Evaluating of the effect of sub-inhibitory concentrations of alcohol on *Pseudomonas aeruginosa* across different biofilm models

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A thesis submitted in fulfilment of the requirements of Manchester Metropolitan University for the degree of Master of Science (by Research)

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Table of Contents

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Abstract

Pseudomonas aeruginosa is a major cause of life-threatening nosocomial infections, especially in immunocompromised individuals, and therