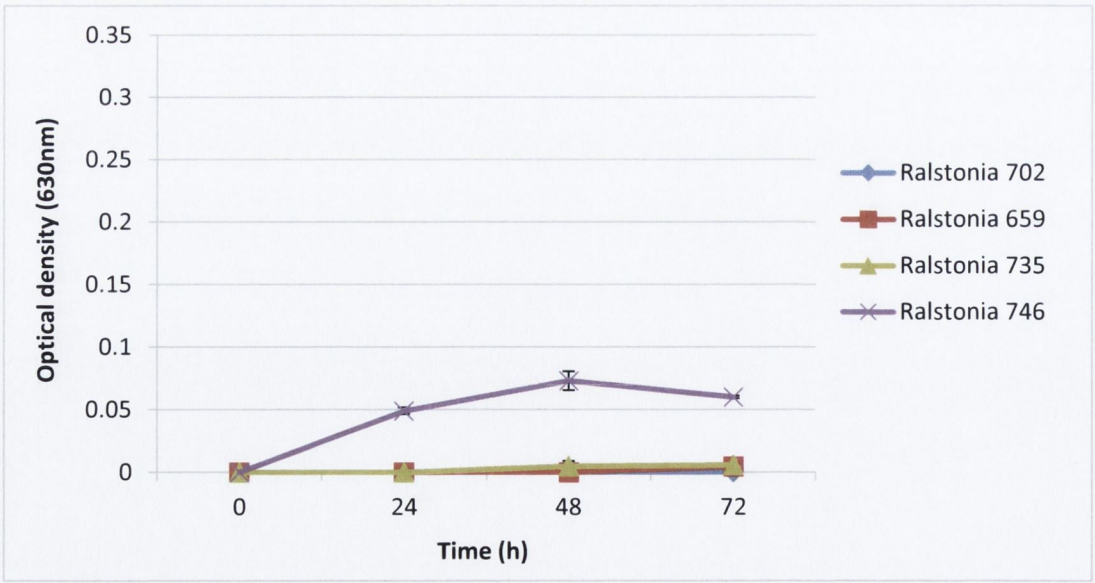
(C) *Acinetobacter* 736 + *P. aeruginosa* ATCC27853

Data represent the mean ± standard deviation of three independent experiments.

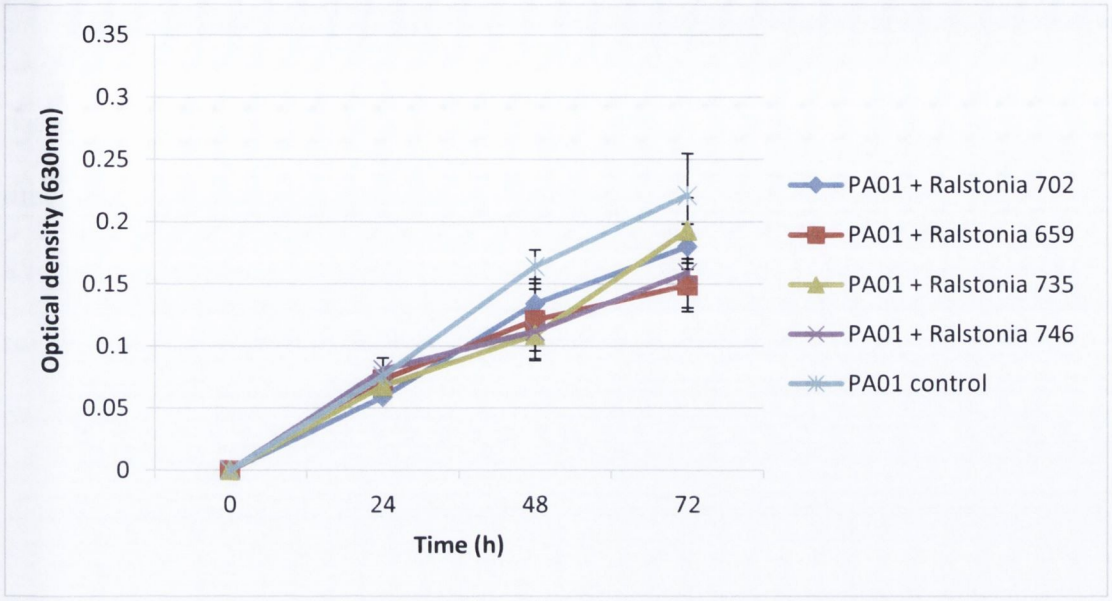
4.3.4 Adherence of single and mixed species of *Ralstonia* sp. and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.

Ralstonia species have been recovered from a variety of clinical samples including blood and sputum. This species has been described as difficult to differentiate and misidentification can often occur with other species of the same genera as well as *Burkholderia* sp. (Coeyne *et al.*, 1999). Individual species of *Ralstonia* did not form strong biofilms under the experimental conditions, with the exception of one isolate *Ralstonia* 746. This strain showed strong adherence at 48 hours and a decrease in biofilm growth at 72 hours at an $OD_{630} \geq 0.05$. There was no difference in biofilm adherence of *P. aeruginosa* PA01 when grown in mixed culture with all four *Ralstonia* species. Similarly, *P. aeruginosa* ATCC27853 showed no difference in the level of adherence at 24 and 72 hours when combined with these strains.

(A)



(B)



(C)

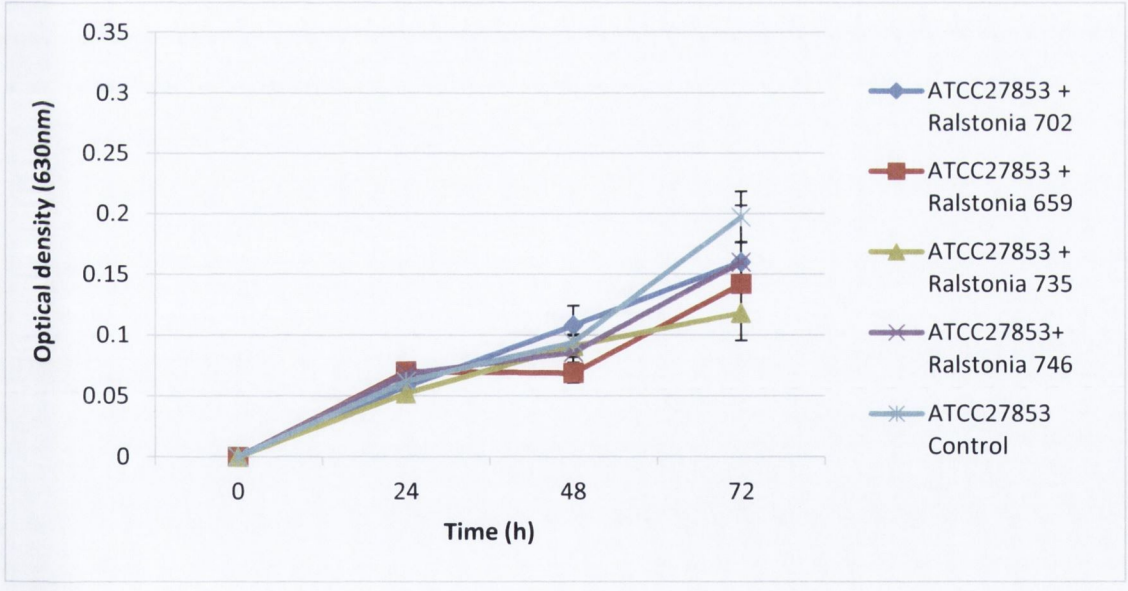


Figure 4.4: Comparison of growth of single and mixed species biofilms of *Ralstonia* sp.

(A) *Ralstonia* sp. controls

(B) *Ralstonia* sp. + *P. aeruginosa* PA01

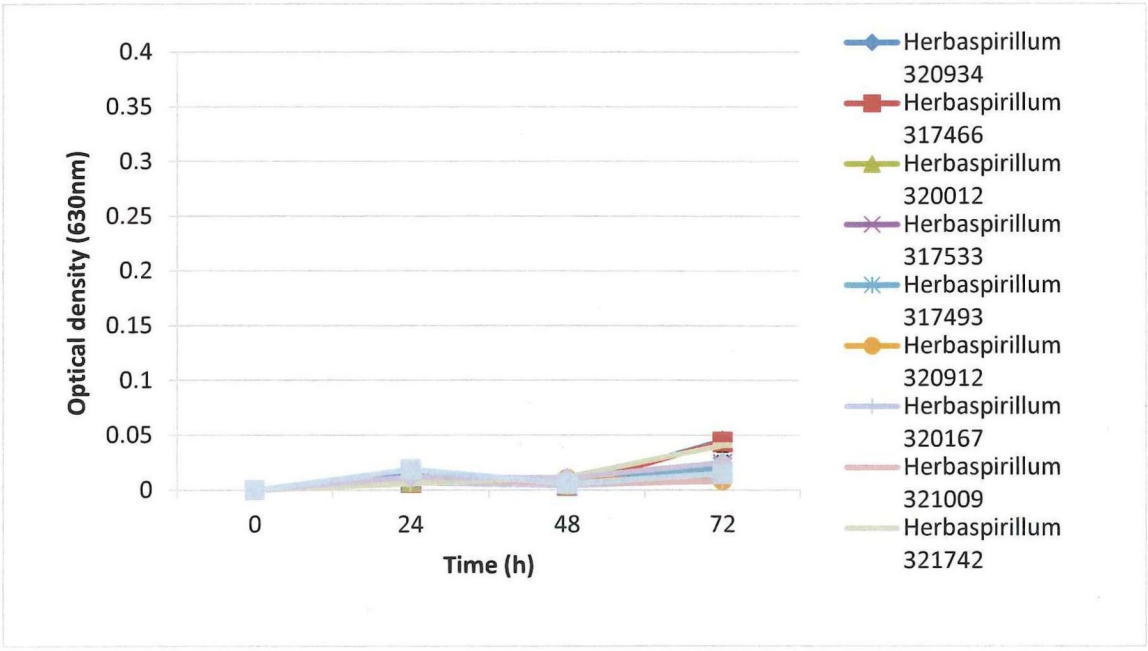
(C) *Ralstonia* sp. + *P. aeruginosa* ATCC27853

Data represent the mean ± standard deviation of three independent experiments.

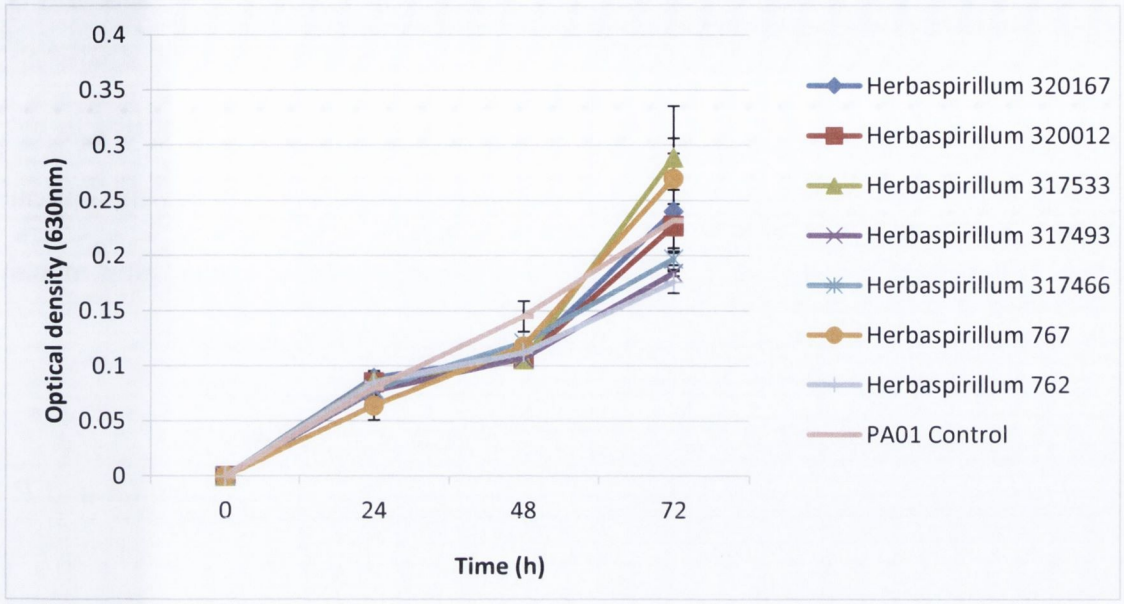
4.3.5 Adherence of single and mixed species of *Herbaspirillum* sp. and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.

Herbaspirillum strains were generally not strong biofilm formers as growth did not reach an $OD_{630} \geq 0.05$, under the conditions tested. Selected isolates grown in conjunction with *P. aeruginosa* PA01 did not alter the normal pattern of biofilm growth following 72 hours incubation. The study demonstrated that mixed biofilm growth of *P. aeruginosa* ATCC27853 and *Herbaspirillum* isolates was also comparable to the *P. aeruginosa* ATCC27853 control.

(A)



(B)



(C)

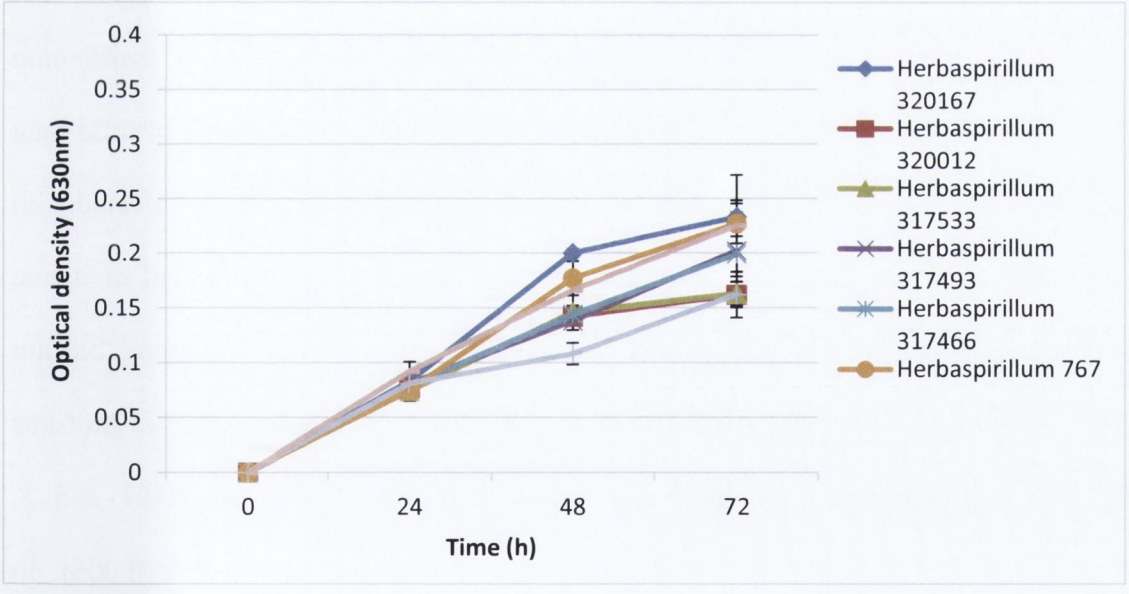


Figure 4.5: Comparison of growth of single and mixed species biofilms with *Herbaspirillum* sp.

(A) *Herbaspirillum* sp. controls

(B) *Herbaspirillum* sp. + *P. aeruginosa* PA01

(C) *Herbaspirillum* sp. + *P. aeruginosa* ATCC27853

Data represent the mean ± standard deviation of three independent experiments.

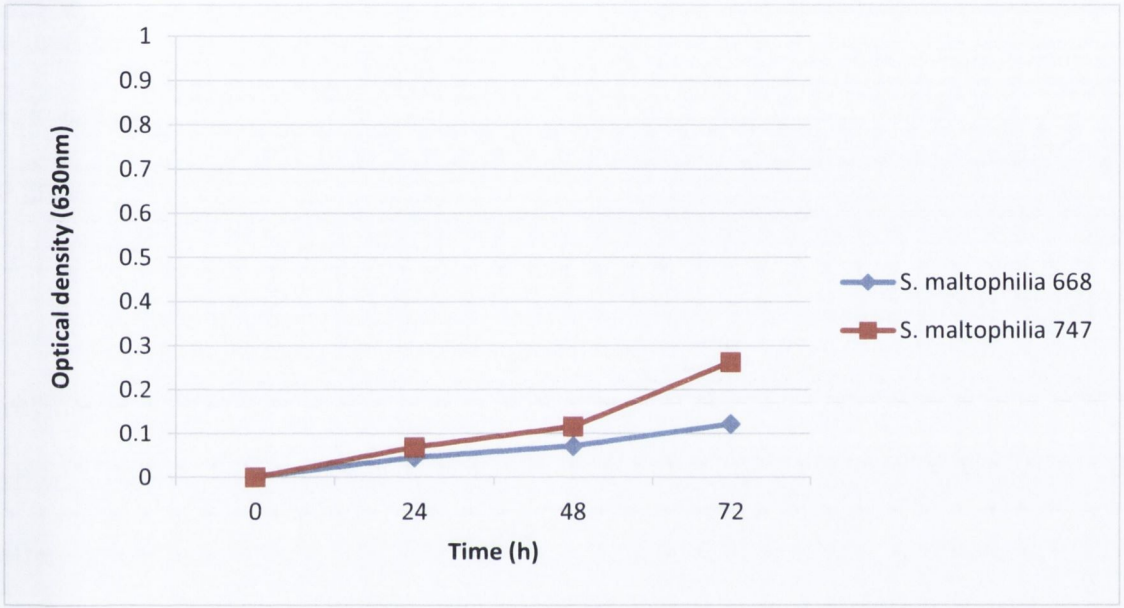
The *in vitro* model for studying biofilms formed on polystyrene pegs has many disadvantages for mixed cultures. The quantity of each species colonising the surface of the pegs or any structural changes in biofilm morphology could not be identified in the current study. As a result, a comparative analysis was carried out between the optical densities obtained from this assay and total viable counts of several interesting mixed biofilm cultures as described in **Sections 4.2.4** and **4.2.5**.

4.3.6 Adherence of single and mixed species of *Stenotrophomonas maltophilia* and *P. aeruginosa* (PA01 and ATCC27853) to polystyrene pegs.

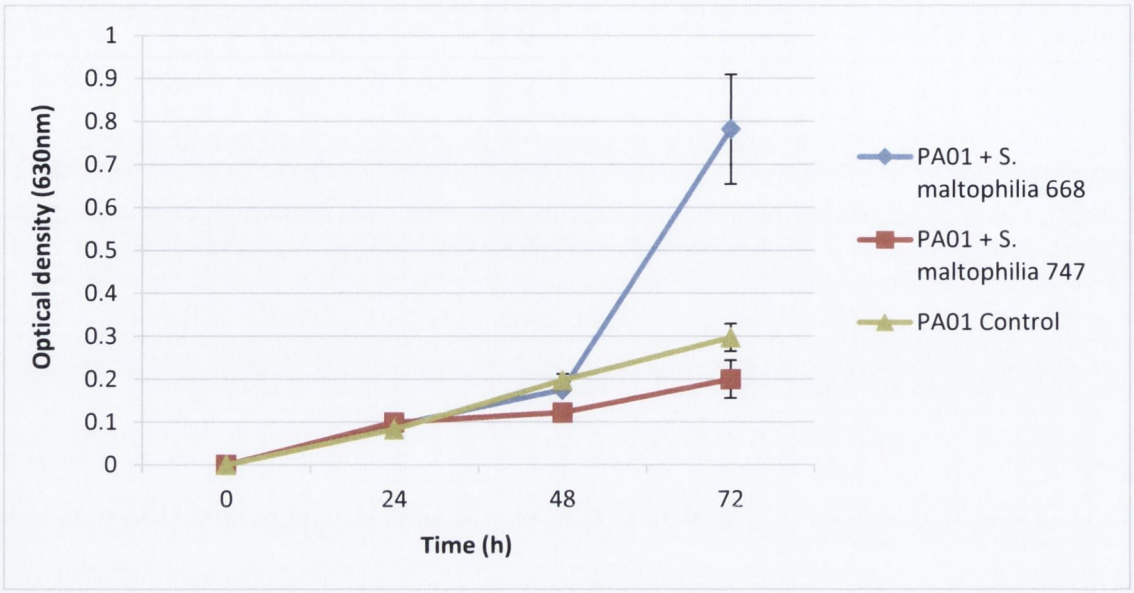
Stenotrophomonas maltophilia is an emerging nosocomial bacterial pathogen and has shown to form complex biofilm structures (Waters *et al.*, 2007; Pompilio *et al.*, 2010) although very little is known about the mechanisms of biofilm formation. *S. maltophilia* is frequently cultured from sputum of CF patients. There have been reports of mild lung function decline with this species, however, patients tend to be older and co-infected with *P. aeruginosa* (Goss *et al.*, 2002). The isolates used in the current study had moderate levels of adherence at 24 hours showing evidence of progression to strong biofilm formation at 72 hours on polystyrene pegs with $OD_{630} \geq 0.05$. Mixed species biofilms showed a significant increase in OD_{630} values when *P. aeruginosa* PA01 and *S. maltophilia* 668 were combined in comparison to the single strains ($P = 0.006$). In addition, mixed biofilm cultures of *S. maltophilia* (668, 747) and *P. aeruginosa* ATCC27853 displayed strong adherence to polystyrene pegs with $OD_{630} \geq 0.05$, with an increase in OD_{630} at 48 and 72 hours ($P = 0.001$, $P = 0.017$). Results are consistent with other studies that have identified these species to adhere to abiotic surfaces (deOliverira-

Garcia *et al.*, 2003), however, it remains unclear if *P. aeruginosa* and *S. maltophilia* are mixing or if one isolate is dominant.

(A)



(B)



(C)

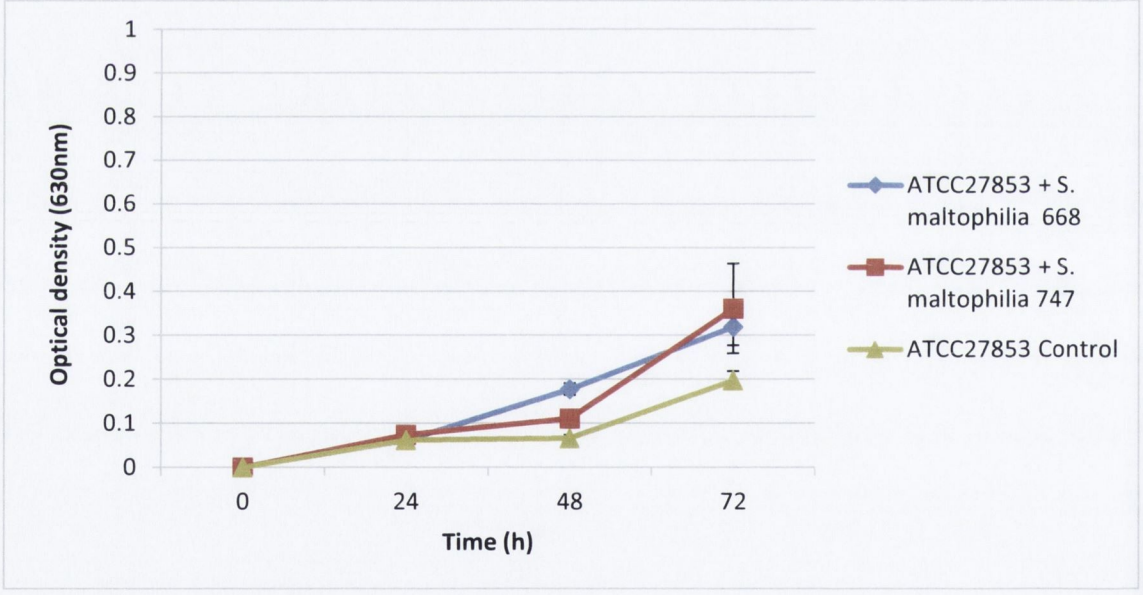


Figure 4.6: Comparison of growth of single and mixed species biofilms with *Stenotrophomonas maltophilia.*

(A) *S. maltophilia* controls

(B) *S. maltophilia* + *P. aeruginosa* PA01

(C) *S.maltophilia* + *P. aeruginosa* ATCC27853

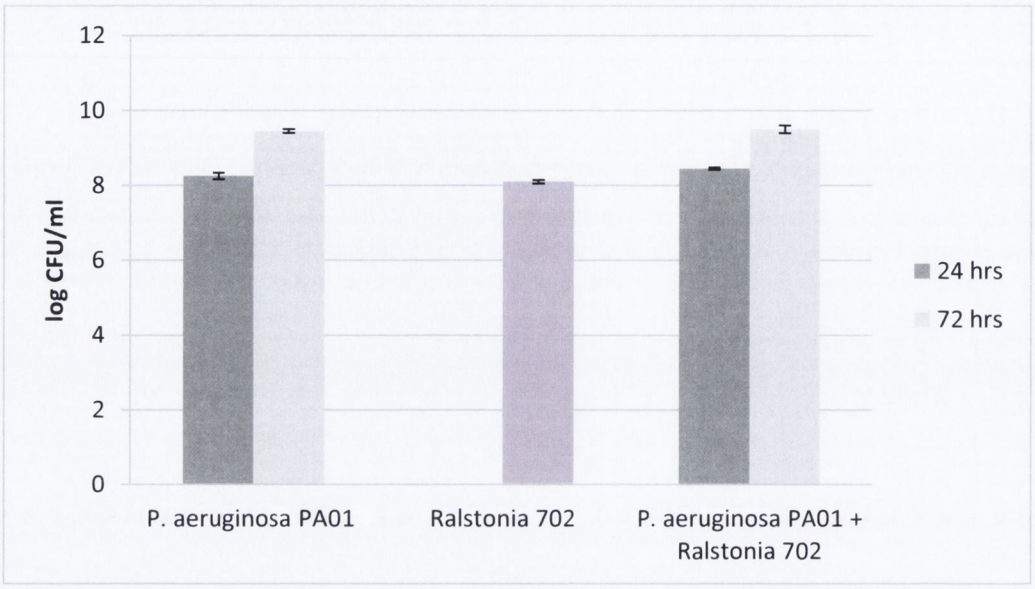
Data represent the mean ± standard deviation of three independent experiments.

4.3.7 Viability of *P. aeruginosa* PA01 biofilms in conjunction with unusual CF strains.

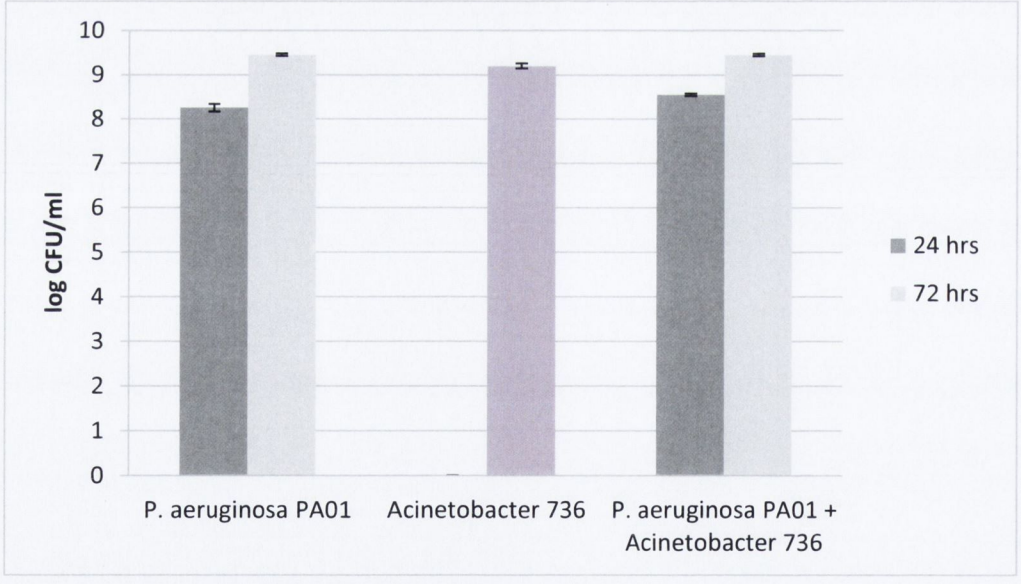
Biofilm growth of selected unusual CF strains was monitored at at 24 and 72 hours. No biofilm growth was observed for *Ralstonia* 702 and *Paracoccus* 1968 which is in agreement with the previous assay. *Acinetobacter* 736 established a stable biofilm following 72 hours incubation. In keeping with biofilms formed on polystyrene pegs, *S. maltophilia* exhibited greater biomass than the other species at 24 hours with an increase in growth at 72 hours.

Results presented in **Figure 4.7** show biofilms consisting of two species differed to the previous assay for isolates *Ralstonia* 702, *P. yeeii* 1968 and *Acinetobacter* 736 in which growth rates seemed to be identical to the *P. aeruginosa* PA01 control. No observable difference to the growth of *P. aeruginosa* PA01 was detected with *S. maltophilia* 747. Mixed growth of *P. aeruginosa* PA01 and *S. maltophilia* 668 resembled biofilm growth and colony morphology of the *S. maltophilia* 668 control. This affect was seen at 24 hours and maintained until 72 hours. Results suggest that *S. maltophilia* may out-compete certain strains of *P. aeruginosa* for nutritional requirements and dominate mixed cultures in certain *in vitro* models.

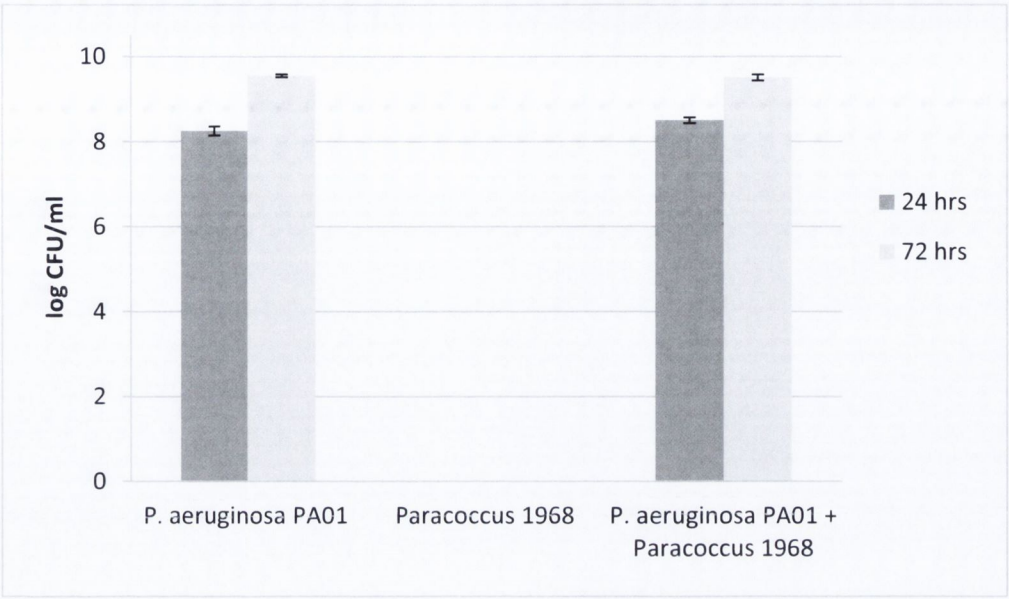
(A)


12
10
8
6
4
2
0
log CFU/ml
24 hrs
72 hrs
P. aeruginosa PA01
Ralstonia 702
P. aeruginosa PA01 + Ralstonia 702

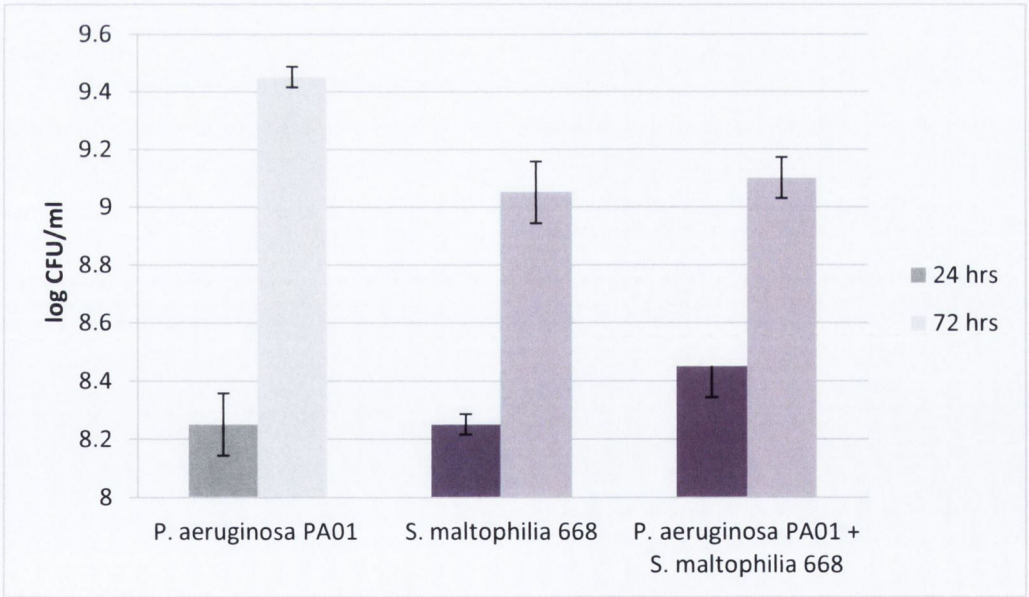
(B)


10
9
8
7
6
5
4
3
2
1
0
log CFU/ml
24 hrs
72 hrs
P. aeruginosa PA01
Acinetobacter 736
P. aeruginosa PA01 + Acinetobacter 736

(C)


10
8
6
4
2
0
log CFU/ml
24 hrs
72 hrs
P. aeruginosa PA01
Paracoccus 1968
P. aeruginosa PA01 + Paracoccus 1968

(D)


9.6
9.4
9.2
9
8.8
8.6
8.4
8.2
8
log CFU/ml
24 hrs
72 hrs
P. aeruginosa PA01
S. maltophilia 668
P. aeruginosa PA01 + S. maltophilia 668

(E)

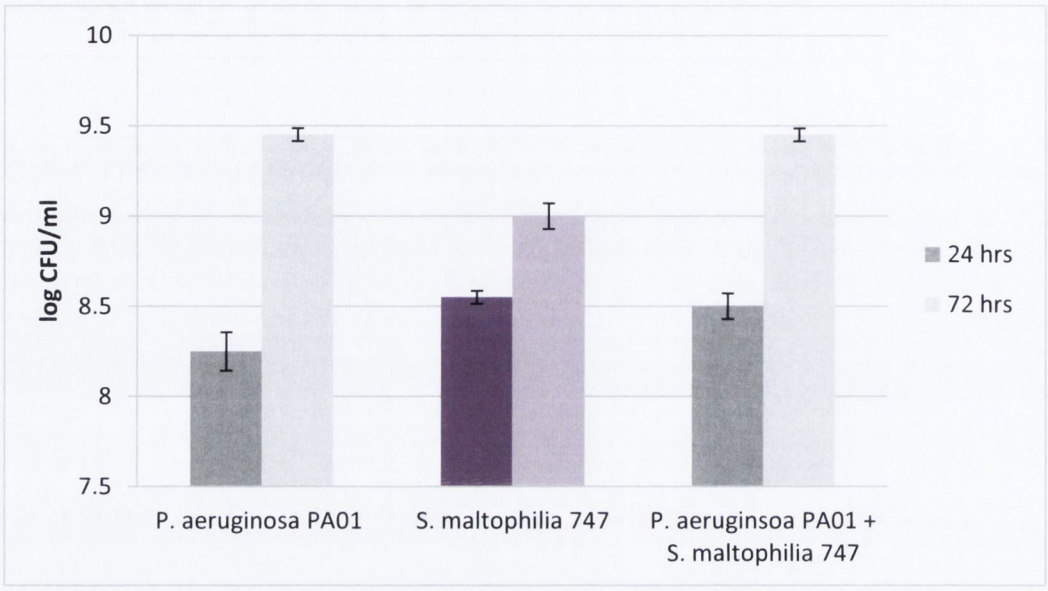
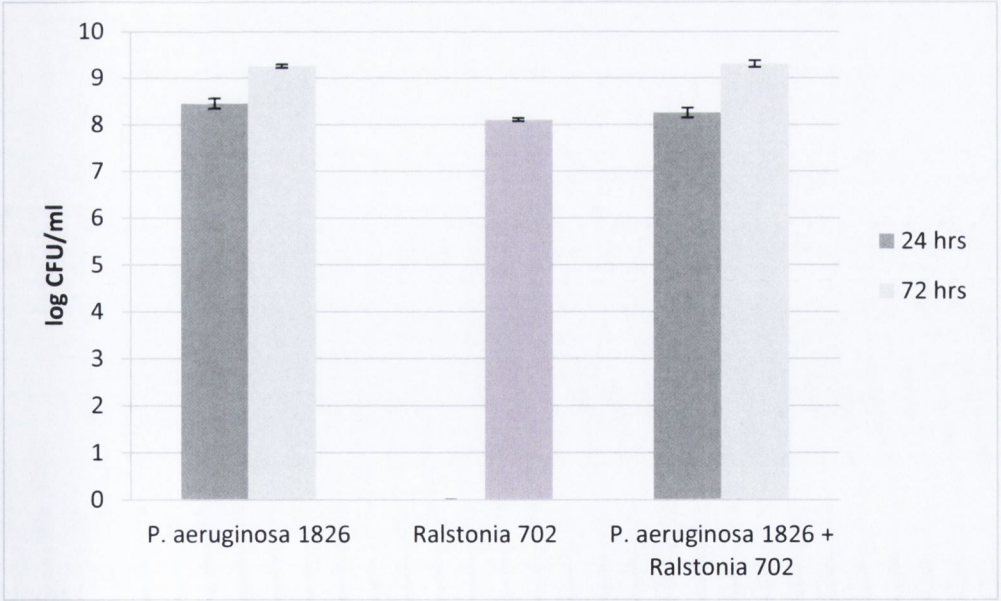


Figure 4.7: Effect of unusual CF strains on the biofilm growth of *P. aeruginosa* PA01 measured as log CFU/ml. Results are based on different colony morphologies on TSA. The controls represent samples that are not in mixed culture. As a single species, *Paracoccus* 1968 did not show any biofilm formation. Results represent the mean ± standard deviation of three independent experiments.

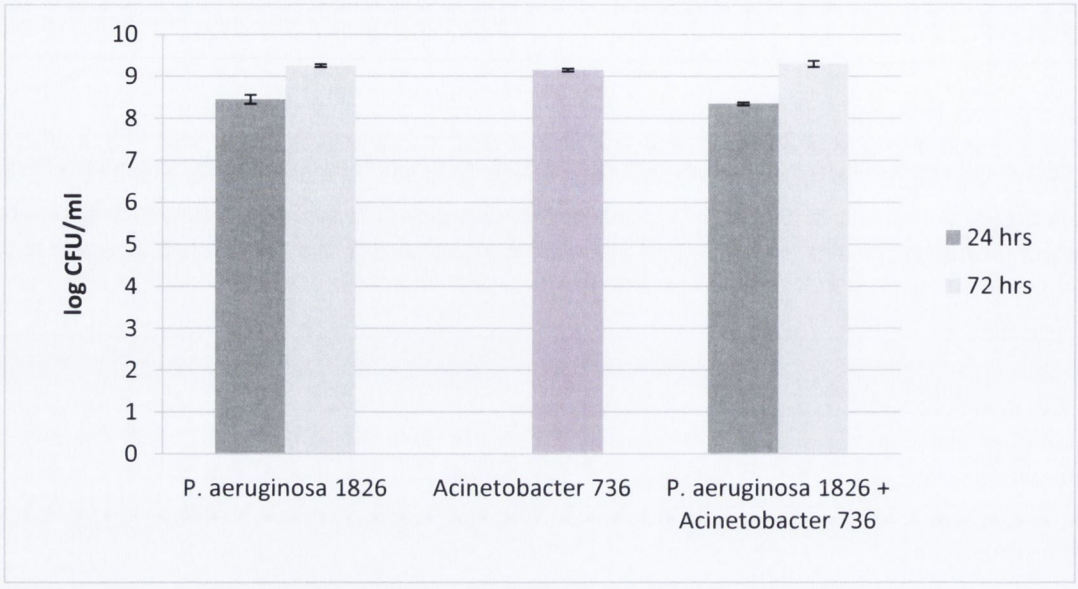
4.3.8 Viability of clinical isolate, *P. aeruginosa* 1826 biofilms in conjunction with unusual CF strains.

Mixed species biofilm development was assessed with clinical isolate *P. aeruginosa* 1826 as there is a possibility that clinical samples may interact with CF isolates in a different manner to control strains. The results presented in **Figure 4.8** differed with previous experiments. *P. aeruginosa* 1826 represented the dominant pathogen in mixed culture with the other species. The study also found that *P. aeruginosa* 1826 was the only viable isolate after 24 and 72 hours incubation with *S. maltophilia*. Findings suggest that clinical *P. aeruginosa* isolates may persist in mixed-culture biofilms with other CF pathogens.

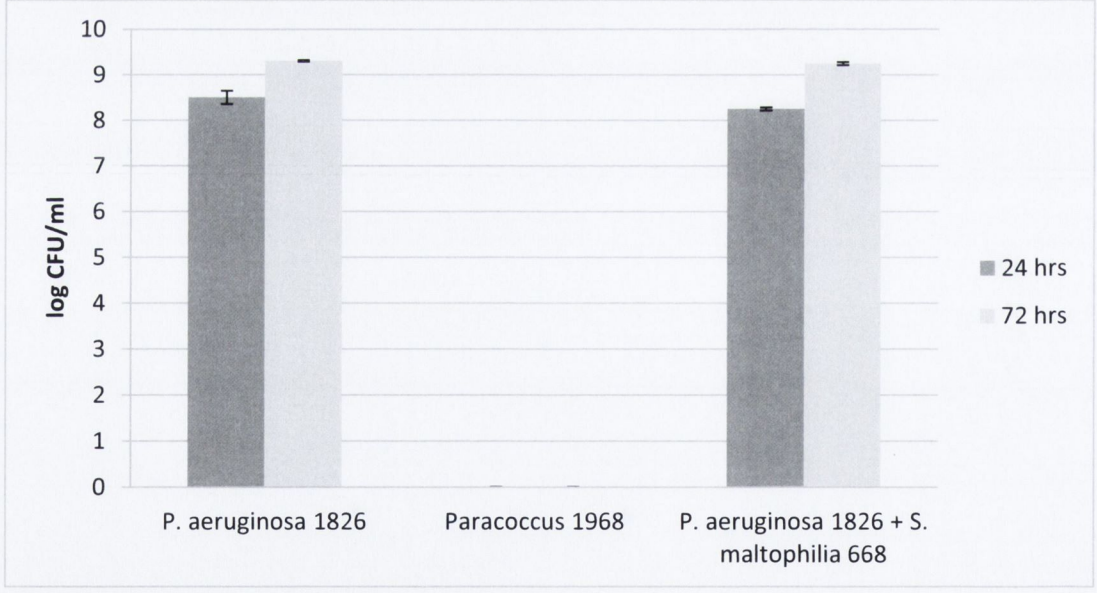
(A)



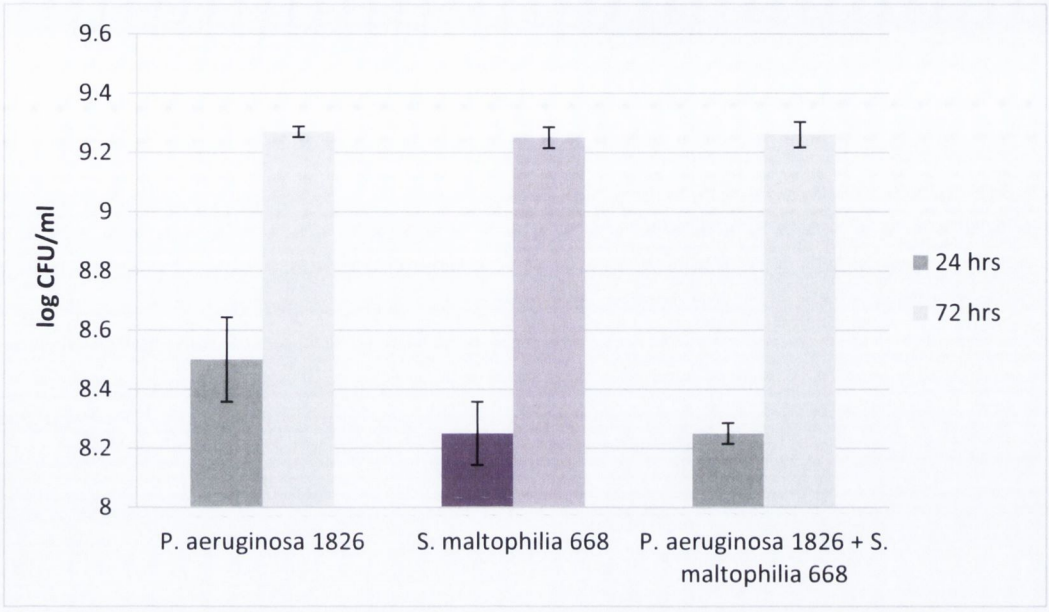
(B)


log CFU/ml
10
9
8
7
6
5
4
3
2
1
0
24 hrs
72 hrs
P. aeruginosa 1826
Acinetobacter 736
P. aeruginosa 1826 + Acinetobacter 736

(C)


log CFU/ml
10
9
8
7
6
5
4
3
2
1
0
24 hrs
72 hrs
P. aeruginosa 1826
Paracoccus 1968
P. aeruginosa 1826 + S. maltophilia 668

(D)



(E)

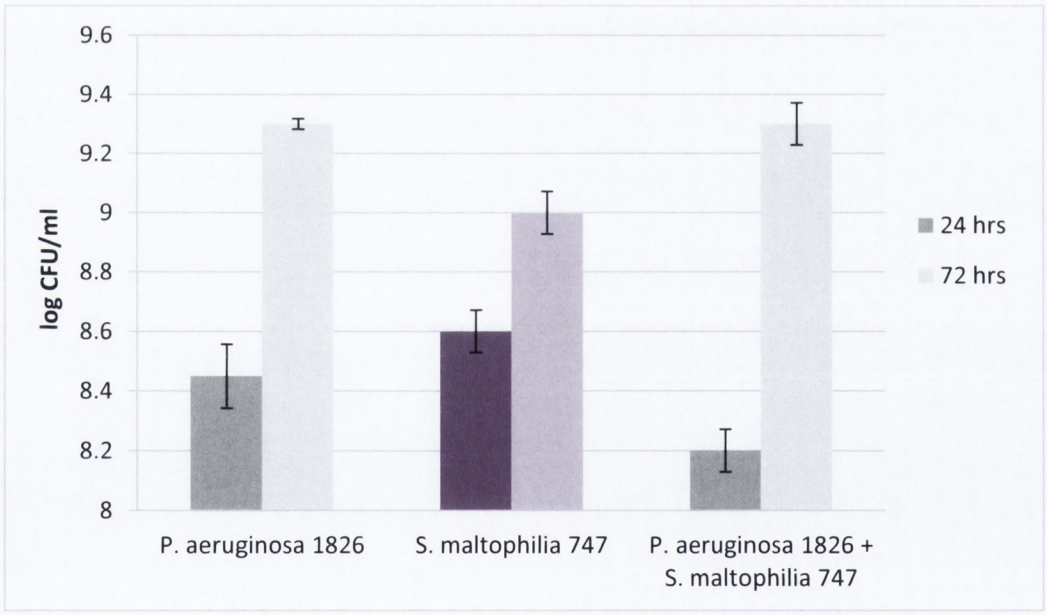


Figure 4.8: Effect of unusual CF strains on the biofilm growth of *P. aeruginosa* 1826 measured as log CFU/ml. Results are based on different colony morphologies on TSA. The controls represent samples that are not in mixed culture. *Paracoccus* 1968 did not develop any biofilm at 24 or 72 hours. Results represent the mean ± standard deviation of

three independent experiments.

4.3.9 Effect of *S. maltophilia* cell-free supernatants and whole cells on *P. aeruginosa* biofilm development.

To examine the effect of *S. maltophilia* on the ability of *P. aeruginosa* to form biofilms two assays were carried out. *P. aeruginosa* and *S. maltophilia* cell-free supernatants or whole cells were grown together at an equal (1:1) ratio. In addition, *P. aeruginosa* biofilms were established for 24 hours and exposed to *S. maltophilia* culture supernatants or whole cells for an additional 24 hours to investigate any biofilm impairment during early developmental stages. Pre-grown *P. aeruginosa* (PA01 and 4505.2) biofilms treated with *S. maltophilia* supernatants showed a decrease in growth compared to the untreated control. These results did not reach statistical significance, with the exception of two isolates, *P. aeruginosa* PA01 ($P = 0.032$) and clinical isolate *P. aeruginosa* 4505.2 ($P = 0.036$). Since *P. aeruginosa* 4505.2 produced substantially more biofilm than the other strains as quantified by crystal violet staining the results suggest that the effect of the *S. maltophilia* supernatant does not depend on the level of biofilm produced.

Upon co-culturing *P. aeruginosa* and *S. maltophilia* at an equal (1:1) ratio, the results revealed *S. maltophilia* cell-free supernatants and whole cells did not promote or decrease *P. aeruginosa* growth in most cases. Virulence factors produced by *P. aeruginosa* such as phenazines have shown to possess toxic effects towards other bacterial species (Kerr *et al.*, 1999). This observation suggests *P. aeruginosa* may be inhibitory towards the other species, however, it remains unclear if changes occur during structural development of *P.*

aeruginosa biofilms with this species. The strains selected for the study secrete pigments which may be enabling *P. aeruginosa* to persist in this multi-species environment.

Table 4.3: Summary of phenotypic characteristics of selected *P. aeruginosa* reference and clinical isolates.

***P. aeruginosa* strain**	**Pigment/No pigment**	**Mucoid/Non-mucoid**
P. aeruginosa PA01	pigment	non-mucoid
P. aeruginosa 1826	pigment	non-mucoid
P. aeruginosa 4505.2	pigment	mucoid

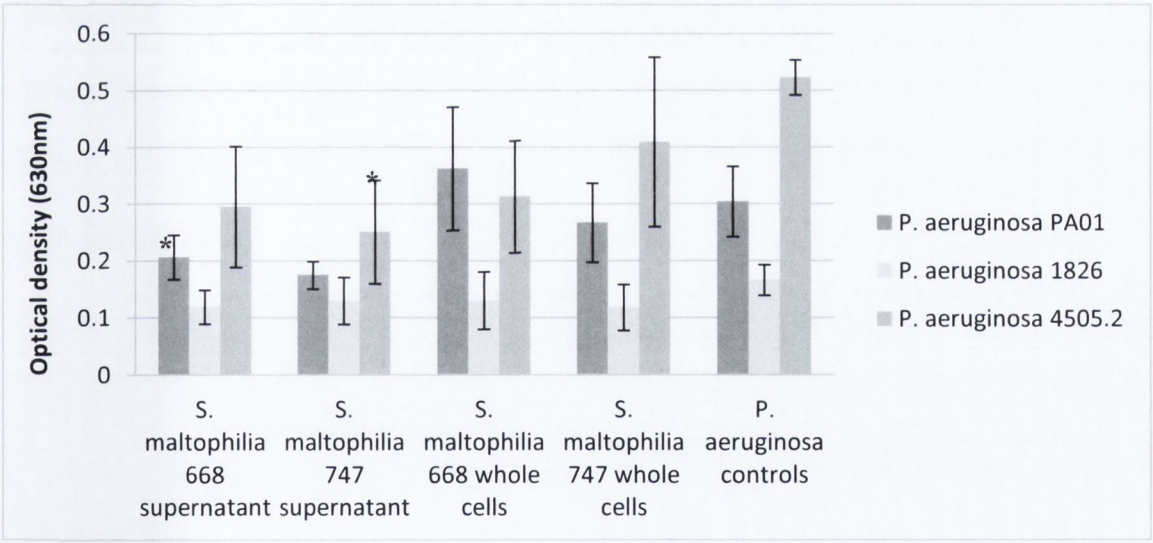
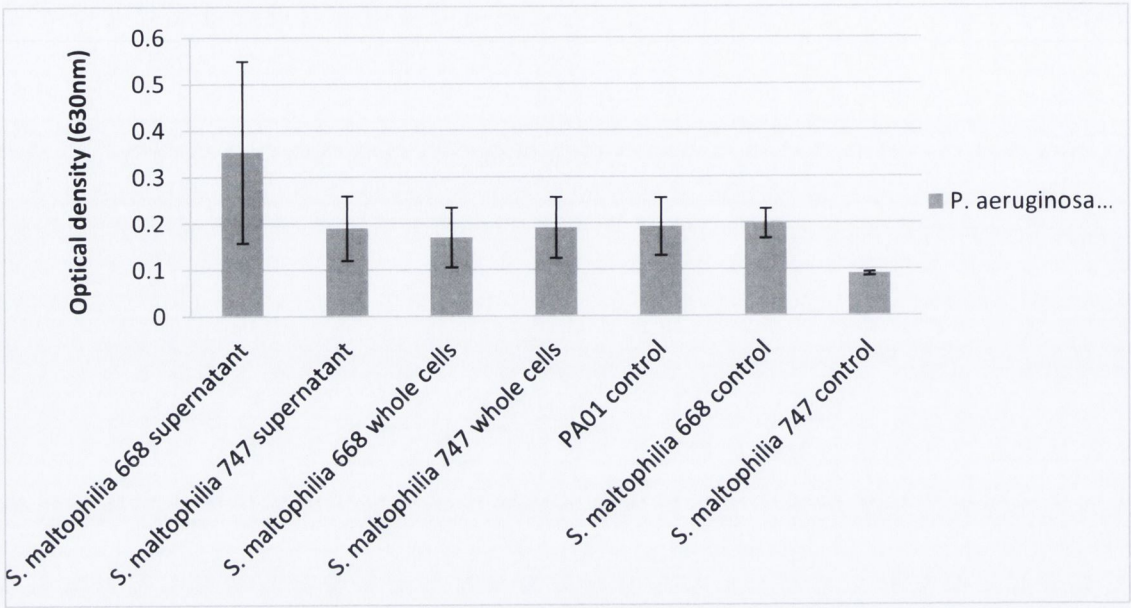
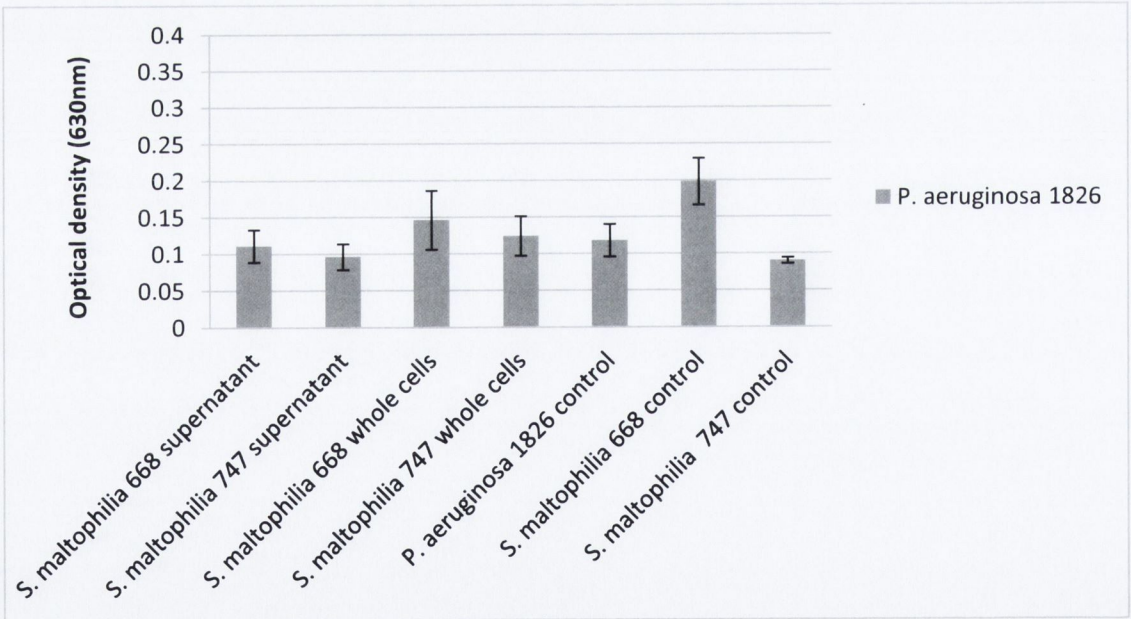


Figure 4.9: The effect of *S. maltophilia* supernatants or whole cells on established biofilms of *P. aeruginosa*. After 24 hours of growth, *P. aeruginosa* biofilms were exposed to *S. maltophilia* for an additional 24 hours. Values represent the mean ± standard deviation of three independent experiments.

(A) *P. aeruginosa* PA01



(B) *P. aeruginosa* 1826



(C) *P. aeruginosa* 4505.2

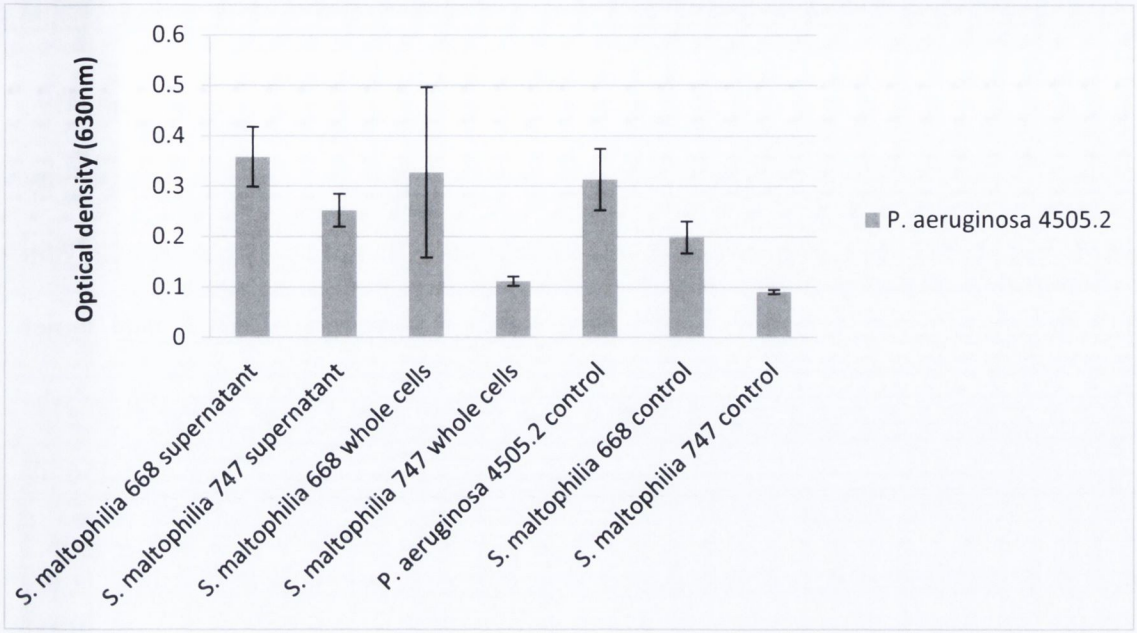


Figure 4.10: Mixed species biofilms of *P. aeruginosa* with *S. maltophilia* supernatants and whole cells. Bacteria were inoculated at a 1:1 ratio and incubated for 24 hours, 35°C. Control values represents biofilm formation of each single species. Values represent the mean ± standard deviation of three independent experiments.

4.3.10 Effect of antibiotics on the growth of mixed-culture biofilms of *P. aeruginosa* and *S. maltophilia.*

The aim of this study was to determine the antibiotic sensitivity of *P. aeruginosa* and *S. maltophilia* isolates grown singly and in co-culture. To assess antibiotic sensitivity biofilms were grown as described in **Section 4.2.5**, treated with antibiotics (tobramycin and ceftazidime) for a further 24 hours and biomass was quantified by crystal violet staining. The effect of the antibiotics against *P. aeruginosa* PA01 in mixed culture was comparable to response of each single species at concentrations ranging from 8 to 256 µl. The activity of tobramycin against mixed cultures of clinical isolate *P. aeruginosa* 4505.2 and *S. maltophilia* 668 strains showed that the mixed culture was more sensitive to lower concentrations of tobramycin (one-way ANOVA). The results indicate that *S. maltophilia* may be increasing the sensitivity of *P. aeruginosa* 4505.2 to this antibiotic.

To further monitor if *P. aeruginosa* biofilms treated with *S. maltophilia* cell-free supernatants were more susceptible to antibiotic exposure, biofilms were developed as described in **Section 4.2.5**. *P. aeruginosa* PA01 treated with the supernatant of *S. maltophilia* 668 responded more effectively to ceftazidime treatment than the *P. aeruginosa* PA01 control. The susceptibility of *P. aeruginosa* PA01 treated with *S. maltophilia* 668 and 747 supernatants to tobramycin was comparable to the *P. aeruginosa* PA01 contol. When clinical isolate *P. aeruginosa* 4505.2 was treated with *S. maltophilia* (668, 747) under the same conditions, a slight increase in sensitivity to tobramycin of *P. aeruginosa* 4505.2 treated with *S. maltophilia* 747 supernatant was observed. Similarly, an increase in sensitivity was detected when *P. aeruginosa* 4505.2 exposed to *S.*

maltophilia 668 supernatant was treated with ceftazidime (student's t-test). Findings indicate the supernatants of *S. maltophilia* isolates used in the current study are rendering *P. aeruginosa* 4505.2 more suscepetible to antibiotic treatment.

Mixed species biofilms of *P. aeruginosa* (PA01, 4505.2) and *S. maltophilia* 668

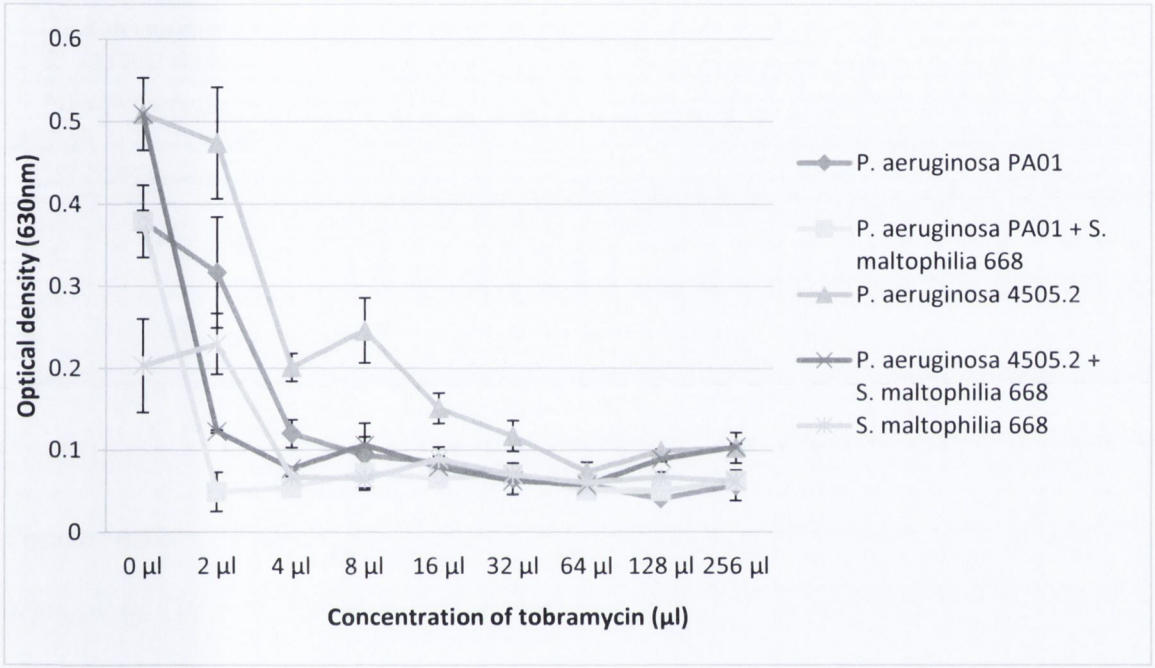
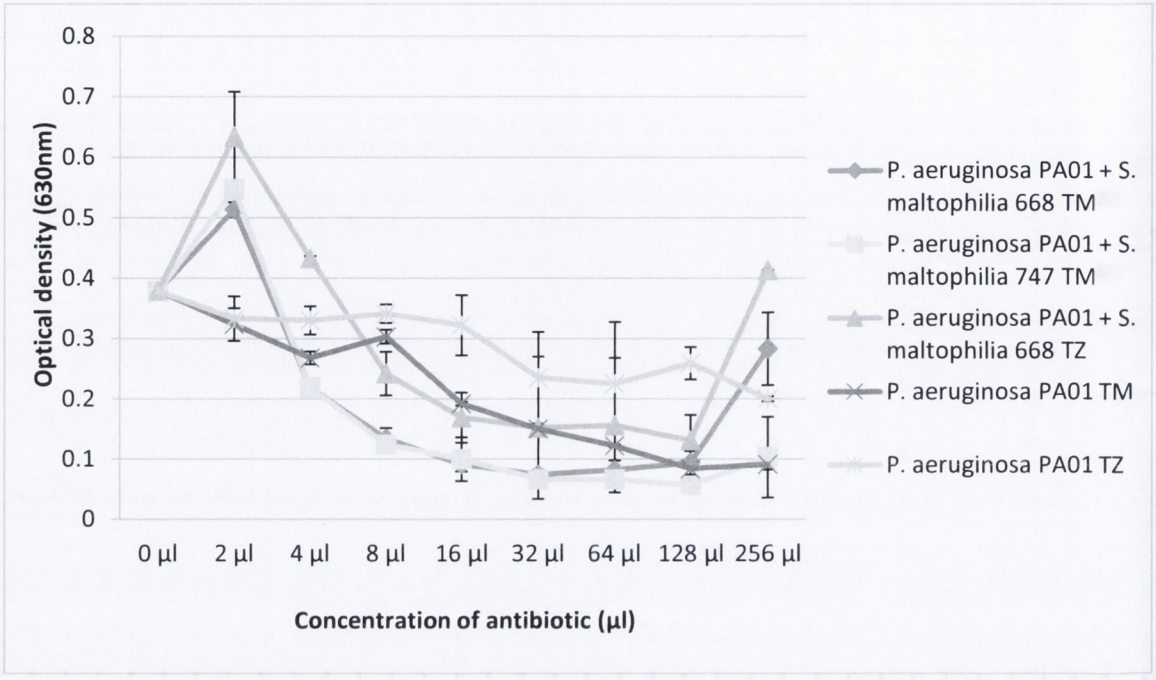


Figure 4.11: The effect of antibiotics (tobramycin) against mixed species biofilms. Mixed species biofilms of *P. aeruginosa* and *S. maltophilia* were grown as described in **Section 4.2.5**. Mixed and single species cultures were exposed to various concentrations of tobramycin for an additional 24 hours. Results represent the average of two independent experiments.

(A) *P. aeruginosa* PA01



(B) *P. aeruginosa* 4505.2

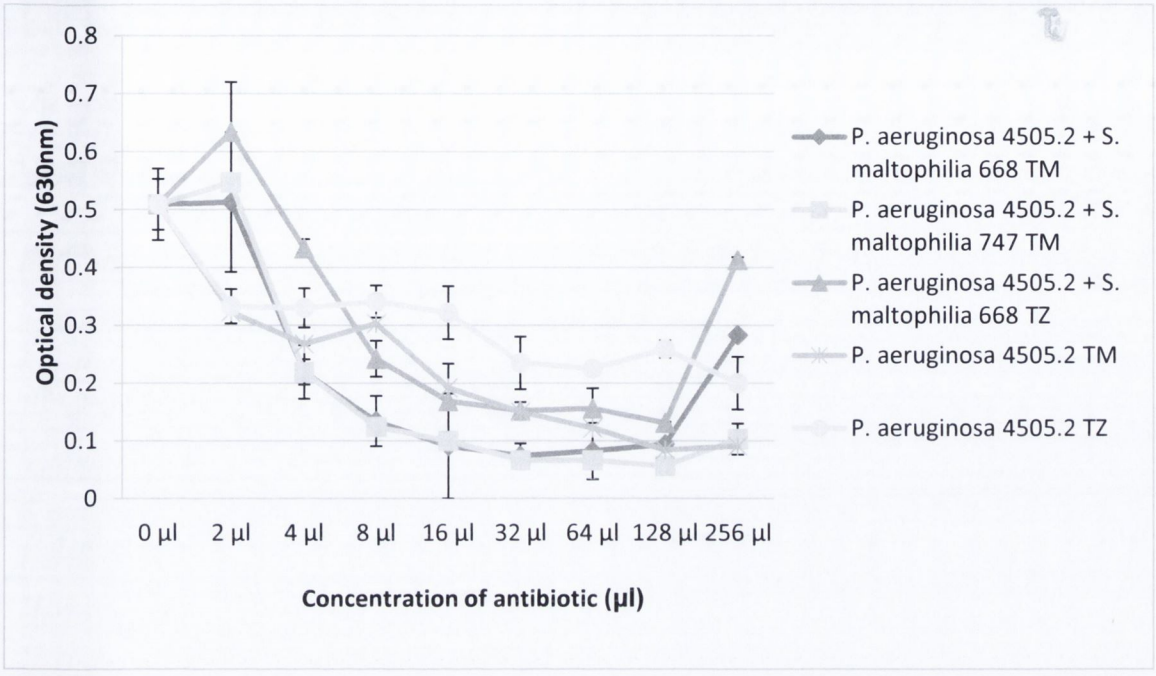


Figure 4.12: The effect of antibiotics (tobramycin, ceftazidime) biofilms of *P. aeruginosa* (PA01, 4505.2) treated with *S. maltophilia* (668, 747) supernatants as described in **Section 4.2.5**. Biofilms were exposed to various concentrations of tobramycin and ceftazidime for an additional 24 hours. Results represent the average of two independent experiments.

4.3.11 Susceptibility testing of cystic fibrosis species to Tobramycin.

Table 4.4 illustrates the sensitivity of planktonic cultures of selected unusual species to tobramycin at concentrations corresponding to traditional MIC's. *Paracoccus* sp. displayed high sensitivity to tobramycin in which the MIC and MBC values were generally not greater than a concentration of 1 μg/ml. The sensitivity of *Ralstonia* sp. to tobramycin appeared to be specific to each individual strain. Three strains were weakly sensitive in which MIC and MBC values were > 64 μg/ml. One isolate, *Ralstonia* 746 displayed moderate sensitivity to aminoglycoside therapy at an MIC value of 4 μg/ml and MBC value of 16 μg/ml. Additionally, weak bactericidal activity was observed against *S. maltophilia* strains at an MBC > 64 μg/ml. Similarly, the antibiotic exhibited weak bactericidal activity against *Acinetobacter* 736 and *Herbaspirillum* isolates at MBC > 64 μg/ml. Results from the present study indicate alternative antibiotics to commonly used aminoglycosides may be required for CF patients infected with these bacterial infections.

Table 4.4: Comparison of susceptibilities of cystic fibrosis species grown planktonically to aminoglycoside antibiotic, Tobramycin (μg/ml).

Unusual cystic fibrosis species	MIC	MBC
Ralstonia 702	>64	>64
Ralstonia 659	>64	>64
Ralstonia 735	>64	>64
Ralstonia 746	4	16
S. maltophilia 668	64	>64
S. maltophilia 747	>64	>64
Herbaspirillum 767	4	>64
Herbaspirillum 762	64	64
Herbaspirillum 320167	>64	>64
Herbaspirillum 320012	>64	>64
Herbaspirillum 317533	>64	>64
Herbaspirillum 317493	>64	>64
Herbaspirillum 317466	16	>64
Acinetobacter 736	64	64
Paracoccus 1426	<0.125	<0.125
Paracoccus 1429	0.25	0.25
Paracoccus 1968	0.25	0.25
Paracoccus 9205	0.25	0.25
Paracoccus 6155	1	1
Paracoccus 4446	0.5	0.5

4.3.12 SDS-page and LC/MS analysis of *S. maltophilia* proteins.

The quantification of protein was determined using the nanodrop^{TM} 1000. The protein concentrations of culture supernatants of *S. maltophilia* 668 and *S. maltophilia* 747 were 2.8 mg/ml and 3.7 mg/ml, respectively. The proteins of these culture supernatants were separated by SDS-page followed by LC/MS analysis of any different protein bands. *S. maltophilia* has previously shown to interfere with the growth of *P. aeruginosa* biofilms. The results presented in **Figure 4.13** show similar protein bands between the *S. maltophilia* strains at 130 kDa, 100 kDa and 70 kDa. Other findings were protein band at approximately 63 kDa which was found to be unique to *S. maltophilia* 668 and 55 kDa protein band was unique to *S. maltophilia* 747. These bands were excised from the gel, trypsin digested and analysed by LC/MS. *S. maltophilia* 668 (70 kDa) was selected as it was one of the common proteins to both strains.

The proteins identified by MASCOT are listed in **Table 4.5** with sequence coverages. The three proteins of *S. maltophilia* 668 (band A) were identified as dihydrolipoamide dehydrogenase, cytosol aminopeptidase and s-adenosyl-L-homocysteine. Other proteins that were identified in *S. maltophilia* 747 (band C) were matched to type II citrate synthase, chaperonin GroEL and elongation factor G. The protein at band B was identified as 60 kDa chaperonin which was a common peptide to both *S. maltophilia* strains.

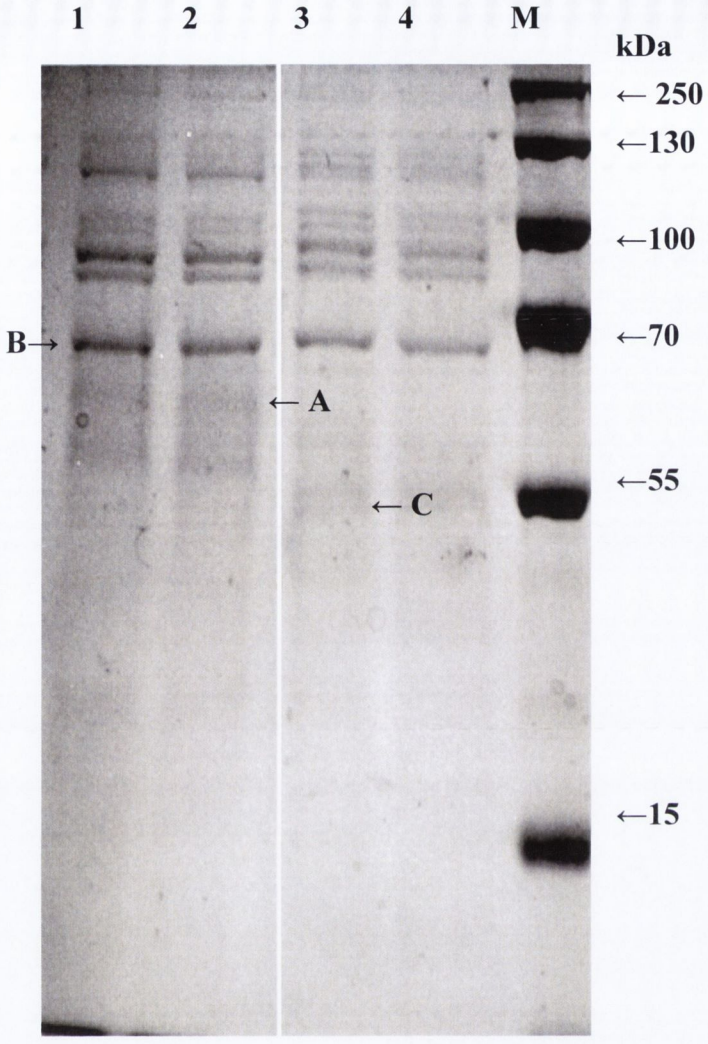


Figure 4.13: Protein analysis of culture supernatants of *S. maltophilia* strains 668 and 747. Proteins were separated by 10% SDS-page and stained with coomassie blue. The migration of molecular weight (kDa) standards is shown on the right of the image.

1. *S. maltophilia* 668
2. *S. maltophilia* 668
3. *S. maltophilia* 747
4. *S. maltophilia* 747
5. Marker

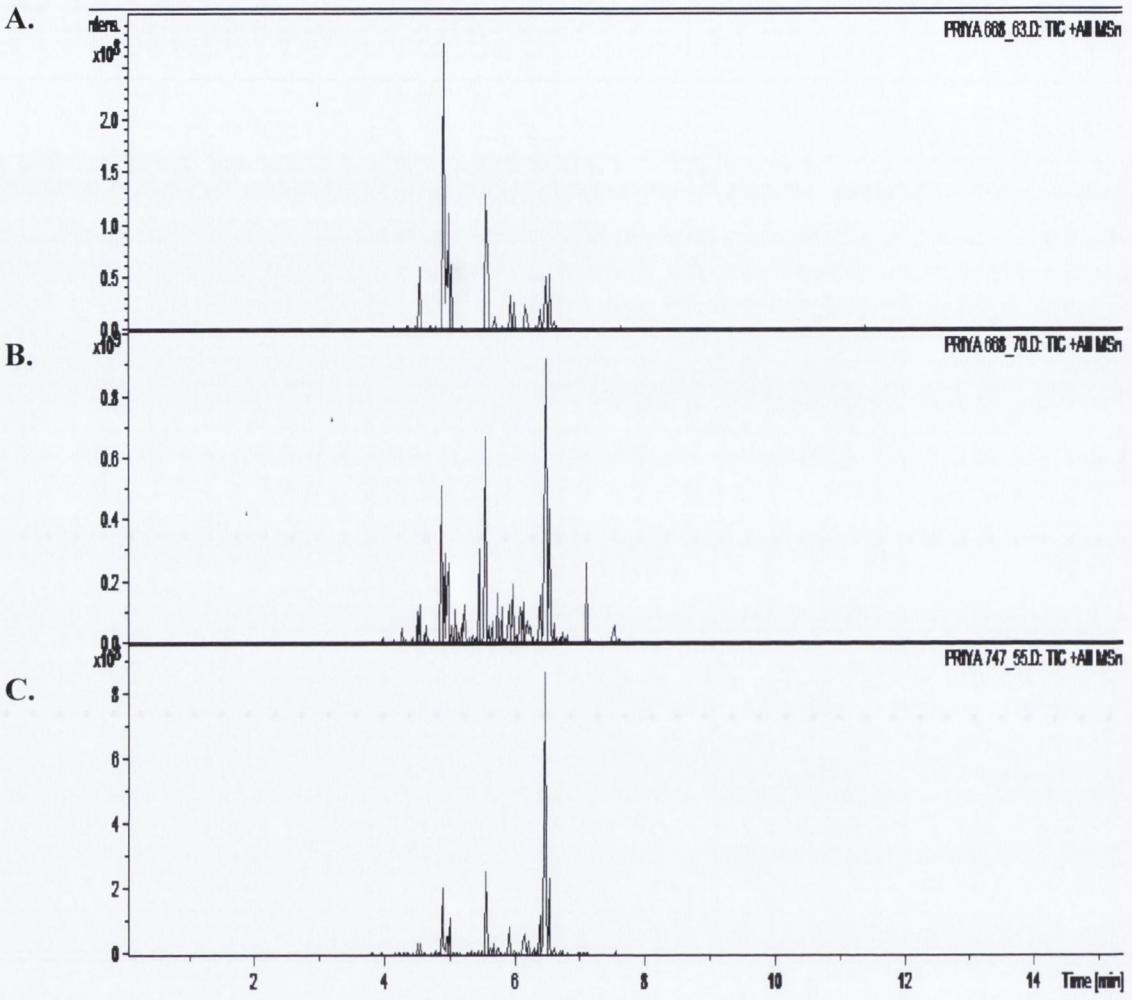


Figure 4.14: Total ion chromatograms from the LC/MS analysis of bands A, B and C of *S. maltophilia* proteins.

Table 4.5: Summary of identified proteins of *S. maltophilia* by LC/MS analysis.

S. maltophilia proteins from bands shown in **Figure 4.13** were excised and trypsin digested prior to LC/MS identification.

Band:	**Identification:**	**Mass (Da)**	**Sequence coverage (%)**	**Peptides matched**	**gi**
A	Dihydrolipoamide dehydrogenase	50683	20	7	344208053
A	Cytosol aminopeptidase	51519	9	4	344205968
A	S-adenosyl-L-homocysteine	53144	6	3	194364404
B	60 kDa chaperonin	57281	65	34	344209060
C	Type II citrate synthase	46461	40	11	491553279
C	Chaperonin GroEl	57312	12	3	194367398
C	Elongation factor G	77980	7	3	190572941

Band A: Dihydrolipamide dehydrogenase

MAEQFDVVVI GAGPAGYHAA IRAAQLGLKT ACIDAALGKD GKPALGGTCL
RVGCIPSKAL LDSSRQFWNM GHIFGDHGIS FKDAK**IDVEA** **MVGR**KDKIVK
QFTGGIGMLF KANKVAAYYG FGELQPGNVV KVTQHDGSIV ELK**GTNVIIA**
AGSDSIELPF **AKFDGETIVD** **NVGGLDFTEV** **PNR**LAVIGAG VIGLELGSVW
K**RLGAEVTIL** **EALPEFLAVA** **DAEVAK**TAAK EFKKQGLDIR LGAKVSKTEI
TGKGKKK**EVV** **VTYTDSEGEK** TLTVDKLLVA VGRRAATKGL LAEGTGVKIN
ERGQIEVDAH CHTGVNGVWA VGDCVRGPML AHKGFEEGIA VAELIAGLPG
HVNFDTIPWV IYTEPELAWV GKTEAQLKAE GIPYK**AGSFP** **FAANGR**AVAM
IEPAGFVKIL AHAETDRILG MHLVGANVSE LVHEGVLTME FSGSADDLAR
ICHAHPSLSE VIHDAAMAVS KRAIHKAN

Band A: Cytosol aminopeptidase

MALEFTLNHV APAAATVDCL VVGAYADHTL TPAAQALDAA SGGRLAALAQ
RGDLSGKTGA TTLLHDLPGV TAPRVLVVGL GEAARFGVPQ YLKAVGDAVR
ALKAGAARSA LFTLSEVAIK DRDAAWAIRQ AVIAADHAAY RYTATLGKKK
ADDAGLAQLA VAGDDAQALA QGQAIAAGVE FARELGNLPP NYCTPAYLAE
VGVKFAGEHD GAEAEILDET QMEALGMGSL LAVARGSANR PRLVVLKWTG
AGDAKPYVLV GKGITFDTGG VNLKTQGGIE EMKYDMCGGA NVIGTFVAAV
KAKLPLNLVV VVPAVENAID GNAYRPSDVI TSMSGKTIEV GNTDAEGRLI
LCDALTYAQR FEPAALVDVA TLTGACMVAL GHQTAGLMSK HDDLANELLA
AGEHVFDRAW RLPLWDEYQP MLDSTFADVY NIGGRWAGAI TAGCFLSRFA
EGQRWAHLDI AGVASDEGKR GMATGRPVGL LSQWLLDQVA RA

Band A: S-adenosyl-L-homocysteine

MNAVAKTFST EGDYKIRDIT LADWGRKELD IAEHEMPGLM SIRRKHAASL
PLKGVRVTGS LHMTIQTAVL IETLKDIGAD VRWASCNIFS TQDHAAAAIA
ATGTPVFAWK GESLEEYWDC TLDALTFTLA DGTLTGPELV VDDGGDVTLL
IHKGYELENG STWVNEPAAS HEEQVIKNLL KRVATERPGY WGRVVKDWKG
VSEETTTGVH RLYQLAQAGI LLIPAINVND SVTKSKFDNL YGCRESLADG
LKRAMDVMLA GKVAVVCGYG DVGKGCAASL RAYGARVIVT EIDPICALQA
AMEGYEVNTI ESTLGRADLY VTTTGNKDII RIEHLSAMKD QAIVCNIGHF
DNEIQVDALV GFKGVQHVNI KPQVDKYIFP NGNAIFLLAE GRLVNLGCAT
GHPSFVMSNS FANQTLAQID LWANKDSYEN KVYLLPKKLD EEVARLHLEK
IGVKLTTLSQ EQADYIGVPV EGPFKPDHYR Y

Band B: 60 kDa chaperonin

MAAKDIRFGE DARSRMVRGV NVLANAVKAT LGPKGRNVVL EKSFGAPTIT
KDGVSVAKEI ELADKFENMG AQMVKEVASR TNDDAGDGTT TATVLAQALI
REGAKAVAAG MNPMDLKRGI DKAVVAAVAE LKTISKPTAD DKAIAQVGTI
SANSDESIGQ IIADAMKEVG KEGVITVEEG SGLDNELDVV KGMQFDRGYL
SPYFINNQQS QTADLDDPFI LLHDKKISNV RDLLPVLEGV AKAGKPLLIV
AEEVEGEALA TLVVNTIRGI VKVVAVKAPG FGDRRKAMLE DMAVLTGGTV
ISEEVGLSLE KATIKDLGRA KKVQVSKENT TIIDGVGDKA AVDARVGQIK
TQIQDTSSDY DREKLQERVA KLAGGVAVIK VGASTEIEMK EKKDRVDDAL
HATRAAVEEG VVPGGGVALV RAVTALAGLK GANEDQNHGI QIALRAMEAP
LREIVANAGE EPSVIINKVK EGTGSFGYNA ATGEFGDMLQ FGILDPTKVT
RSALQNAASI AGLMITTEAM VAEAPKKDEP AMGGAGGMGG MGGMGGMDF

Band C: Type II citrate synthase

MSDLDQVTLN AGDKSVVLPV IKPTLGNDCV DIAKLTKETG FFTYDSGFTA
TASCKSAITY IDGDKGVLLY RGYPIEQLSE KSSYVEVAYL LINGERPSAE
QLKAFTDELT AEANVDESIN TLIGSFAKDA HPMAILAAAI AQLSAIYHDS
LDLSDAEQRR QAAVRLIAKV PTLSALIYRH GKGLPANKPD TSLDYVSRFL
KQTFESADGQ YDLNPDVVKA LDLLFILHAD HEQNASTSTV RLVGSTGANP
YASVAAGVTA LWGPAHGGAN EAVLKMLEEI GSADNVESAV VKAKDKTSGF
RLMGFGHRVY KNFDPRAKVI GEMTSKVLKQ LGVQDPLLDV AVKLEQAALQ
DEYFVARKLY PNVDFYSGII YKALQIPTEM FTVMFALGRT SGWVSHWLEQ
QVDPEMKIGR PRQVYTGSDV RDYQG

Band C: Chaperonin GroEL

MAAKDIRFGE DARSRMVRGV NVLANAVKAT LGPKGRNVVL EKSFGAPTIT
KDGVSVAKEI ELADKFENMG AQMVKEVASR TNDDAGDGTT TATVLAQALI
REGAKAVAAG MNPMDLKRGI DKAVVAAVNE LKSISKPTAD DKAIAQVGTI
SANSDESIGQ IIADAMKEVG KEGVITVEEG SGLDNELDVV KGMQFDRGYL
SPYFINNQQS QTADLDDPFI LLHDKKISNV RDLLPVLEGV AKAGKPLLIV
AEEVEGEALA TLVVNTIRGI VKVVAVKAPG FGDRRKAMLE DMAVLTGGTV
ISEEVGLSLE KATIKDLGRA KKVQVSKENT TIIDGVGDKA AVDSRVAQIK
TQIQDTSSDY DREKLQERVA KLAGGVAVIK VGASTEIEMK EKKDRVDDAL
HATRAAVEEG VVPGGGVALV RAVSALAGLK GANEDQNHGI QIALRAMEAP
LREIVANAGE EPSVIVNKVK EGTGSFGYNA ATGEFGDMLQ FGILDPTKVT
RSALQNAASI AGLMITTEAM VAEAPKKDEP AMGGAGGMGG MGGMGGMDF

Band C: Elongation factor G

MARSTPIERY RNFGIMAHID AGKTTTSERI LFYTGKSHKI GEVHDGAATM
DWMEQEQERG ITIQSAATTA FWKGMDKSLP EHRFNIIDTP GHVDFTIEVE
RSLRVLDGAV FVLCAVGGVQ PQSETVWRQA NKYHVPRIAF VNKMDRTGAN
FQKVVGQLKA KLGAVAVPMQ LPIGAEDNFK GVVDLLKMKA IHWDEASQGM
KFEYSDIPAD LQAAAEEARQ FMVETAAEAS EELMEKYLGG EELAEAEIIN
ALRTRTLATE IVPMYCGSAF KNKGVQAMLD GVIQLLPSPV DVPDVKGVDV
DDDTVEMTRK SDDKAPFSSL AFKIITDPFV GALTFFRVYS GTLNGGDTVL
NSVKGKKERI GRILQMHSNN REEIKEVLAG DIAAAVGLKD TTTGDTLCAV
DAPIILERMT FPEPVISMAV EPKTKSDQEK MGLALGRLAQ EDPSFRVKTD
EESGQTIISG MGELHLDIIV DRLKREFNVE ANVGAPQVAY RETITLADVK
SDYKHAKQSG GKGQYGHVVI ELSPITAADR ADPKLAPAIK DDFLFINDIT
GGVIPKEFIP SVEKGLRETI TSGPLAGFPV VDVKVKLVFG SYHDVDSSEM
AFKLASSMAF KQGFAKAKPV LLEPIMKVEI VTPEDYQGDV MGDVSRRRGV
LQGSDTTGDG SASIINAMIP LGEMFGYATA LRSQTQGRAT FTMEFDHYEP
APTNIAEAVM KKG

Figure 4.15: The seven peptides sequenced of *S. maltophilia* proteins identified from bands A, B and C. All peptides that have been matched by LC/MS are highlighted in red.

4.4 Discussion

Mixed species biofilm interactions have been the focus of many literature studies since the airways of CF patients have been known to harbour many diverse bacterial communities (Zemanick *et al.*, 2011). Although *P. aeruginosa* accounts for approximately 80% of CF related infection, other strains are frequently identified within the same environment (LiPuma *et al.*, 2010). Many reports indicate unusual species can often be misidentified as those that are more common (Coeyne *et al.*, 1999). This study provides an indication of the characteristics of unusual CF species that have been isolated from the CF lung and their ability to form biofilms.

Findings from the current study indicate that while the biofilm forming capacity of clinical CF *P. aeruginosa* strains remains strong, adherence of other clinical CF species to polystyrene pegs remains much weaker. *Paracoccus* sp. and *Herbaspirillum* sp. did not develop biofilms under the experimental conditions suggesting biofilm formation is not a specific virulence factor for colonisation in the CF lung. In addition, *Acinetobacter* 736 strain used in this study was viable at 72 hours but did not form biofilm on polystyrene pegs at any time point. Recent studies have shown that the amount of exopolysaccharide produced is involved in biofilm formation of this species (Tomaras *et al.*, 2003). Often bacteria that do not form biofilms require different adaptive mechanisms for colonisation which have not been identified in this study. Recently, Caraher *et al.*, (2007a), found *Pandoraea* sp., which have been associated with deterioration of CF lung function was not attributed to biofilm formation. *Pandoraea* sp. most likely acts as an inflammatory

stimulant for other pathogens that cause infection.

Among the *Ralstonia* sp., tested, strain 746 adhered to polystyrene pegs with an OD value $\geq$ 0.05 within 72 hours indicating biofilm formation may be a specific virulence trait to each individual strain. Previously, Anderson *et al.*, (1990), identified biofilm formation of this genus with *Ralstonia picketti* isolates. Recovery of *Ralstonia* sp., in CF sputum samples in generally low compared to other species (Coeyne *et al.*, 2002) and occasionally misidentification can occur (Henry *et al.*, 2001). Nosocomial outbreaks of the species are rare and have been mainly linked to contamination by hospital equipment (Roberts *et al.*, 1990; Gardner *et al.*, 1984). A clinical study by Burns *et al.*, (1998), found a total of 2 out of 559 CF patients were infected with the bacterium. Previous studies have described *Ralstonia solanacearum* as motile when transferred into water or a suitable medium (Kelman *et al.*, 1973). Overall, limited information is available about the virulence factors of this species.

Although *S. maltophilia* is a prominent CF pathogen (Fitzsimmons *et al.*, 1993) there have been limited studies of an increased rate in lung function decline. The CFRI reports approximately 2.7% of CF patients tested positive for *S. maltophilia* in 2011. Other studies have reported that *S. maltophilia* has been isolated from the CF lungs in approximately 30% of CF patients (Steinkamp *et al.*, 2005). *S. maltophilia* used in this study showed strong adherence to polystyrene pegs with high $OD_{630} \geq 0.05$ at 72 hours suggesting biofilm formation may be an important virulence trait of this species. This was consistent with the study by Pompilio *et al.*, (2011), by which CF patients' established *S.*

maltophilia infection by biofilm formation and diversity in phenotypic properties during the course of infection. Studies have reported that flagella may allow bacterial adhesion of this species to various abiotic surfaces (Sampaio *et al.*, 2009). Exoproducts produced by *P. aeruginosa* that cause damage to the epithelial mucosa may enhance the ability of *S. maltophilia* colonisation (DeAbreu *et al.*, 2001; Karpati *et al.*, 1994).

The present study focused on mixed biofilm interactions between *P. aeruginosa* and the unusual CF species. According to Spilker *et al.*, (2008), species that are less commonly found in the CF lungs are less frequently identified as the main cause of chronic lung disease but may be contributing to lung deterioration and tolerance to antibiotic treatment. *Paracoccus* sp. and *Herbaspirillum* sp. which did not readily form single species biofilms did not appear to influence *P. aeruginosa* biofilm formation on polystyrene pegs. Similarly, Spilker *et al.*, (2008), found a reduced frequency of *Herbaspirillum* isolates in the CF lungs likely to be caused by other more common strains overtaking *Herbaspirillum*. A clinical study by Filkins *et al.*, (2012), found *P. aeruginosa* was the main organism in the majority of CF patients. Tomlin *et al.*, (2001), described co-colonisation of the lungs with *P. aeruginosa* and other strains such as *B. cepacia* can lead to more serious pulmonary decline than *P. aeruginosa* alone. Interestingly, *Acinetobacter* 736 which did not develop biofilm on the pegs reduced the biomass of both *P. aeruginosa* strains (PA01 and ATCC27853) at 72 hours. When viable counts were obtained of the same strains to quantify the biofilm produced by each species, no difference in the growth of *P. aeruginosa* was observed suggesting that *Acinetobacter* 736 may be influencing the attachment of *P. aeruginosa*.

Many patients who acquire *S. maltophilia* have a higher risk of contracting *P. aeruginosa* and *B. cepacia* (Goss *et al.*, 2002). A recent clinical CF study by Saminos *et al.*, (2012), found 33.8% of *S. maltophilia* patients were co-infected with other pathogens. An increase in biofilm growth was observed with *P. aeruginosa* PA01 and *S. maltophilia* 668 on polystyrene pegs at 72 hours which may indicate one species is enhancing biofilm formation of the other. Mixed biofilm cultures of *P. aeruginosa* ATCC27853 and *S. maltophilia* 668 and 747 showed an increase in biofilm growth at 72 hours in the same model. Additionally, the cell-free supernatants showed a significant reduction in biofilm growth of *P. aeruginosa* PA01 and clinical isolate *P. aeruginosa* 4505.2. It was interesting that *P. aeruginosa* 4505.2 was mucoid and formed a biofilm of greater cell density than any other strain used in this part of the study. There is a possibility that extracellular products secreted by *S. maltophilia* are able to disrupt established *P. aeruginosa* biofilms. Previous reports have suggested that extracellular products such as polysaccharides produced by some bacteria are able to control biofilm development during infection (Pihl *et al.*, 2010). Recently, Ryan *et al.*, (2008), indicated the cell-cell signalling system of *S. maltophilia,* known as diffusible signal factor (DSF) is not restricted to xanthomonads. The study found *S. maltophilia* alters the biofilm structure of *P. aeruginosa* through DSF signalling. Resistance of *P. aeruginosa* to cationic antimicrobial peptides (CAMP) may be allowing growth and survival of CF strains in the presence of species that produce DSF since CAMP is a component of the innate immune system. The other *P. aeruginosa* strains (PA01 and 1826) remained unaffected in co-culture at 24 hours. Since pigment production was a virulence factor of the selected *P. aeruginosa* isolates used in the current study, this may have allowed *P. aeruginosa* to

gain a competitive advantage during dual-species biofilm formation with *S. maltophilia* supernatants and whole cells.

Recent studies have demonstrated quorum sensing influences signalling between bacterial communities (Reidel *et al.*, 2001; Eberl *et al.*, 2004). An *et al.*, (2006), reported flagella and type-IV pili influence interactions between *P. aeruginosa* PA01 and *Agrobacterium*. Reidel *et al.*, (2001), found the N-acyl-homoserine lactone (HSL) quorum sensing system enhances synergistic interactions between *B. cepacia* and *P. aeruginosa* during biofilm formation. The study by Yang *et al.*, (2011), showed type-IV pili facilitated mixed species aggregate formation of *P. aeruginosa* and *S. aureus* under flow conditions. Harriott *et al.*, (2009), suggested that the up-regulation of matrix production by *S. aureus* may occur during co-culture with *C. albicans* since differences between single and mixed structural components were observed.

The bacterial interactions that were found in the current study highlight the importance of examining the response of these species to antibiotics. Carmeli *et al.*, (1997), suggested the use of broad spectrum antibiotics in CF patients could lead to acquisition of *S. maltophilia*, in particular the use of carbapenem antibiotics. Since biofilm formation of *P. aeruginosa* and *Burkholderia* sp., is associated with an increase in antibiotic resistance (Cos *et al.*, 2010), susceptibility of tobramycin was assessed for planktonic cultures of this collection of species. Aminoglycosides such as tobramycin are one of the main antibiotic classes for treating CF infections. Interestingly, strains that did not form biofilms were not sensitive to antibiotic treatment such as *Ralstonia*, *Acinetobacter* and

Herbaspirillum species. *Paracoccus* sp. displayed the most sensitivity at MIC and MBC ranging 0.25 – 1 µg/ml. *S. maltophilia* strains resistant to aminoglycosides are known to be substrates of aminoglycoside phosphotransferase (APH) enzymes (Okazaki *et al.*, 2007). Otkun *et al.*, (2005), found antimicrobial resistance of *S. maltophilia* significantly higher than other species, mainly mediated by intrinsic factors such as multidrug efflux pumps. Further testing of the antimicrobial susceptibility of mixed species biofilms found *P. aeruginosa* 4505.2 showed greater sensitivity to tobramycin indicating *S. maltophilia* can reduce the tolerance of mucoid *P. aeruginosa* isolates. The study by Skillman *et al.*, (1999), described interactions between the matrixes of two species may result in a thicker and more protective barrier to foreign agents, particularly between *Enterobacter agglomerans* and *Klebsiella pneumonia.*

Previous studies have suggested that expression of proteins secreted by CF bacteria may vary during initial and chronic infection. The results presented in this section of the study found *S. maltophilia* strains may interfere with the growth of *P. aeruginosa* biofilms. SDS-page was employed to detect any differences between the CF *S. maltophilia* strains. A protein at approximately 63 kDa was detected in *S. maltophilia* 668 and a protein at approximately 50 kDa in *S. maltophilia* 747. According to Hanna *et al.*, (2000), these differences among specific strains may be influencing their virulence during CF lung infection. Further information of these protein bands was carried out by LC/MS analysis. A peptide at 70 kDa which was common to both *S. maltophilia* strains was recognised as a 60 kDa chaperonin. Hartl *et al.*, (1996), described the chaperones to assist in the folding of newly synthesised polypeptides and folding of proteins that have denatured. The *S.*

maltophilia 668 proteins identified at a unique band A were dihydrolipoamide dehydrogenase, cytosol aminopeptidase and s-adenosyl-l-homoserine. Dihydrolipoamide dehydrogenases are homodimeric flavoproteins that are known to enhance virulence and multiplication of infection of pneumococcal disease (Smith *et al.*, 2002). The aminopeptidases have been shown to act as repressors or activators in the operon regulation of virulence genes in many bacterial species (Woolwine *et al.*, 1999). In addition, s-adenosyl-l-homoserine has an important role in cellular homeostasis (Loenen *et al.*, 2006). This identification provides an insight into the proteins that may be allowing certain *S. maltophilia* strains to gain a competitive advantage with other CF bacteria. Other proteins identified in *S. maltophilia* 747 were elongation factor G and type II citrate synthase. Kunert *et al.*, (2007), showed that elongation factor G is a surface protein of *P. aeruginosa* that enhances attachment and immune evasion and limited information was available on the role of type II citrate synthase. A previous study by Kelly *et al.*, (1990), found *P. aeruginosa* strains that have the mucoid phenotype expressed different proteins to non-mucoid strains. In addition, Karaba *et al.*, (2013), found that protein secretion by *S. maltophilia* which caused the supernatants to have protease activity was dependent on the type 2 secretion system. Another study by deOliveira-Garcia *et al.*, (2002), found flagellin protein at 38 kDa was common to *S. maltophilia* strains but the molecular mass differed among strains. From the data presented it is evident that there are differences between CF *S. maltophilia* isolates. The peptides that have been characterised will allow further studies to detect the proteins that may be acting as virulence factors during co-infection.

Chapter 5: General Discussion and Conclusions

Biofilms are communities of bacterial cells growing on a surface encased within an exopolysaccharide matrix. It has been estimated that approximately 65% of bacterial infections are related to biofilm formation. Antibiotic resistance of bacteria growing in biofilms is dependent on a number of factors such as the exopolysaccharide polymers composed of alginate which reduces the ability of antimicrobials to penetrate the cells. *P. aeruginosa* isolates from cystic fibrosis patients can be highly antibiotic resistant due to biofilm formation (Costerton *et al.*, 2003). The main aim of this study was to explore the activity of potential anti-biofilm treatments such as antibiotic combination therapy and synthesised metal-based complexes with the aim of identifying novel agents that may be inhibiting *P. aeruginosa* reference and clinical strains. Mixed species biofilm interactions were also explored to identify any species that may be altering the growth of *P. aeruginosa*. The clinical isolates recovered from sputum samples from different Irish CF centres were useful in understanding the responses of *P. aeruginosa* in a clinical context.

The results showed that clinical isolates produced different phenotypic characteristics which may be influencing specific adaptive behaviour and response to treatment. The results in Chapter 2 demonstrated that phenotypic characteristics are important components for biofilm formation, *in vivo* pathogenesis and antibiotic susceptibility of *P. aeruginosa*. Biofilm density differed with each isolate and each model. Many strains that adhered weakly to polystyrene pegs showed greater biomass determined by total viable counts, indicating that biofilms formed on pegs are not representing fully developed

biofilm structures. Houry *et al.*, (2009), described motility as a major factor influencing the ability of cells to find suitable places to settle. It was evident from the *in vivo* characterisation of *P. aeruginosa* that phenazines are major virulence factors of CF isolates influencing *G. mellonella* mortality.

The results also suggested that strains may have different susceptibilities to antibiotic single and combination therapy. Resistance to antimicrobial agents is generally higher in *P. aeruginosa* biofilms than in planktonic cells (Nickel *et al.*, 1985). Antibiotics such as aminoglycosides have been shown to diffuse into the biofilm at a slower rate due to binding with the exopolysaccharide matrix (Hogdes and Gordon, 1991). In the current study most of the antibiotic combinations against the biofilms were similar to the activity of the antibiotics used as single agents, however, this study evaluated the importance of choosing the most suitable treatment for a particular patient. E-test results showed all *P. aeruginosa* isolates were susceptible to colistin, however, this effect did not translate against the biofilms. *P. aeruginosa* also displayed resistance to antibiotic combinations involving colistin. The OprH gene increases membrane permeability of biofilms maintaining a higher rate of resistance to colistin therapy (MacFarlane *et al.*, 1999; Bell *et al.*, 1991).

Tobramycin and ciprofloxacin were examined in combination with other antipseudomonal agents as they displayed the greatest inhibitory activity as single antibiotics. Synergistic interactions of tobramycin-ceftazidime and tobramycin-meropenem (FIC ≤ 0.5) were observed against clinical CF isolate *P. aeruginosa* 1826 and

tobramycin-ceftazidime against clinical CF isolate *P. aeruginosa* 4505.2. In addition, tobramycin-ceftazidime was also synergistic against *P. aeruginosa* 1826 but had a reduced effect against *P. aeruginosa* 4505.2. Although synergistic antibiotic interactions were limited in this study, findings suggest that two or more antibiotics that have different mechanisms of action may be used as alternative treatments to single antibiotics. The results showed the synergistic activities of antibiotic combinations seen in 24 hour biofilms may be reduced against 72 hour biofilms. In addition, the efficacy of tobramycin-ceftazidime may not be influenced by the phenotypic characteristics of *P. aeruginosa* strains since these stains differed in exopolysaccharide production. Based on the study by Ciofu *et al.*, (2001), the mucoid phenotype may ensure survival of the biofilm, while the non-mucoid cells act as protective barriers against antimicrobial agents. Overall, findings reveal antibiotic combination therapy is specific to each individual *P. aeruginosa* isolate and antibiotics administered during the early stages of CF infection may be more effective to control infection. The mechanisms that are influencing synergistic interactions warrant further investigation.

A similar response was seen in Chapter 3 following treatment of *P. aeruginosa* biofilms with metal-based agents, in particular silver-coumarin compounds. The activity of silver against *P. aeruginosa* has been studied by many researchers with promising results (Kalishwaralal *et al.*, 2010; Bjarnsholt *et al.*, 2007). Synthesised silver-coumarin compounds reduced the rate of respiration by disrupting the synthesis of cytochromes in the mitochondrion of *C. albicans* (Thati *et al.*, 2007). *P. aeruginosa* planktonic cells and biofilms formed under static conditions displayed sensitivity to these agents, however,

results were dependent on the response of each strain to each individual compound. MIC and MBC values of the agents against an array of phenotypically diverse *P. aeruginosa* planktonic isolates revealed that silver exhibits a broad range of activity irrespective of these differences. In addition, mature biofilms of certain *P. aeruginosa* strains formed in static models were more resistant following exposure to the novel compounds. Exopolysaccharide has been shown to play a major role in the absorption of heavy metals (Kocberber and Donmez, 2008). Exopolysaccharide production and its binding with silver may be reducing the activity of the silver-coumarin compounds against mature biofilms. Other metals including copper-benzotriazole compounds and gallium nitrate had a reduced effect against planktonic cells and biofilms suggesting *P. aeruginosa* is resistant to certain metals-based agents.

The study of bacterial biofilm formation is of major significance in understanding the pathogenesis of *P. aeruginosa* during CF lung infection. Arrays of different *in vitro* models have been designed to study biofilm formation in great detail. As described previously, biofilm formation was assessed using a variety of different *in vitro* models. Many authors have suggested that biofilms formed on polystyrene pegs are suitable for rapid screening of antimicrobial agents but the assay has a number of limitations. Smith *et al.*, (2008), described gravitational forces may be depositing cells on the bottom of the wells leading to structures formed on polystyrene pegs that may not be representative of a genuine biofilm. Other studies have suggested that *in vitro* biofilms may not be an accurate representation of biofilms formed in an *in vivo* environment (Aaron *et al.*, 2002). The flowchamber biofilm system which was used as part of this study allowed direct

visualisation of biofilm structures over a specific period of time. The replenishment of nutrients and elimination of metabolic waste from bacterial cultures is a continuous process in order to develop phenotypic characteristics of each individual strain (Nichols *et al.*, 1999). This model enables the structural development of biofilms to be observed over time, however, a large volume of samples cannot be analysed simultaneously. The effect of silver-coumarin compounds against *P. aeruginosa* biofilms examined in this model showed that all cells were inhibited following 24 hours exposure. Results suggest that shear force from this model may be rendering the biofilms more susceptible to the antimicrobial properties of the silver-coumarin compounds.

The unusual CF bacteria from the CF reference laboratory at AMNCH expanded this study to explore the possible biofilm forming ability of these species and any potential interactions with *P. aeruginosa* as seen in Chapter 4. Sibley *et al.*, (2011), described the airways of CF patients that consist of many bacterial species and the contribution of some less commonly isolated species may lead to a rapid decline in lung function. It was demonstrated that *P. aeruginosa* remained the dominant pathogen when grown in mixed culture with several unusual CF species including *Paracoccus* sp., *Herbaspirillum* sp. and *Ralstonia* sp. Since the *P. aeruginosa* isolates used for this study produced virulence factors such as phenazines, that may be exerting toxic effects towards other bacterial species as previously demonstrated by Kerr *et al.*, (1999). It was reported that *S. maltophilia* and *Acinetobacter sp.* influenced the attachment of *P. aeruginosa* to polystyrene pegs. Viability testing also showed that *S. maltophilia* 668 was capable of out-competing *P. aeruginosa* PA01 when grown as mixed species biofilms. Additionally,

exposure to cell-free supernatants of *S. maltophilia* 668 and 747 showed a significant reduction in biofilm formation of *P. aeruginosa* strains. Alternatively, not all *P. aeruginosa* biofilms were sensitive to treatment with *S. maltophilia* whole cells and supernatants. *Stenotrophomonas maltophilia* is gaining much attention as an emerging nosocomial pathogen in CF patients but the clinical outcome of infected individuals has not been studied extensively (Waters *et al.*, 2007; Steinkamp *et al.*, 2005).

Proteomic studies have previously been used to detect a number of virulence associated proteins of *P. aeruginosa* (Hanna *et al.*, 2000). To assess the protein profiles of the CF *S. maltophilia* strains that were virulent in the co-culture model, LC/MS analysis on tryptic digests of excised SDS-page bands was conducted. The selected bands corresponded to proteins that were common and different to each strain. Chaperonins were a common peptide group in *S. maltophilia*. This group in involved in the folding of newly generated polypeptides in the bacterial cytosol by an ATP-dependent reaction (Hartl *et al.*, 1996). The proteins of *S. maltophilia* 668 that were identified at a unique band (63 kDa) included dihydrolipoamide dehydrogenase, cytosol aminopeptidase and s-adenosyl-l-homocysteine. This strain was of interest as it was able to out-compete *P. aeruginosa* PA01 and the cell-free supernatants reduced the biofilm growth of the same strain. As described in Chapter 4, Section 4.4 studies have shown that these proteins regulate virulence factor production in other bacterial species. Dihydrolipoamide dehydrogenases are known to enhance virulence of pneumococcal disease (Smith *et al.*, 2002). Aminopeptidases found in *E. coli* and *P. aeruginosa* may act as repressors or activators in the operon regulation of virulence genes (Woolwine *et al.*, 1999). Finally, s-adenosyl-l-

homoserine is involved in cellular homeostasis (Loenen *et al.*, 2006).

As mentioned previously by Sibley *et al.*, (2011), CF lung infection can often involve numerous bacterial species. The current study indicated that some species may be dominant pathogens in a multi-species environment. The results suggest that extracellular proteins of *S. maltophilia* may be related to persistent, chronic infection and antibiotic resistance. Langford *et al.*, (1989), described the outer membrane proteins of *P. aeruginosa* increased resistance to aminoglycosides. Ciofu *et al.*, (2001), described the outer membrane proteins may differ among mucoid and non-mucoid *P. aeruginosa* isolates. Examining mixed species interactions under *in vivo* conditions and the response to antibiotics would be required for a more accurate analysis. Finally, a larger amount of diverse strains would be useful for examining the interactions of *P. aeruginosa* and *S. maltophilia* during lung infection in CF patients.

Chapter 6: References

Aaron, S. *et al.* (2002). "Single and combination antibiotic susceptibilities of planktonic, adherent and biofilm-grown *Pseudomonas aeruginosa* isolates cultures from sputa of adults with cystic fibrosis." *Clinical Microbiology* **40**(11): 4172-4179.

Achtman, M. *et al.* (2008). "Microbial diversity and genetic nature of microbial species." *Nature Reviews Microbiology* **6**: 431-440.

Adam, B. *et al.* (2002). "Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*." *Medical Microbiology* **51**: 344-349.

Akiyama, H. *et al.* (2003). "Assessment of *Streptococcus pyogenes* microcolony formation in infected skin by confocal laser scanning microscopy." *Journal of Dermatological Science* **32**(3): 193-199.

Al-Bakri, A. G. *et al.* (2008). "The assessment of the antibacterial and antifungal activities of aspirin, EDTA and aspirin-EDTA combination and their effectiveness as antibiofilm agents." *Applied Microbiology* **107**: 280-286.

Alhede, M. *et al.* (2011). "Phenotypes of non-attached *Pseudomonas aeruginosa* aggregates resemble surface attached biofilm." *PloS ONE* **6**(11): 1-12.

Allegrucci, M. *et al.*, (2006). "Phenotypic characteristics of *Streptococcus pneumonia* biofilm development." *Journal of Bacteriology* **188**(7): 2325-2335.

Allison, C. and Hughes, C. (1991). "Bacterial swarming: an example of prokaryotic differentiation and multicellular behavior." *Science progress* **75**(298): 403-427.

Allison, D. G. *et al.* (1992). "Effect of polysaccharide interactions on antibiotic susceptibility of *Pseudomonas aeruginosa.*" *Applied Bacteriology* **73**: 484-488.

Allison, D. G. *et al.* (2000). "Biofilms: problems of control. In Community Structure and Co-operation in Biofilms." 309-327.

Ammons, M. C. *et al.* (2008). "*In vitro* susceptibility of established biofilms composed of a clinical wound isolate of *Pseudomonas aeruginosa* treated with

lactoferrin and xylitol." *Antimicrobial Agents* **33**(3): 230-236.

An, D. *et al.* (2006). "Quorum sensing and motility mediate interactions between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* in biofilm co-culture." *PNAS* **103**: 3828-3833.

An, Y. H. *et al.* (2000). Mechanisms of bacterial adhesion and pathogenesis of implant and tissue infections. Handbook of Bacterial Adhesion 1-27.

Anderl, J. N. *et al.* (2000). "Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin." *Antimicrobial Agents and Chemotherapy* **44**: 1818-1824.

Anderson, R. L. *et al.* (1990). "Effects of disinfectants on *Pseudomonas* colonised on the interior surfaces of PVC pipes." *American Journal of Public Health* **80**: 17-21.

Ankri, S. *et al.* (1999). "Antimicrobial properties of allicin from garlic." *Microbes and Infection* **2**(1): 125-129.

Anwar, H. M. *et al.* (1989). "Tobramycin resistance of mucoid *Pseudomonas aeruginosa* biofilm grown under iron limitation." *Antimicrobial Agents and Chemotherapy* **24**: 647-655.

Aperis, G. *et al.* (2007). "*Galleria mellonella* as a model host to study infection by the *Francisella tularensis* live vaccine strain." *Microbes and Infection* **9**: 729-734.

Armstrong, D. S. *et al.* (1996). "Bronchoalveolar lavage or oropharyngeal cultures to identify lower respiratory pathogens in infants with Cystic Fibrosis." *Paediatrics Pulmonology* **21**: 267-275.

Bagge, N. *et al.* (2004). "*Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and *B. lactamase* and alginate production." *Antimicrobial agents and Chemotherapy* **48**(4): 1175-1187.

Baker, J. *et al.* (2010). "Copper stress induces a global stress response in *Staphylococcus aureus* and represses *sae* and *agr* expression and biofilm formation." *Applied and Environmental Microbiology* **76**(1): 150-160.

Balsalobre, L. *et al.* (2003). "Occurrence of yeasts in municipal wastes and their behaviour in presence of cadium copper and zinc." *Journal of Basic Microbiology* **43**: 185-193.

Bandara, M. *et al.* (2009). "*Escherichia coli* and its lipopolysaccharide modulate *in vitro Candida* biofilm formation." *General Microbiology* **58**: 1623-1631.

Banjeree, D. and Stableforth, D. (2000). Treatment of respiratory *Pseudomonas* infection in CF: What drug and which way? Drugs, Adis International. **60:** 1053-1064.

Banin, E. *et al.* (2006). "Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm." *Applied and Environmental Microbiology* **72**(3): 2064-2069.

Barclay, M. L. *et al.* (1996). "Adaptive resistance to Tobramycin in *Pseudomonas aeruginosa* lung infection in cystic fibrosis." *Journal of Antimicrobials and Chemotherapy* **37**: 1155-1164.

Beardsmore, C. S. *et al.* (1994). "Pulmonary function in infants with cystic fibrosis: the effect of antibiotic treatment." *Disease in Childhood* **71**: 133-137.

Beiser, S. *et al.* (2007). "Prevalence and clinical significance of *Staphylococcus aureus* small-colony variants in Cystic Fibrosis lung disease." *Journal of Clinical Microbiology* **45**: 168-172.

Bell, A. *et al.* (1991). "*Pseudomonas aeruginosa* outer membrane protein OprH: expression from the cloned gene and function in EDTA and gentamicin resistance." *Journal of Bacteriology* **173**: 6637-6664.

Bergin, D. *et al.* (2006). "Pre-exposed to yeast protects larvae of *Galleria mellonella* from subsequent lethal infection by *Candida albicans* and is mediated by the increased expression of antimicrobial peptides." *Microbes and Infection* **8**(8): 2105-2112.

Bernstein, R. L. *et al.* (1998). "Mechanisms of therapeutic activity of gallium." *Pharmacology Reviews* **50**: 665-682.

Beyenal, H. and Lewandowski, Z. (2002). "Internal and external mass transfer in biofilms at various flow velocities." *Biotechnology Progress* **18**: 55-61.

Bigger, J. W. *et al.* (1944). "Treatment of *staphylococcus* infection with penicillin." *Lancet* **II**: 497-500.

Bjarnsholt, T. *et al.* (2007). "Silver and *Pseudomonas aeruginosa* biofilms." *APMIS* **115**(8): 921-928.

Blunders, C. A. *et al.* (2010). "Identification of aryl 2-aminoimidazoles as biofilm inhibitors in gram-negative bacteria." *Bioorganic and Medicinal Chemistry Letters* **20**(12): 3797-3800.

Boland, T. *et al.* (2000). "Molecular basis of cell adhesion to polymers characterised by AFM." *Critical Reviews in Biomedical Engineering* **28**: 195-198.

Boles, B. R. *et al.* (2008). "*agr*-mediated dispersal of *Staphylococcus aureus* biofilms." **4**(4).

Bonfield, T. L. *et al.* (1995). "Inflammatory cytokines in cystic fibrosis lungs." *Respiratory Critical Care Medicine* **2**(15): 111-118.

Boucher, R. C. *et al.* (2007). "Cystic Fibrosis: A disease of vulnerability to airway surface dehydration." *Trends in Molecular Medicine* **13**(6): 231-240.

Bouts, B. A. *et al.* (1999). "Images in clinical medicine. Argyia." *New England Journal of Medicine* **340**(20): 1554.

Boyd, A. *et al.* (1995). "*Pseudomonas aeruginosa* biofilms: role of exopolysaccharide." *Industrial Microbiology* **15**: 162-168.

Bridges, K. *et al.* (1979). "Gentamicin-silver-resistant *Pseudomonas* in a burn unit." *Journal of Medicine* **1**: 446-449.

Brisou, J. *et al.* (1995). "Biofilms: Methods for Enzymatic Release of microorganisms".

Brooun, A. *et al.* (2000). "A dose-response study of antibiotic resistance in

Pseudomonas aeruginosa biofilms." *Antimicrobial Agents and Chemotherapy* **44**: 640-646.

Bullen, J. J. *et al.* (1972). "Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants." *Royal Society of Medicine* **65**: 1968.

Burgel, P. R. *et al.* (2011). "High prevalence of azole-resistant *Aspergillus fumigatus* in adults with cystic fibrosis exposed to itraconazole." *Antimicrobial Agents and Chemotherapy* **56**(2): 869-874.

Burns, J. L. *et al.* (2000). "Comparison of agar diffusion methodologies for antimicrobial susceptibility testing for *Pseudomonas aeruginosa* isolates from cystic fibrosis patients." *Clinical Microbiology* **38**: 1818-1822.

Burton, E. *et al.* (2007). "A microplate spectrofluorometric assay for bacterial biofilms." *Journal of Industrial Microbiology and Biotechnology* **34**(1): 1-4.

Caiazza, N. C. *et al.* (2007). "Inverse regulation of biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14." *Bacteriology* **189**(9): 3603-3612.

Campana, S. *et al.* (2007). "Emergence of an Epidemic Clone of Community Associated Methicillin Resistant Panton-Valentine Leucocidin-Negative *Staphylococcus aureus* in a Cystic Fibrosis Patient Population." *Clinical Microbiology* **45**(9): 3146-3147.

Caraher, E. *et al.* (2007a). "Evaluation of *in vitro* virulence characteristics of the genus *Pandoraea* in lung epithelial cells." *Journal of Medical Microbiology* **57**: 15-20.

Caraher, E. *et al.* (2007b). "The effect of recombinant human lactoferrin on growth and the antibiotic susceptibility of the cystic fibrosis pathogen *Burkholderia cepacia* complex when cultures planktonically or as biofilms." *Antimicrobial Chemotherapy* **60**: 546-554.

Carmeli, Y. *et al.*, (1997). "Comparison of treatment with imipenem vs. ceftazidime as a predisposable factor for nosocomial acquisition of *Stenotrophomonas maltophilia*: a historical cohort study." *Clinical Infectious Disease* **24**: 1131.

Carpentier, B. *et al.* (1993). "Biofilms are their consequences, with particular reference to hygiene in the food industry." *Applied Bacteriology* **75**: 499-511.

Ceri, H. *et al.* (1999). "The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms." *American Society of Microbiology* **37**(6): 1771-1776.

Chan, W. C. *et al.* (2004). "Virulence regulation and quorum sensing in staphylococcal infections: competitive AgrC antagonists as quorum sensing inhibitors." *Journal of Medicinal Chemistry* **47**: 4633-4641.

Chang, W. *et al.*, (2007). "Alginate production by *Pseudomonas putida* creates a hydrated microenvironment and contributes to biofilm architecture and stress tolerance under water-limiting conditions." *Journal of Bacteriology* **189**(22): 8290-8299.

Chaudhary, M. *et al.* (2009). "Evaluation of efficacy and safety of fixed dose combination of ceftazidime-tobramycin in comparison with ceftazidime with lower respiratory tract infections." *Current Clinical Pharmacology* **4**: 1-5.

Chernish, R. N. and Aaron, S. D. (2007). "Approach to resistant gram-negative bacterial pulmonary infection in patients with cystic fibrosis." *Current Opinions in Pulmonary Medicine* 9: 509-515.

Chitambar, C. R. *et al.* (1988). "Inhibition leukemic HL60 cell growth by transferrin gallium: effects on ribonuclease reductase and demonstration of drug synergy and hydroxyurea." *Blood* **72**: 1930-1936.

Cimon, B. *et al.* (2001). "Molecular epidemiology of airway colonisation by *Aspergillus fumiagatus* in cystic fibrosis patients." *Journal of Medical Microbiology* **50**: 367-374.

Ciofu, O. *et al.* (2001). "Characterisation of paired mucoid and non-mucoid *P. aeruginosa* isolates from Danish CF patients: antimicrobial resistance, β-lactamase activity and ribotyping." *Journal of Antimicrobial Chemotherapy* **28**(3): 391-396.

Ciofu, O. *et al.* (2005). "Occurrence of *Pseudomonas aeruginosa* in cystic fibrosis patients is associated with oxidative stress caused by chronic lung inflammation."

Antimicrobial Agents and Chemotherapy **49**: 2276-2282.

Clode, F. E. *et al.* (2000). "Distribution of genes encoding putative transmissibility factors among epidemic and non-epidemic strains of *Burkholderia cepacia* from cystic fibrosis patients in the UK." *Journal of Clinical Microbiology* **38**: 1763-1766.

Coenye, T. *et al.* (2002). "Infection by *Ralstonia* species in cystic fibrosis patients: identification of *R. pickettii* and *R. mannitolilytica* by polymerase chain reaction." *Emerging Infectious Diseases* **8**: 692-696.

Coeyne, T. *et al.* (1999). "Classification of some Alcaligenes faecalis-like isolates from the environment and human clinical samples of *Ralstonia gilardii* sp." *International Journal of Systemic Bacteriology* **49**: 405-413.

Coeyne, T. *et al.* (2008). "Use of the modified Robbins Device to study the *in vitro* biofilm removal efficacy of NitrAdine, a novel disinfecting formula for the maintenance of oral medical devices." *Applied Microbiology* **105**: 733-740.

Colombo, C. *et al.* (2005). "Cytokine levels in sputum of cystic fibrosis patients before and after antibiotic therapy." *Paediatric Pulmonology*: 15-21.

Cos, P. *et al.* (2010). "Review: Biofilms: an extra hurdle for effective antimicrobial therapy." *Current Opinion in Pharmacology* **16**(20): 2279-2295.

Costerton, J. W. *et al.* (1994). "Biofilms: a customised microniche." *Journal of Bacteriology* **176**: 2137-2142.

Costerton, J. W. and Stewart, P. S. (2001). "Battling biofilms." *Scientific American* **285**(1): 74-81.

Costerton, J. W. *et al.* (1999). "Bacterial Biofilms: A common cause of persistent infections." *Science* **284**: 1318-1322.

Costerton, J. W. *et al.* (1987). "Bacterial biofilms in nature and disease" *Annual Reviews of Microbiology* 41: 435-464.

Costerton, J. W. *et al.* (2003). "The application of biofilm science to the study and control of chronic bacterial infections." *Clinical Investigation* **112**: 1466-1477.

Damerson and Harrison, (1998). "Mechanisms of protection against copper toxicity." *American Journal of Clinical Nutrition* **67**(5): 1091-1097.

Danese, P. N. *et al.* (2000). "Exopolysaccharide production required for development of *E. coli* K-12 biofilm architecture." *Journal of Bacteriology* **182**(12): 3593-3596.

Darouiche, R. O. *et al.* (1999). "Anti-infective efficacy of silver-coated medical prostheses." *Clinical Infectious Diseases* **29**(6): 1371-1377.

Davey, M. E. *et al.* (2003). "Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PA01." *Journal of Bacteriology* 185: 1027-1036.

Davies, D. G. *et al.* (1993). "Exopolysaccharide production in biofilms: substratum activation of alginate gene expression." *Applied and Environmental Microbiology* **59**: 1181-1186.

Davies, D. G. *et al.* (1998). "The involvement in cell-to-cell signals in the development of bacterial biofilms." *Science* **280**: 295-298.

DeAbreu, V. L. *et al.* (2001). "*Stenotrophomonas maltophilia* interaction with human epithelial respiratory cells *in vitro*." *Microbiology and Immunology* **45**: 563-569.

DeKievit, T. R. *et al.* (2001). "Multidrug Efflux Pumps: Expression Patterns and Contribution to Antibiotic Resistance in *Pseudomonas aeruginosa* biofilms." *Antimicrobial Agents and Chemotherapy* **45**: 1761-1770.

Deligianni, E. *et al.* (2010). "*Pseudomonas aeruginosa* cystic fibrosis isolates of similar RAPD genotype exhibit diversity in biofilm forming ability *in vitro*." *BMC Microbiology* **10**: 38.

Demirag, M. K. *et al.* (2007). "The effect of aspirin on adherence of slime producing coagulase negative staphylococci to vascular grafts." *Annals of Vascular Surgery* **21**: 464-467.

Denning, D. *et al.* (1995). "Commentary: unusual manifest of *Aspergillosis*." *Thorax* **50**: 812-813.

Denton, M. *et al.* (1998). "Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*." *Clinical Microbiology Reviews* (11): 57-80.

deOliveira-Garcia, *et al.* (2002). "Characterisation of flagella produced by clinical strains of *Stenotrophomonas maltophilia*." *Emerging Infectious Diseases* **8**(9): 918-923.

deOliveira-Garcia, *et al.* (2003). "Fimbriae and adherence of *Stenotrophomonas maltophilia* to epithelial cells and to abiotic surfaces." *Cell Microbiology* **5**(9): 625-636.

Desai, M. *et al.* (1998). "Increasing resistance of planktonic and biofilm cultures of *Burkholderia cepacia* to ciprofloxacin and ceftazidime during exponential phase." *Antimicrobial Chemotherapy* **42**: 153-160.

deValk, H. A. *et al.* (2005). "Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates." *Journal of Clinical Microbiology* **43**: 4112-4120.

deValk, H. A. *et al.* (2009). "Molecular typing and colonising patterns of *Aspergillus fumigatus* in patients with cystic fibrosis." *Journal of Cystic Fibrosis* **8**: 110-114.

Deziel, E. *et al.* (2003). "*rhlA* is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids." *Microbiology* **149**: 2005-2013.

Dodge, J. A. *et al.* (1995). "Male infertility in Cystic Fibrosis." *Lancet* **346**: 587-588.

Dong, Z. *et al.* (1997). "Inhibition of activator protein I activity and neoplastic transformation by aspirin." *Journal of Biological Chemistry* **272**: 9962-9970.

Donmez, G. and Kocberber N. (2008). "Environmental conditions affect exopolysaccharide production by *Pseudomonas aeruginosa*, *Micrococcus* sp. and *Ochrobactium* sp." *Journal of Hazard Materials* **154**: 1019-1024.

Douglas, L. J. *et al.* (2006). "Biofilm matrix of *C. albicans* and *C. tropicals*: chemical

composition and role in drug resistance." *Journal of Medical Microbiology* **55**: 999-1008.

Drake, D. R. *et al.* (2002). Continuous-culture chemostat systems and flowcells as methods to investigate microbial interactions. *Polymicrobial Diseases*. Washington, ASM Press.

Drenkard, E. *et al.* (2002). "*Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation." *Nature* **416**: 740-743.

Dubin, P. J. *et al.* (2007). "IL-23 mediated inflammatory responses to mucoid *Pseudomonas aeruginosa* lung infection in mice." *Journal of Physiology-Lung Cellular and Molecular Physiology* **292**(1): 519-528.

Dunne, M. *et al.* (1993). "Diffusion of rifampin and vancomycin through *Staphlyococcus epidermidis* biofilms." *Antimicrobial agents and Chemotherapy* **37**: 2522-2526.

Dupont, M. J. *et al.* (1990). "Quantitative effect of roxithromycin and rifampicin on mucoid cultures from directly plated sputum of cystic fibrosis patients chronically colonised with *Pseudomonas aeruginosa*." *Drugs Experimental and Clinical Research* **16**: 597-605.

Eberl, L. *et al.* (2001). "The *cep* quorum sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility." *Microbiology* **147**: 2517-2528.

Eberl, L. *et al.* (2004). "*Pseudomonas aeruginosa* and *Burkholderia cepacia* in cystic fibrosis: genome evolution, interactions and adaptation." *Medical Microbiology* **294**: 123-131.

El-Azizi, M. A. *et al.* (2004). "Interactions of *Candida* with other *Candida* spp. and bacteria in biofilms." *Applied Microbiology* **96**: 1067-1073.

El-Laboudi, A. H. *et al.* (2009). "Acute *Burkholderia cenocepacia* pyomyositis in a patient with cystic fibrosis." *Cystic Fibrosis* **8**(4): 273-275.

Ellison, R. T. *et al.* (1994). "The effects of lactoferrin on gram-negative bacteria."

Advances in Experimental Medicine and Biology **357**: 71-90.

Evans, A. K. *et al.* (2001). "The impact of meconium ileus on the clinical course of children with Cystic Fibrosis." *European Respiratory Journal* **18**: 784-789.

Evans, R. C. *et al.* (1987). "Effect of vancomycin hydrochloride on *Staphylococcus epidermidis* biofilms associated with silicone elastomer." *Antimicrobial Agents and Chemotherapy* **31**: 889-894.

Farber, B. *et al.* (1992). "The use of non-steroidal anti-inflammatory drugs to prevent adherence of *Staphylococcus epidermidis* to medical polymers." *Infectious Diseases* **166**: 861-865.

Fazli, M. *et al.* (2009). "Non-random distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in chronic wounds." *Journal of Clinical Microbiology* **47**(12): 4084-4089.

Feng, Q. L. *et al.* (2000). "A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*." *Journal of Biomedical Material Research* **52**: 662-668.

Ferretti, S. *et al.* (2003). "IL-17 produced by the lymphocytes and neutrophils is necessary for lipopolysaccharide-induced airway neutrophilia: IL-5 as a possible trigger." *Journal of Immunology* **170**: 2106-2112.

Filkins, L. M. *et al.* (2012). "Prevalence of *streptococci* and increased polymicrobial diversity associated with cystic fibrosis patient stability." *Journal of Bacteriology* **194**(17): 4709-4717.

Fitzsimmons, S. C. *et al.* (1993). "The changing epidemiology of cystic fibrosis." *Journal of Paediatrics* **22**: 1-9.

Franklin, H. J. *et al.* (1993). "Identification of algF in the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation." *Journal of Bacteriology* **175**: 5057-5065.

Fredricks, D. N. *et al.* (2005). "Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR." *Journal of Clinical*

Microbiology **53**: 5122-5128.

Gardner, S. *et al.* (1984). "A nosocomial common source outbreak caused by *Pseudomonas pickettii*." *Paediatric Infectious Diseases* **3**: 420-422.

Geller, D. E. *et al.* (2009). "Review aerosol antibiotics in Cystic Fibrosis." *Respiratory Care* **54**(5): 658-670

Gensberg, K. *et al.* (1999). "Identification of *oprG*, a gene encoding a major outer membrane protein of *Pseudomonas aeruginosa*." *Antimicrobial Agents and Chemotherapy* **43**: 607-608.

Gibson, R. L. *et al.* (2003). "Pathophysiology and management of pulmonary infection in cystic fibrosis." *American Journal of Respiratory and Critical Care Medicine.* **168**: 918-951.

Gilchrist, F. J. *et al.* (2011). "Bronchoalveolar lavage in children with cystic fibrosis. How many lobes should be sampled?" *Archives of Disease in Childhood* **96**: 215-217.

Gillis, R. J. *et al.* (2004). "Azithromycin retards *Pseudomonas aeruginosa* biofilm formation." *Journal of Clinical Microbiology* **42**(12): 5842-5845.

Gjodsbol, K. J. J. *et al.* (2006). "Multiple bacterial species reside in chronic wounds: a longitudional study." *International Wound Journal* **3**(3): 225-231.

Goodman, A. E. and Geesey, G. G. (2001). Biofilms: Recent Advances in their Study and Control. London, Harwood Academic Publishers. 71-80.

Goss, C. H. *et al.* (2002). "Detecting *Stenotrophomonas maltophilia* does not reduce survival of patients with cystic fibrosis." *American Journal of Respiratory and Critical Care Medicine* **166**: 356-361.

Gotz, F. *et al.* (2002). "Staphylococcus and biofilms." *Molecular Microbiology* **43**: 1367-1378.

Govan, J. R. *et al.* (1993). "Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis." *Lancet* **342**: 15-19.

Govan, J. R. W. *et al.* (1996). "Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia.*" *Microbiological Reviews* **60**(3): 539-574.

Greenfield, J. L. *et al.* (1995). "Decreased bacterial adherence and biofilm formation on chlorhexidine and silver sulfadiazine-impregnated central venous catheters implanted in swine." *Critical Care Medicine* **23**: 894-900.

Grisaru-Soen, G. *et al.* (2007). "Nosocomial bloodstream infection in a paediatric intensive care unit: 3 year study." *Medical Science Monitor* **13**: 250-257.

Groneberg, D. A. *et al.* (2002). "Expression of MUC5AC and MUC5B mucins in normal and cystic fibrosis lung." *Respiratory Medicine* **96**(2): 81-86.

Gu, F. *et al.* (2005). "*In situ* and non-invasive detection of specific bacterial species in oral biofilms using fluorescently labeled monoclonal antibodies." *Journal of Microbiological Methods* **62**: 145-160.

Gupta, A. *et al.* (1999). "Molecular basis for resistance to silver cations in *Salmonella.*" *Nature* **5**(2): 183-188.

Gutierrez, J. P. *et al.* (2001). "Interlobar differences in bronchoalveolar lavage fluid from children with cystic fibrosis." *European Respiratory Journal* **17**: 281-286.

Haagensen, J. A. J. *et al.* (2007). "Differentiation and distribution of colistin/SDS tolerant cells in *Pseudomonas aeruginosa* biofilms." *Bacteriology* **189**(1): 28-37.

Halwani, M. *et al.* (2008). "Co-encapsulation of gallium and gentamicin in liposomes enhances antimicrobial activity of gentamicin against *Pseudomonas aeruginosa.*" *Antimicrobial Chemotherapy* **62**(6): 1291-1297.

Hanna S. L. *et al.* (2000). "Comparison of proteins expressed by *Pseudomonas aeruginosa* strains representing initial and chronic isolates from a cystic fibrosis patient: an analysis by 2-D gel electrophoresis and capillary column liquid chromatography-tandem mass spectrometry." *Microbiology* **146**, 2495-2508.

Hansen, S. K. *et al.* (2007). "Characteristics of *Pseudomonas putida* rough variant evolved in a mixed-species biofilm with *Acinetobacter* sp. Strain C6." *Journal of*

Bacteriology **189**(13): 4932-4943.

Harbitz, O. *et al.* (1984). "Lysozyme and lactoferrin in sputum from patients with chronic obstructive lung disease." *European Journal of Respiratory Disease* **65**: 512-520.

Hartl M. K. *et al.* (1996). "Mechanism of chaperonin action: GroES binding and release can drive GroEL-mediated protein folding in the absence of ATP hydrolysis." *EMBO* **15**(22): 6111-6121.

Harris, J. K. *et al.* (2007). "Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis." *Proceedings of the National Academy of Sciences, USA* **104**(51): 20529-20533.

Harrison, D. *et al.* (1998). "Mechanisms of protection against copper toxicity." *American Journal of Clinical Nutrition* **67**(5): 1091-1097.

Harrison, J. J. *et al.* (2008). "Copper and Quaternary Ammonium Cations exert synergistic bactericidal and antibiofilm activity against *Pseudomonas aeruginosa.*" *Antimicrobial Agents and Chemotherapy* **52**(8): 2870-2881.

Hasset, D. J. *et al.* (2009). "*Pseudomonas aeruginosa* hypoxic or anaerobic biofilm infections with cystic fibrosis airways." *Trends in Microbiology* **17**(3): 130-138.

Heath, D. G. *et al.* (2002). "Six-year molecular analysis of *Burkholderia cepacia* complex isolates among cystic fibrosis patients at a referral centre for lung transplantation." *Journal of Clinical Microbiology* **40**(4): 1188-1193.

Heidelberg, J. F. *et al.* (2002). "Genome sequence of dissimilatory metal ion-reducing bacteria *Shewanella oneidensis*." *Nature Biotechnology* **20**(11): 1118-1123.

Heijerman, H. *et al.* (2009). "Inhaled medication and inhalation devices for lung disease in patients with cystic fibrosis: a European consensus." *Journal of Cystic Fibrosis* **8**(5): 295-315.

Heilmann, C. *et al.* (1996). "Molecular basis of intercellular adhesions in the biofilm-formation *Staphylococcus epidermidis*." *Molecular Microbiology* **20**: 1083-1091.

Hendrickson, E. L. *et al.* (2001). "Differential roles of *Pseudomonas aeruginosa* PA14 *rpoN* gene in pathogenicity in plants, nematodes, insects and mice." *Journal of Bacteriology* **183**(24): 7126-7134.

Henig, N. R. *et al.* (2001). "Sputum induction as a research tool for sampling the airways of subjects with Cystic Fibrosis." *Thorax* **56**: 306-311.

Henrichsen, J. *et al.* (1972). "Bacterial surface translocation – a survey and a classification." *Bacteriology Reviews* 36: 478-503.

Henry, D. A. *et al.* (2001). "Phenotypic methods for determining genomovar status of the *Burkholderia cepacia* complex." *Clinical Microbiology* **39**(3): 1073-1078.

Herbert, D. *et al.* (1956). "The continuous culture of bacteria: a theoretical and experimental study." *General Microbiology* **14**: 601-622.

Hermann, G. *et al.* (2010). "Colistin-tobramycin combinations are superior to monotherapy concerning the killing of biofilm *Pseudomonas aeruginosa*." *Infectious Diseases* **202**: 1585-1592.

Hodges, N. A. and Gordon C. A. (1991). "Protection of *Pseudomonas aeruginosa* against ciprofloxacin and beta-lactam by homologus alginate." *Antimicrobial agents and Chemotherapy* **35**: 2450-2452.

Hodges, C. A. *et al.* (2008). "Infertility in females with cystic fibrosis is multifactorial: evidence from mouse models." *Endocrinology* **6**(149): 2790-2797.

Hodson, M. E. *et al.* (1995). "Maintenance treatment with antibiotics in cystic fibrosis patients. Sence or nonsence?" *The Netherlands Journal of Medicine* **46**(6): 288-292.

Hoffmann, J. A. *et al.* (1995). "Review Innate Immunity of Insects." *Current opinions in Immunology* **7**(1): 4-10.

Hoiby, N. *et al.* (2002). "New antimicrobials in the management of cystic fibrosis." *Antimicrobial Chemotherapy* **49**: 235-238.

Hoiby, N. *et al.* (2005). "Eradication of early *Pseudomonas aeruginosa* infection"

Journal of Cystic Fibrosis **2**: 49-54.

Horrevorts, A. M. *et al.* (1987). "Checkerboard titrations: the influence of the composition of serial dilutions of antibiotics on the fractional inhibitory concentration index and fractional bactericidal concentration index." *Antimicrobial agents and Chemotherapy* **19**: 119-125.

Hoult, J. R. *et al.* (1996). "Pharmacological and biochemical actions of simple coumarins: natural products with therapeutic potential." *General Pharmacology* **27**(4): 713-722.

Houry, A. *et al.* (2009). "Involvement of motility and flagella in *Bacillus cereus* biofilm formation." *Microbiology* **156**(4): 1009-1018.

Hubbard, J. A. *et al.* (1986). "Effects of iron limitation of *Escherichia coli* on growth, the respiratory chains and gallium uptake." *Archives in Microbiology* **146**: 80-86.

Ichikawa, Y. *et al.* (1992). "Erythromycin reduces neutrophils and neutrophil-derived elastolytic-like activity in the lower respiratory tract of bronchiolitis patients." *American Review of Respiratory Disease* **146**: 196-203.

Ito, A. *et al.* (2009). "Increased antibiotic resistance of *Escherichia coli* in mature biofilms." *Applied and Environmental Microbiology* **75**(12): 4093-4100.

Jaffe, *et al.* (1998). "Long-term azithromycin may improve lung function in children with cystic fibrosis." *Lancet* 351-420.

Jander, G. *et al.* (2000). "Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects." *Bacteriology* **182**: 3843-3845.

Jayaraman, S. *et al.* (1999). "Long-wavelength iodide-sensitive fluorescent indicators for measurement of functional CFTR expression in cells." *American Journal of Physiology* **277**: 1008-1018.

Jurivich, D. A. *et al.* (1995). "Salicylate triggers heat shock factor differently than heat." *Journal of Biological Chemistry* **270**: 24489-24495.

Kalishwaralal, K. *et al.* (2010). "Silver nanoparticles impede the biofilm formation of

Pseudomonas aeruginosa and *Staphylococcus epidermidis*." *Colloids and Surfaces. B: Biointerfaces* **79**(2): 340-344.

Karaba, S. M. *et al.*, (2013). "*Stenotrophomonas maltophilia* encodes a type II protein secretion system that promotes detrimental effects on lung epithelial cells." *Infection and Immunity* **81**(8).

Karpati, F. *et al.*, (1994). "Bacterial colonization with *Xanthomonas maltophilia*: a retrospective study in cystic fibrosis patient population." *Infection* 22:258-263.

Kelman, A. *et al.* (1973). "The role of motility and aerotaxis in the selective increase of avirulent bacteria in still broth cultures of *Pseudomonas solanacearum*." *Journal of General Microbiology* **76**: 177-188.

Kelly, N. M. *et al.* (1990). "Comparison of the outer membrane protein and lipopolysaccharide profiles of mucoid and nonmucoid *Pseudomonas aeruginosa*. *Clinical Microbiology*. **28**: 2017-2021.

Keren, I. *et al.* (2004). "Persister cells and tolerance to antimicrobials." *Microbiological Letters* **230**: 13-18.

Kerr, J. R. *et al.* (1999). "*Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth." *Journal of Clinical Pathology* **52**(385): 385-387.

Khan, T. Z. *et al.* (1995). "Early pulmonary inflammation in infants with cystic fibrosis." *American Journal of Respiratory Critical Care Medicine* **151**: 1095-1082.

Khot, P. D. *et al.* (2008). "Development and optimisation of quantitative PCR for the diagnosis of invasive aspergillosis with bronchoalveolar lavage fluid." *BMC Infectious Diseases* **8**: 73.

Kim, E. *et al.* (2003). "Iron deficiency leads to inhibition of oxygen transfer and enhanced formation of virulence factors in cultures of *Pseudomonas aeruginosa* PA01." *Microbiology* **149**: 2627-2634.

Kim, J. S. *et al.* (2007). "Antimicrobial effects of silver nanoparticles." *Nanomedicine* **3**(1): 95-101.

Klausen, M. *et al.* (2003). "Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms." *Molecular Microbiology* **50**: 61-68.

Kohler, T. H. K. *et al.* (2000). "Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signalling and requires flagella and pili." *Bacteriology* **182**: 5990-5996.

Konstan, M. W. *et al.* (1993). "Leukotriene B4 markedly elevated in the epithelial lining fluid of patients with cystic fibrosis." *Respiratory Disease* **8**(14): 896-901.

Konstan, M. W. *et al.* (1994). "Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation." *Respiratory Critical Care Medicine*: 448-454.

Kopp, E. *et al.* (1994). "Inhibition of NKkB by sodium salicylate and aspirin." *Science* **265**: 956-959.

Korber, D. R. *et al.* (1989). "Effects of laminar flow velocity on the kinetics of surface colonisation by mot$^+$ and mot$^-$ *Pseudomonas fluorescens*." *Microbial Ecology* **18**: 1-19.

Kostova, I. *et al.* (2007). "Studying plant derived coumarins for their pharmacological and therapeutic properties as potential anti-cancer agents." *Expert Opinions in Drug Discovery* **2**: 1605-1618.

Kreth, J. J. *et al.* (2005). "Competition and co-existence between *Streptococcus mutans* and *Streptococcus sangiuires* in the dental biofilm." *Journal of Bacteriology* **187**: 7193-7203.

Krista, D. L. *et al.* (1996). "Accuracy of four commercial systems for identification of *Burkholderia cepacia* and other gram-negative non-fermenting bacilli recovered from patients with cystic fibrosis." *Clinical Microbiology* **34**: 886-891.

Kumon, H. *et al.* (1994). "A sandwich cup method for the penetration assay of antimicrobial agents through *Pseudomonas* exopolysaccharides." *Microbiology and Immunology* **38**: 615-619.

Kunert, A. *et al.* (2007). "Immune evasion of the human pathogen *Pseudomonas*

aeruginosa: elongation factor Tuf is a factor H and plasminogen binding protein." *Journal of Immunology* **179**(5): 2979-2988.

Kuramitsu, H. K. *et al.* (2007). "Interspecies interactions within oral microbial communities." *Microbiology and Molecular Biology Reviews* **71**(4): 653-670.

Lam, J. *et al.* (1980). "Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis." *Infection and Immunity* **28**: 546-556.

Lambert, R. J. W. *et al.* (2004). "The synergistic effect of EDTA/antibiotic combinations of *Pseudomonas aeruginosa*." *Journal of Applied Microbiology* **96**(2): 244-253

Langford, *et al.* (1989). "Outer membrane proteins of gentamicin induced small colony variants of *Pseudomonas aeruginosa.*" *FEMS Microbiology Letters* **52**(1-2): 33-36.

Lansdown, A. B. G. and Williams, A., (2007). "Bacterial resistance to silver in wound care and medical devices." *Journal of Wound Care* **16**(1): 15-19.

Latifi, A. *et al.* (1996). "A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS." *Molecular Microbiology* **21**: 1137-1146.

LeBlanc, L. *et al.* (2006). "Fluoroquinolones and risk for methicillin-related *Staphylococcus aureus*, Canada." *Emerging Infectious Diseases* **12**: 1398-1405.

LeGrys, V. A. *et al.* (1996). "Sweat testing for the diagnosis of CF practical considerations." *Journal of Paediatrics* **129**(6): 892-7.

Leitch, E. C. *et al.* (1999). "Lactoferrin increases the susceptibility of *S. epidermidis* biofilms to lysozyme and vancomycin." *Current Eye Research* **19**: 12-19.

Lembke, C. *et al.* (2006). "Characterisation of biofilm formation by clinically relevant serotypes of group A streptococci." *Applied and Environmental Microbiology* **72**: 2864-2875.

Lentsch, A. B. *et al.* (1997). "*In vivo* suppression of NBkB and preservation of IkB alpha by interleukin-10 and interleukin-13." *Journal of Clinical Investigation* **100**: 2443-2448.

Lewenza, S. *et al.* (2001). "Regulation of ornibactin synthesis and N-acyl-l-homoserine lactone production by CepK in *Burkholderia cepacia*." Journal of Bacteriology **183**: 2212-2218.

Lewis, H. A. *et al.* (2004). "Structure of nucleotide-binding domain 1 of the cystic fibrosistransmembrane conductance regulator." *EMBO Journal* **2**(23): 282-293.

Lewis, K. and Speoring, A. L. (2001). "Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials." *Bacteriology* **183**: 1262-1268.

Li, X. Z. *et al.* (1995). "Role of MexA-MexB-OprM in antibiotic efflux in *Pseudomonas aeruginosa*." *Antimicrobial Agents and Chemotherapy* **39**: 1948-1953.

Li, X. Z. *et al.* (1997). "Silver-resistant mutants of *Escherichia coli* display active efflux of Ag+ and are deficient in porins." *Journal of Bacteriology* **179**: 6127-6132.

LiPuma, J. *et al.* (1990). "Person-to-person transmission of *Pseudomonas cepacia* between patients with cystic fibrosis." *Lancet* **336**: 1094-1096.

LiPuma, J. *et al.* (2001). "*Burkholderia cepacia* complex: a contradiction to lung transplantation in cystic fibrosis." *Transplant Infectious Disease* **3**(3): 149-160.

LiPuma, J. *et al.* (2010). "The changing microbial epidemiology in Cystic Fibrosis." *Clinical Microbiology Reviews* **23**: 299-323.

Loenen, W. A. *et al.* (2006). "S-adenosyl-l-methionine: jack of all trades and master of everything?" *Biochemical Society transactions* **34**(part2): 330-333. .

Looney, W. J. *et al.* (2005). "Role of *Stenotrophomonas maltophilia* in hospital-acquired infection." *Biomedical Science* **62**: 145-154.

Lukacs, G. L. *et al.* (1994). "Conformational maturation of CFTR but not its mutant counterpart (delta F508) occurs in the endoplasmic reticulum and required ATP."

EMBO Journal **24**(13): 6076-6086.

Lyczak, J. B. *et al.* (2000). "Establishment of *Pseudomonas aeruginosa* infection from a versatile opportunist." *Microbes and Infection* **2**(9): 1051-1060.

MacFarlane, E. L. *et al.* (1999). "PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer membrane protein OprH and polymyxin B resistance." *Molecular Microbiology* **34**: 305-316.

Malic, B. *et al.* (2009). "Detection and identification of specific bacteria in wound biofilms using peptide nucleic acid fluorescent *in situ* hydridization (PNA FISH)." *Microbiology* **155**: 2603-2611.

Manz, W. *et al.* (1999). "Phylogenetic composition, spatial structure and dynamic of lotic bacterial biofilms investigated by fluorescent *in situ* hybridization and confocal laser scanning microscopy." *Microbial Ecology* **37**: 225-237.

Markowitz, S. M. *et al.* (1979). "Antimicrobial susceptibility of mucoid *Pseudomonas aeruginosa* and their spontaneously occurring non-mucoid derivatives." *Journal of Antimicrobial Chemotherapy* **6**(2): 251-260.

Marvin, M. J. *et al.* (1989). "Release of outer membrane fragments from wild-type *Escherichia coli* and several *E. coli* lipopolysaccharide mutants by EDTA and heat shock treatments." *Journal of Bacteriology* **171**(10): 5262-5267.

Marshall, K. C. *et al.* (1985). "Mechanisms of bacterial adhesion at solid-water interfaces." *Bacterial Adhesion: Mechanisms and Physiological significance.* New York, Plenum Press. 133-167.

Masten, J. A. *et al.* (1981). "Rapid reporting of results impact on patients and the laboratory." *American Society of Microbiology* **98**: 98-102.

Matsukawa, M. *et al.* (2004). "Putative exopolysaccharide synthesis genes influence *Pseudomonas aeruginosa* biofilm development." *Journal of Bacteriology* **186**: 4449-4456.

Maxwell, A. *et al.* (1993). "The interaction between coumarin drugs and DNA gyrase." *Molecular Microbiology* **9**(4): 681-686.

McAllister, F. *et al.* (2005). "Role of IL-17A and IL-17F and the IL-17 receptor in regulating growth related oncogene-alpha and granulocyte colony-stimulating factor in bronchial epithelium: implications for airway inflammation in cystic fibrosis." *Journal of Immunology* **175**: 404-412.

McPhee, J. B. *et al.* (2003). "Cationic antimicrobial peptides activate a two-compliment regulatory resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*." *Molecular Microbiology* **50**: 205-217.

Mhanna, M. J. *et al.* (2001). "Nitric oxide deficiency contributes to impairment of airway relaxation in cystic fibrosis mice." *Respiratory Cell and Molecular Biology* **24**: 621-626.

Mizukane, R. Y. *et al.* (1994). "Comparative *in vitro* exo-enzyme-suppressing activities of azithromycin and other macrolide antibiotics against *Pseudomonas aeruginosa*." *Antimicrobial Agents and Chemotherapy* **38**: 528-533.

Molinari, G. C. *et al.* (1994). "Inhibition of *Pseudomonas aeruginosa* virulence factors by sub-inhibitory concentrations of azithromycin and other macrolide antibiotics." *Antimicrobial Agents and Chemotherapy* **31**: 681-688.

Monden, K. *et al.* (2002). "Role of fosfomycin in a synergistic combination with ofloxacin against *Pseudomonas aeruginosa* growing in a biofilm." *Infection and Chemotherapy* **8**(3): 218-226.

Moriarty, T. F. *et al.* (2007). "Sputum antibiotic concentrations: Implications for treatment of cystic fibrosis lung infection." *Paediatric Pulmonology* **42**: 1008-1017.

Morris, C. E. *et al.* (1997). "Methods of observing microbial biofilms directly on leaf surfaces and recovering them for isolation and culturable microorganisms." *Applied and Environmental Microbiology* **63**(4): 1570-1576.

Mortensen, K. L. *et al.* (2011). "A prospective survey of *Aspergillus* spp. in respiratory tract samples: prevalence, clinical impact and anti-fungal susceptibility." *European Journal of Clinical Microbiology Infections and Disease* **30**: 1355-1363.

Moskowitz S. M. *et al.* (2005). "Use of Pseudomonas biofilm susceptibilities to assign simulated antibiotic regimens for cystic fibrosis airway infection."

Antimicrobial agents and Chemotherapy **56**: 876-886.

Moskowitz, S. M. *et al.* (2004). "Clinically Feasible Biofilm Susceptibility Assay for Isolates of *Pseudomonas aeruginosa* from Patients with Cystic Fibrosis." *Clinical Microbiology* **42**(5): 1915-1922.

Moter, A. *et al.* (2000). "Fluorescent *in situ* hybridisation (FISH) for direct visualisation of microorganisms." *Journal of Microbiological Methods* **41**: 85-112.

Mousa, S. *et al.* (2002). "Anticoagulants and Thrombosis and Cancer. The missing link." *Expert Reviews in Anticancer Therapy* **2**: 227-233.

Muhlebach, M. *et al.*, (1999). "Quantitation of inflammatory responses to bacteria in young CF and control patients." *American Journal of Respiratory Critical Care Medicine* **160186**: 191.

Mulet, X. *et al.* (2009). "Azithromycin in *Pseudomonas aeruginosa* biofilms: bactericidal activity and selection of nfxB mutants." *Antimicrobial Agents and Chemotherapy* **53**: 1552-1560.

Musk, D. J. *et al.* (2005). "Iron salts perturb biofilm formation and disrupt existing biofilms of *Pseudomonas aeruginosa*." *Chemico-Biological Interactions* **12**: 789-796.

Mylonakis, E. *et al.* (2005). "*Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis." *Infection and Immunity* **73**: 3842-3850.

NCCLS, (2000). Sweat testing: Sample collection and quantitative analysis: Appoved guidelines, Second edition. Wayne, the Committee.

Ng, T. B. *et al.* (1996). "Examination of coumarins, flavonoids and polysaccharides for antibacterial activity." *General Pharmacology* **27**(7): 1237-1240.

Nickel, J. C. *et al.* (1985). "Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material." *Antimicrobial Agents and Chemotherapy* 17: 619-624.

Nies, D. H. *et al.* (1999). "Microbial heavy-metal resistance." *Applied Microbiology and Biotechnology* **51**: 730-750.

Nies, D. H. *et al.* (2003). "Efflux-mediated heavy metal resistance in prokaryotes." *Microbiology Reviews* **27**: 313-339.

Nivens, D. E. *et al.* (2001). "Role of alginate and its *O*-acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms." *Bacteriology* **183**: 1047-1057.

O' Sullivan, C. *et al.* (2009). "Application of flowcell technology for monitoring biofilm development and cellulose degradation in leachate and rumen systems." *Bioresourse Technology* **100**(1): 492-496.

O'Toole, G. A. *et al.* (1999). "Genetic Approaches to study biofilms." *Methods in Enzymology* **310**: 91-109.

O'Toole, G. *et al.* (2000). "Biofilm formation as microbial development." *Annual Review of Microbiology* **54**: 49-79.

Ogle, J. W. *et al.* (1988). "Development of resistance in *Pseudomonas aeruginosa* to imipenem, norfloxacin and ciprofloxacin during therapy: proof provided by typing with a DNA probe." *Journal of Infectious Diseases* **157**: 743-748.

Okazaki, A. *et al.* (2007). "Aph(3')-IIc, an aminoglycoside resistance determinant from *Stenotrophomonas maltophilia*." *Antimicrobial Agents and Chemotherapy* **51**(1): 359-360.

Olakanmi, O. *et al.* (2000). "Gallium disrupts iron metabolism of mycobacteria residing within human macrophages." *American Society of Microbiology* **68**(10): 5619-5627.

Oliver, A. *et al.* (2010). "Mutators in cystic fibrosis chronic lung infection: Prevalence, mechanisms and consequences for antimicrobial therapy." *International Journal of Medical Microbiology* **300**: 563-572.

Oliver, A. *et al.* (2000). "High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection." *Science* **288**: 1251-1254.

Otkun, M. T. *et al.* (2005). "The antimicrobial susceptibility of *Stenotrophomonas maltophilia* isolates using three different methods and their genetic relatedness." *BMC Microbiology* **5**: 1-6.

Outten, F. W. *et al.* (2001). "The independent *cue* and *cus* systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*." *Biological Chemistry* **276**: 30670-30677.

Palmer, R. L. *et al.* (1999). "Microscopy flowcells: perfusion chambers for real-time study of biofilms." *Methods in Enzymology* **310**: 160-166.

Pamp, S. J. *et al.* (2008). "Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes." *Molecular Microbiology* **68**(1): 223-240.

Parad, R. B. *et al.* (1999). "Pulmonary outcome in cystic fibrosis is influenced primarily by mucoid *Pseudomonas aeruginosa* infection and immune status and only modestly by genotype." *Infection and Immunity* **9**: 4744-4750.

Parsek, M. R. *et al.* (2003). "Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*." *Applied and Environmental Microbiology* **69**: 2313-2320.

Parson, D. *et al.* (2006). "Silver: clarifying the claims." *Ostomy Wound Management* **52**: 34-41.

Paugam, A. *et al.* (2010). "Characteristics and consequences of airway colonisation by filamentous fungi in 201 adult patients with Cystic Fibrosis on France." *Medical Mycology* **48**: 32-36.

Pearson, J. P. *et al.* (1994). "Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes." *Proceedings of the National Academy of Sciences*, USA **91**: 197-201.

Peeters, E. *et al.* (2009). "Resistance of planktonic and biofilm-grown *Burkholderia cepacia* complex isolates to the transition metal gallium." *Antimicrobial Agents and Chemotherapy* **63**(5): 1079.

Peleg, A. Y. *et al.* (2009). "*Galleria mellonella* as a model system to study *Acinetobacter baumannii* pathogenesis and therapeutics." *Antimicrobial Agents and Chemotherapy* **53**: 2605-2609.

Perez, A. *et al.*, (2007). "CFTR inhibition mimics the cystic fibrosis inflammatory profile." *American Journal of Physiology, Lung cellular and Molecular physiology* **292**(2): 383-395.

Percival, S. L. *et al.* (2008). "Prevalence of silver resistance in bacteria isolated from diabetic foot ulcer and efficacy of silver-containing wounds." *Wound Management* **54**(3): 30-40.

Perdreau-Remington, F. *et al.* (1998). "The abilities of a *Staphylococcus epidermidis* wild-type strain and its slime-negative mutant to induce endocarditis in rabbits are comparable." *Infection and Immunity* **66**: 2778-2781.

Perks, B. *et al.* (2000). "DNA and actin bind and inhibit interleukin-8 function in cystic fibrosis sputa: *In vitro* effects of mucolytics." *American Journal of Respiratory Critical Care Medicine* **162**: 1767-1772.

Pesci, E. C. *et al.* (1997). "Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*." *Bacteriology* **179**: 3127-3132.

Pihet, M. *et al.* (2009). "Melanin is an essential component for the integration of the cell wall of *Aspergillus fumigatus* conidia." *BMC Microbiology* **9**: 177.

Pihl, M. *et al.* (2010). "Effects of clinical isolates of *Pseudomonas aeruginosa* on *Staphylococcus aureus* biofilm formation." *Immunology and Medical Microbiology* **59**: 504-512.

Pitt, T. L. *et al.* (1996). "Type characterisation and antibiotic susceptibility of *Burkholderia* (Pseudomonas) *cepacia* isolates from patients with cystic fibrosis in the UK and Republic of Ireland." *Journal of Microbiology* **44**: 203-210.

Pompilio, A. *et al.* (2011). "Phenotypic and genotypic characterisation of *Stenotrophomonas maltophilia* isolate from patients with cystic fibrosis: genome, diversity, biofilm formation and virulence." *BMC Microbiology* **11**: 1-16.

Poole, K. *et al.* (2001). "Multi-drug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* related organisms." *Journal of Molecular Microbiology and Biotechnology* **3**: 255-264.

Prigent-Cambaret, C. *et al.* (2000). "Development pathway for biofilm formation in curli-producing *E. coli* strains role of flagella, curli and colanic acid." *Environmental Microbiology* **2**(4): 3593-3596.

Prithiviraj, B. *et al.* (2005). "Down regulation of virulence factors of *Pseudomonas aeruginosa* by salicylic acid attenuates its virulence on *Arabidopsis thaliana* and *Caenorhabditis elegans*." *Infection and Immunity* **73**: 5319-5328.

Prunier, A. L. *et al.* (2003). "High rate of macrolide resistance in *Staphylococcus aureus* strains from patients with cystic fibrosis reveals high proportions of hypermutable strains." *Journal of Infectious Diseases* **187**: 1709-1716.

Prunier, A. L. *et al.* (2005). "Role of mutS and mutL genes in hypermutability and recombination in *Staphylococcus aureus*." *Journal of Bacteriology* **187**: 3455-3464.

Quinton, P. M. *et al.* (2008). "Cystic Fibrosis: impaired bicarbonate secretion and mucoviscidosis." **9636**(372): 415-417.

Raad, I. *et al.* (1996). "Silver iontophoretic catheter: a prototype of a long-term anti-infective vascular access device." *Journal of Infectious Diseases* **173**: 495-498.

Rabodonirina, M. *et al.* (1997). "Rapid detection of *Pneumocystis carinii* in bronchoalveolar lavage specimens from human immunodeficiency virus-infected patients: use of a simple DNA extraction and nested PCR." *Journal of Clinical Microbiology* **35**: 2748-2751.

Rainey, P. B. *et al.* (1998). "Adaptive radiation in a heterogeneous environment." *Nature* **394**: 69-72.

Rambaugh, K. P. *et al.* (1999). "Contribution of the regulatory gene lasR to the pathogenesis of *Pseudomonas aeruginosa* infection of burned mice." *Journal of Burn Care and Rehabilitation* **20**: 42-49.

Ramsey, D. M. *et al.*, (2005). "Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects managed of chronic infection in cystic fibrosis." *Molecular Microbiology* 56: 309-322.

Ranieri, E. *et al.*, (1994). "Neonatal screening for cystic fibrosis using

immunoreactive trypsinogen and direct gene analysis. Four year experiment." *British Medical Journal* **308**: 1469.

Rao, D. *et al.* (2005). "Competitive interactions in mixed-species biofilms containing the marine bacterium *Pseudoalteromonas tunicata*." *Applied and Environmental Microbiology* **71**(4): 1729-1736.

Rashid, M. H. *et al.* (2000). "Inorganic polyphosphate is needed for swimming, swarming and twitching motilities of *Pseudomonas aeruginosa*." *Proceedings of the National Academy of Sciences* **97**: 4885-4890.

Rasmussen, T. B. *et al.* (2005). "Screening from QS inhibitors (QSI) by use of a novel genetic system, the QSI selector." *Journal of Bacteriology* **5**(187): 1799-1814.

Ratcliff, N. *et al.* (1985). "Invertebrate immunity-a primer for the non-specialist." *Immunology Letters* **10**(5): 253-270.

Ratjen, F. and Doring, G. (2003). "Cystic Fibrosis." *Lancet* **361**: 681-689.

Reeves, E. P. and Kavanagh K. (2004). "Correlation of Glicotoxin production and virulence in *Aspergillus fumigatus* in *Galleria mellonella.*" *Micopathologica* **158**(1): 73-79.

Rensing, C. *et al.* (2003). "*Escherichia coli* mechanisms of copper homeostasis in a changing environment." *FEMS Microbiology Reviews* **27**: 197-213.

Rice, S. A. *et al.* (2005). "Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutritional cues." *Journal of Bacteriology* **187**(10): 3477-3485.

Riedel, K. *et al.* (2001). "N-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms." *Microbiology* **147**: 3249-3262.

Riordan, J. R. *et al.* (2008). "CFTR function and prospects for therapy." *Annual Review of Biochemistry* **77**: 701-726.

Riordan, J. R. *et al.* (1989). "Identification of cystic fibrosis gene: cloning and

characterisation of complementary DNA." *Science* **245**: 1066-1073.

Roberts, L. A. *et al.* (1990). "An Australia-wide epidemic of *Pseudomonas pickettii* bacteraemia due to contaminated 'sterile' water for injection." *Medical Journal Australia* **152**: 652-655.

Rogers, G. B. *et al.* (2004). "Characterisation of bacterial community diversity in CF lung infections by use of 16S Ribsomal DNA terminal restriction fragment length polymorphism profiling." *Clinical Microbiology* **42**(11): 5176-5183.

Rohr, U. *et al.* (1999). "Four years of experience with silver-copper ionization for control of *Legionella* in a German university hospital hot water plumbing system." *Clinical Infectious Diseases* **29**(6): 1507-1511.

Rosenfeld, *et al.* (1999). "Diagnostic accuracy or oropharyngeal cultures in infants and young children with Cystic Fibrosis." *Paediatric Pulmonology* **28**: 321-328.

Rosselli, S. *et al.* (2007). "Antibacterial and anticoagulant activities of coumarins isolated from flowers of Magydaris tomentosa." *Planta Medica* **73**(2): 116-120.

Rowe, S. M. *et al.* (2005). "Cystic Fibrosis." *New England Journal of Medicine* **352**: 1992-2001.

Ruiz-Ponte, C. *et al.* (1998). "*Roseobacter gallaeciensis* sp. nov., a new marine bacterium isolated from rearing and collectors of the scallop *Pecten maximus*." *Systemic and Evolutionary Microbiology* **48**: 537-542.

Ryan, R. *et al.* (2008). "Interspecies signalling via the *Stenotrophomonas maltophilia* diffusible signal factor influences biofilm formation and polymyxin tolerance in *Pseudomonas aeruginosa.*" *Molecular Microbiology* **68**: 75-86.

Ryan, R. J. *et al.* (1995). "Treatment of pulmonary *aspergillosis* in cystic fibrosis by percutaneous installation of amphothercin B via catheters." *Thorax* **50**(7).

Sagel, S. D. *et al.* (2001). "Airway inflammation in children with cystic fibrosis and healthy children assessed by sputum induction." *Respiratory Critical Care Medicine* **6**(16): 1425–1431.

Saha, S. *et al.* (2001). "Rapid identification of antibiotic susceptibility testing of *Salmonella enterica* Serovar Typhi isolated from blood: Implications for therapy." *Journal of Clinical Microbiology* **39**(10): 3583-3585.

Saiman, L. *et al.* (2004). "Microbiology of early cystic fibrosis lung disease." *Paediatric Respiratory Reviews* **5**: 367-369.

Saiman, L. *et al.* (2003). "Azithromycin in patients with cystic fibrosis chronically infected with *Pseudomons aeruginosa*: a randomised controlled trial. *JAMA* **290**: 1749-1756.

Saiman, L. *et al.* (1999). "Evaluation of reference dilution test methods for antimicrobial susceptibility testing for *Pseudomonas aeruginosa* strains isolated from patients with cystic fibrosis." *Clinical Microbiology* **37**: 2987-2991.

Saiman, L. *et al.* (2003). "Evaluation of MicroScan Autoscan for identification of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients." *Clinical Microbiology* **41**: 492-494.

Salunkhe, P. *et al.* (2005). "A cystic fibrosis epidemic strain of *Pseudomonas aeruginosa* displays enhanced virulence and antimicrobial resistance." *Bacteriology* **187**: 4908-4920.

Samonis, G. *et al.* (2012). "*Stenotrophomonas maltophilia* infections in a general hospital: patient characteristics, antimicrobial susceptibility and treatment outcome." PloSOne **7**(5).

Sampaio, S. C. *et al.* (2009). "The flagella of an atypical enteropathogenic *Escherichia coli* strain are required for efficient interaction with and stimulation of interleukin-8 production by enterocytes *in vitro*." *Infection and Immunity* **77**(10): 4406-4413.

Sauer, K. *et al.* (2002). "*Pseudomonas aeruginosa* displays multiple phenotypes during biofilm development." *Bacteriology* **184**: 1140-1154.

Sauer, K. *et al.* (2004). "Characterisation of nutrient induced dispersion of *Pseudomonas aeruginosa* PA01." *Journal of Bacteriology* **186**(21): 7312-7326.

Schottelius, A. J. *et al.* (1999). "Interleukin-10 signalling blocks inhibitor of kB kinase activity and nuclear factor kB DNA binding." *Journal of Biological Chemistry* **274**: 31868-31874.

Scott, F. W. *et al.* (2004). "Identification and characterisation of transmissible *Pseudomonas aeruginosa* strains in cystic fibrosis patients in England and Wales." *Journal of Medical Microbiology* **53**(7): 609-615.

Seed, K. D. *et al.* (2008). "Development of *Galleria mellonella* as an alternative infection model for the *Burkholderia cepacia* complex." *Infection and Immunity* **76**(3): 1267-1275.

Seibert, G. *et al.* (1992). "The *in vitro* antibiotic activity of a combination of cefpirome or cefoperazone with vancomycin against enterococci and *Staphylococcus aureus*." *Journal of Antimicrobial Chemotherapy* **38**: 1067-1071.

Seifert, H. *et al.* (2003). "Small colony variants of *Staphylococus aureus* and pacemaker-related infection".

Senol, E. *et al.* (2004). "*Stenotrophomonas maltophilia*: the significance and role as a nosocomial pathogen." *Hospital Infection* **57**: 1-7.

Shelly, D. B. *et al.* (2000). "Utility of commercial systems for identification of *Burkholderia cepacia* complex from cystic fibrosis sputum culture." *Clinical Microbiology* **38**: 3112-3115.

Sheppard, D. N. *et al.* (1993). "Mutation in CFTR associated with mild disease form Cl- channels with altered pore properties." *Nature* **6416**(362): 160-164.

Shigeta, M. *et al.* (1997). "Permeation of antimicrobial agents through *Pseudomonas aeruginosa* biofilms: a simple method." *Bacteriology* **43**: 340-345.

Sillankorva, S. *et al.* (2008). "*Pseudomonas fluorescens* biofilm subjected to phage phiIBB PF7A." *Biotechnology* **8**: 1-8.

Silver, S. *et al.* (1996). "Bacterial heavy metal resistance: new surprises." *Annual Review of Microbiology* **50**: 753-789.

Silver, S. *et al.* (1998). "Genes for all metals-a bacterial view of the Periodic Table: The 1996 Thom award lecture." *Industrial Microbiology and Biotechnology* **20**: 1-12.

Singh, P. K. *et al.* (2003). "Bacterial biofilms: An emerging link to disease pathogenesis." *Annual Review of Microbiology* **57**: 677-701.

Singh, P. K. *et al.* (2007). "The transition metal gallium disrupts *Pseudomonas aeruginosa* iron metabolism and has antimicrobial and antibiofilm activity." *American Society for Clinical Investigation* **117**(4): 877-888.

Singh, R. *et al.* (2003). "Microbial diversity of biofilms in dental unit water systems." *Applied and Environmental Microbiology* **69**: 3412-3420.

Skillman, L. C. *et al.* (1999). "The role of exopolysaccharides in dual species biofilm formation." *Applied Microbiology* **85**: 13-18.

Skindersoe, M. E. *et al.* (2008). "Effects of antibiotics on quorum sensing in *Pseudomonas aeruginosa.*" *Antimicrobial Agents and Chemotherapy* **52**(10): 3648-3663.

Slawson, R. M. *et al.* (1992). "Germanium and silver resistance, accumulation and toxicity in microorganisms." *Plasmid* **27**(1): 72-79.

Smith, A. L. *et al.* (1997). "Pathogenesis of bacterial bronchitis in cystic fibrosis." *The Paediatric Infectious Disease Journal* **57**(8): 1018-1023.

Smith, A. L. *et al.* (1988). "Sputum changes associated with therapy for endobronchial exacerbation in cystic fibrosis." *Paediatrics* **112**: 547-554.

Smith, A. L. *et al.* (1999). "Comparison of B-lactam alone versus B-lactam and an aminoglycoside for pulmonary exacerbation in cystic fibrosis." *Paediatrics* **134**: 413-421.

Smith, A. W. *et al.* (2002). "Characterisation of the dihydrolipoamide dehydrogenase from *Streptococcus pneumoniae* and its role in pneumococcal infection." *Molecular Microbiology* **44**(2): 431-448.

Smith, K. *et al.* (2008). "Biofilm formation by Scottish clinical isolates of

Staphylococcus aureus." *Journal of Medical Microbiology* **57**(8): 1018-1023.

Smyth, T. *et al.* (2009). "A study of the antimicrobial activity of selected naturally occurring synthetic coumarins." *Journal of Antimicrobial Agents* **33**(5): 421-426.

Snelders, E. *et al.* (2008). "Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism." *PLOS Medicine* **5**: 219.

Song, Z. *et al* (1997). "Ginseng treatment reduces bacterial load and lung pathology in chronic *Pseudomonas aeruginosa* pneumonia in rats." *Antimicrobial Agents and Chemotherapy* **41**: 961-964.

Song, Z. *et al.* (2010). "Panax ginseng has anti-infective activity against opportunistic pathogen *Pseudomonas aeruginosa* by inhibiting quorum sensing, a bacterial communication process critical for establishing infection." *Phytomedicine* **17**: 1040-1046.

Soong, G. *et al.* (2006). "Bacterial neuraminidase facilitates mucosal infection by participating in biofilm formation." *Journal of Clinical Infection* **116**(8): 2297-2305.

Speert, D. P. *et al.* (2002). "Epidemiology of *Burkholderia cepacia* complex in patients with cystic fibrosis." *Emerging Infectious Diseases* **8**: 181-187.

Spilker, T. *et al.* (2008). "Recovery of *Herbaspirillum* species from Persons with Cystic Fibrosis." *Microbiology* **46**(8): 2774-2777.

Srinivasan, A. *et al.* (2006). "A prospective trial of a novel silicone based, silver coated foley catheter for the prevention of nosocomial urinary tract infections." *Infection Control and Hospital Epidemiology* **27**: 38-43.

Steinkamp, G. *et al.* (2005). "Prospective evaluation of emerging bacteria in cystic fibrosis." *Journal of Cystic Fibrosis* **4**: 41-48.

Stepanovic, S. *et al.* (2004). "Influence of acetylsalicylic acid (aspirin) on biofilm formation production by *Candida* species." *Chemotherapy* **16**: 134-138.

Stephens, S. *et al.* (2002). "Microbiology: Breaking down biofilms." *Current Biology* **12**: 132-134.

Sternberg, C. *et al.* (1999). "Distribution of bacterial growth activity in flow-chamber biofilms." *Applied and Environmental Microbiology* **65**: 4108-4117.

Stevens, D. A. *et al.* (2003). "Allergic aspergillosis in Cystic Fibrosis-State of the art: Cystic Fibrosis foundation Concensius Conference." *Clinical Infectious Disease* **37**: 225-264.

Stewart, P. S. *et al.* (1996). "Theoretical aspects of antibiotic diffusion into microbial biofilms." *Antimicrobial Agents and Chemotherapy* **40**: 2517-2522.

Stoodley, P. *et al.* (1999). "Influence of hydrodynamics and nutrients on biofilm structure." *Applied Microbiology* **85**: 19-28.

Tack, K. J. *et al.* (1985). "Increased minimum inhibitory concentrations with anaerobiasis for tobramycin, gentamicin, amikacin, compared to laxamoxef, piperacillin, chloramphenicol and clindamycin." *Chemotherapy* **31**: 204-210.

Tang, J. X. *et al.* (2005). "Anionic poly(amino acid) dissolve F-actin and viscosity of cystic fibrosis sputum." *American Journal of Physiology-Lung cellular and Molecular physiology* **289**: 599-605.

Tateda, K. Y. *et al.* (1996). "Direct evidence for antipseudomonal activity of macrolides: exposure-dependent bactericidal activity and inhibition of proteins synthesis by erythromycin, clarithromycin and azithromycin." *Antimicrobial Agents and Chemotherapy* **40**: 2271-2275.

Teitzel, G. M. *et al.* (2006). "Survival and growth in the presence of elevated copper: transcriptional profiling of copper stressed *Pseudomonas aeruginosa*." *Bacteriology* **188**: 7242-7256.

Thati, B. *et al.* (2007). "Mechanism of action of coumarin and silver(I)-coumarin complexes against the pathogenic yeast *Candida albicans*." *Toxicology in Vitro.*" **21**(5): 801-808

Thomassen, M. J. *et al.* (1984). "Cultures of thoracatomy specimens confirm usefulness of sputum cultures in cystic fibrosis." *Journal of Paediatrics* **104**: 352-356.

Tilman, D. *et al.* (1994). "Biodiversity and stability in grasslands." *Nature* **367**: 363-

365.

Tomaz, A. *et al.* (1985). "Antibiotic tolerance among clinical isolates of bacteria." *Reviews of Infectious Diseases* **7**: 368-386.

Tomlin, K. L. *et al.*, (2001). "Interspecies biofilms of *Pseudomonas aeruginosa* and *Burkholderia cepacia*." *Canadian Journal of Microbiology* **47**(10): 949-954.

Towner, K. J. *et al.* (1996). "*Acinetobacter* sp. as nosocomial pathogens: microbiological, clinical and epidemiological features." *Clinical Microbiology Reviews* **9**(2): 148.

Tre-Hardy, M. C. *et al.* (2009). "Effect of antibiotic co-administration on young and mature biofilms of cystic fibrosis clinical isolates: the importance of the biofilm model." *Antimicrobial Agents* **33**: 40-45.

Tre-Hardy, M. *et al.* (2010). "Efficacy of the combination of tobramycin and a macrolide in an *in vitro Pseudomonas aeruginosa* mature biofilm model." *Antimicrobial Agents and Chemotherapy* **54**(10): 4409-4415.

Trezise, A. E. *et al.* (1993). "CFTR expression is regulated during both the cycle of semiinferous epithelium and the oestrous cycle of rodents." *Nature Genetics* **3**: 157-164.

Tunney, M. M. *et al.* (2008). "Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis." *American Journal of Respiratory and Critical Care Medicine* **177**: 995-1001.

Tunney, M. M. *et al.* (2013). "Lung microbiota and bacterial abundance in patients with bronchiectasis when clinically stable and during exacerbation." *American Journal of Respiratory and Critical Care Medicine* 187(10): 1118-1126.

Tuomanen, E. *et al.* (1986). "The rate of killing of *Escherichia coli* by B-lactam antibiotics is strictly proportional to the rate of bacterial growth." *General Microbiology* **132**: 1297-1304.

Turner, R. J. *et al.* (2001). "Tellurite toxicity and resistance in gram-negative bacteria." *Recent Research Developments in Microbiology* **5**(69-77): 69.

Usher, L. R. *et al.* (2002). "Induction of neutrophil apoptosis by the *Pseudomonas aeruginosa* exotoxin pyocyanin: a potential mechanism of persistent infection." *Journal of Immunology* **168**: 1861-1868.

Van Deale, S. *et al.* (2006). "Survey of *Pseudomonas aeruginosa* genotypes in colonised cystic fibrosis patients." *The European Respiratory Journal* **28**(4): 740-747.

Van Ewijk, B. E. *et al.* (2005). "Viral respiratory infections in cystic fibrosis." *Cystic Fibrosis* **4**: 31-36.

Van Goor, F., *et al.* (1996). "Rescue of Delta F508-CFTR trafficking and grating in human cystic fibrosis airway primary cultures by molecules." *American Journal of Physiology-Lung Cellular and Molecular Physiology* **6**(209): 1117-1130.

Van Loodsdrecht, M. C. M. *et al.* (1990). "Influence of interfaces on microbial activity." *Microbiological Reviews* **54**: 75-87.

Vandamme, P. *et al.* (1997). "Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp." *International Journal of Systemic and Evolutionary Microbiology* **47**: 1188-1200.

Venugopalan, V. P. *et al.* (2005). "Architecture of nascent *Sphingomonas* sp. biofilm under varied hydrodynamic conditions." *Applied and Environmental Microbiology* **71**: 2677-2686.

Vial, L. *et al.* (2007). *Burkholderia* diversity and versatility: an inventory of the extracellular products." *Journal of Microbiology and Biotechnology* **17**: 1407-1429.

Visca, P. *et al.* (1992). "Metal regulation of siderophore synthesis in *Pseudomonas aeruginosa* and functional effects of siderophore-metal complexes." *Applied and Environmental Microbiology* **58**: 2886-2893.

Vuong, C. *et al.* (2000). "Impact of the *agr* quorum sensing system on adherence to polystyrene in *Staphylococcus aureus*." *Journal of Infectious Diseases* **182**: 1688-1693.

Wagner, V. E. *et al.* (2008). Pseudomonas Virulence and Pathogenesis Issues. Pseudomonas Genomics and Molecular Biology. P. Cornelis. Norfolk, UK, Caister

Academic Press: 129-158.

Wahab, A. A. *et al.* (2004). "Microbiological identification in cystic fibrosis patients with CFTR11234V mutations." *Journal of Tropical Paediatrics* **50**: 229-233.

Waider, B. *et al.* (2005). "The *H. pylori* CrdRS two-component regulation system is required for copper mediation induction to the copper resistance determinant CrdA." *Journal of Bacteriology* **187**(13): 4683-4688.

Wall, M. A. *et al.* (1983). "Inhaled antibiotics in Cystic Fibrosis." *Lancet* **i**: 1325.

Walters, M. C. *et al.* (2002). "Contributions of antibiotic penetration, oxygen limitation and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to Ciprofloxacin and Tobramycin." *Antimicrobial Agents and Chemotherapy* **47**(1): 317-323.

Walters, S. *et al.* (2007). Epidemiology of cystic fibrosis. In: Hodson M, Geddes DM, Bush A, eds. Cystic Fibrosis, 3rd edition, London, Edward Arnold Ltd, pp. 21-45, ISBN 10.

Wang, W. *et al.* (2007). "*In-vitro* anti-cancer activity and structure-activity relationships of natural products isolated from fruits of Panax ginseng." *Cancer Chemotherapy and Pharmacology* **59**: 589-601.

Ward, C. L. *et al.* (1994). "Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins." *Journal of Biological Chemistry* **41**(269): 25710-25718.

Ward, S. K. *et al.* (2008). "The global responses of *Mycobacterium tuberculosis* to physiological levels of copper." *Bacteriology* **190**: 2939-2946.

Wat, D. *et al.* (2003). "Respiratory virus infections in cystic fibrosis." *Paediatric Respiratory Reviews* **4**: 172-177.

Waters, V. J. *et al.* (2007). "Immunostimulatory properties of the emerging pathogen *Stenotrophomonas maltophilia*." *Infection and Immunity* **75**: 1698-1672.

Whiteley, M. *et al.* (2001). "Gene expression in *Pseudomonas aeruginosa* biofilms."

Nature **413**: 860-864.

Wilfond, B. S. *et al.* (2005). "Policy issues for expanding newborn screening programs: the cystic fibrosis newborn screening experience in the United States." *Journal of Paediatrics* **146**(5): 668-674.

Willner, D. *et al.* (2012). "Comparison of DNA extraction methods for microbial community profiling with an application to BAL samples." **7**(4): 1-12.

Woolwine S. C. *et al.* (1999). Identification of an *Escherichia coli pepA* homolog and its involvement in suppression of the *algB* phenotype in mucoid *Pseudomonas aeruginosa.*" *Journal of Bacteriology*, **181**(1): 107-116.

Yang, L. *et al.* (2011). "Pattern differentiation in co-culture biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*." *Immunology and Medical Microbiology* **62**(3): 339-347.

Yarwood, J. M. *et al.* (2004). "Quorum sensing in *Staphylococcus aureus* biofilms." *Journal of Bacteriology* **186**: 1838-1850.

Yasuda, H. Y. *et al.* (1993). "Interactions between biofilms formed by *Pseudomonas aeruginosa* and clarithromycin." *Antimicrobial Agents and Chemotherapy* **37**: 1749-1755.

Young, W. J. *et al.* (2009). "The antimicrobial efficacy of sustained release silver-carbene complex-loaded L-tyrosine polyphosphate nanoparticles: Characterisation *in vitro* and *in vivo* studies." *Biomaterials* **30**(22): 3771-3779.

Yu, Q. *et al.* (2012). "*In vitro* evaluation of tobramycin and aztneonam versus *Pseudomonas aeruginosa* biofilms on cystic fibrosis derived human airway epithelial cells." *Journal of Antimicrobial Chemotherapy*. **67**(10): 2673-2681.

Zemanick, E. T. *et al.* (2011). "The airway microbiome in cystic fibrosis and implications for treatment." *Current Opinions in Paediatrics* **23**: 319-324.

Zeng, Z. *et al.* (2008). "Virtual screening for novel quorum sensing inhibitors to eradicate biofilm formation of *Pseudomonas aeruginosa*." *Applied Microbiology and Biotechnology* **79**(1): 119-126.

Zhai, B. *et al.*, (2010). "Polymyxin B in combination with fluconazole exerts a potential fungicidal effect." *Journal of Antimicrobial Chemotherapy* **65**: 931-938.

Zhang, T. C. *et al.* (1996). "Evaluation of substrate and pH effects in a nitrifying biofilm." *Water Environment Research* **68**: 1107-1115.

Zmeili, O. S. *et al.* (2007). "Pulmonary aspergillosis: a clinical update." *QJM* **100**(6): 317-334.

Zottola, E. A. and Sasahara K. C. (1994). "Microbial biofilms in the food-processing industry-should they be a concern." *International Journal of Food Microbiology* **23**: 125-148.

Chapter 7: Appendices

Appendix 1: Molecular weights and solubility of metal-free coumarin compounds.

Metal-free coumarin ligands	**Molecular Weight**	**Solubility**
Coumarin 2	224.60	Hot water, alcohols, Ethyl acetate, dichloromethane, DMSO
Coumarin 3	220.18	Hot water, alcohols, Ethyl acetate, dichloromethane, DMSO
Coumarin 4	269.05	Hot water, alcohols, Ethyl acetate, DMSO
Coumarin 5	220.18	Hot water, alcohols, Ethyl acetate, DMSO
Coumarin 6	254.62	Hot water, Hot alcohols, Ethyl acetate, DMSO
Coumarin 7	254.62	Hot water, Hot alcohols, Hot Ethyl acetate, DMSO
Coumarin 8	220.18	Hot water, alcohols, Ethyl acetate, DMSO
CCa (coumarin-3-carboxylic acid	190.15	Hot water, alcohols, Ethyl acetate, DMSO

Appendix 2: Effect of metal-free coumarin compounds against *P. aeruginosa*.

Metal-free coumarin ligands (μM)	***P.aeruginosa* PA01**	***P. aeruginosa* ATCC27853**	***P.aeruginosa* 1826**	***P.aeruginosa* 4276.3**
Coumarin 2	>569.9	>569.9	>569.9	>569.9
Coumarin 3	>581.34	>581.34	>581.34	>581.34
Coumarin 4	>475.74	>475.74	>475.74	>475.74
Coumarin 5	>581.34	>581.34	>581.34	>581.34
Coumarin 6	>502.7	>502.7	>502.7	>502.7
Coumarin 7	>502.7	>502.7	>502.7	>502.7
Coumarin 8	>581.34	>581.34	>581.34	>581.34
CCa (coumarin-3-carboxylic acid	>637	>637	>637	>637

Appendix 3: The effect of different concentrations of gallium nitrate against *P. aeruginosa* planktonic cells and biofilms.

Gallium nitrate (μM)	***P.aeruginosa* PA01**		***P.aeruginosa* ATCC27853**		***P.aeruginosa* 1826**		***P.aeruginosa* 4276.3**	
	MIC	**BIC**	**MIC**	**BIC**	**MIC**	**BIC**	**MIC**	**BIC**
2 μM	>2	>2	>2	>2	>2	>2	>2	>2
5 μM	>5	>5	>5	>5	>5	>5	>5	>5
20 μM	>20	>20	>20	>20	>20	>20	>20	>20
50 μM	>50	>50	>50	>50	>50	>50	>50	>50
200 μM	>200	>200	>200	>200	>200	>200	>200	>200
500 μM	>500	>500	>500	>500	>500	>500	>500	>500
Tobramycin	0.5	1	0.5	1	0.5	0.5	4	32

Appendix 4: Calculations of increasing concentrations of antibiotics based on the MIC (μg/ml) values determined by the broth microdilution method.

(A) *P. aeruginosa* PA01

Antibiotic (μg/ml)	**MIC**	**5xMIC**	**10xMIC**	**20xMIC**
Tobramycin	0.25	1.25	2.5	5
Ceftazidime	4	20	40	80

(B) *P. aeruginosa* 1826

Antibiotic (μg/ml)	**MIC**	**5xMIC**	**10xMIC**	**20xMIC**
Tobramycin	0.5	2.5	5	10
Ceftazidime	1	5	10	20

Appendix 5: Calculations of increasing concentrations of silver nitrate and compound 8 based on the MIC (μM) values determined by the broth microdilution method.

(A) *P. aeruginosa* PA01

Antibiotic (μg/ml)	**MIC**	**10xMIC**	**20xMIC**
Silver nitrate	23.5	235	470
Compound 8	12.2	122	244

(B) *P. aeruginosa* 1826

Antibiotic (µg/ml)	**MIC**	**10xMIC**	**20xMIC**
Silver nitrate	23.5	235	470
Compound 8	12.2	122	244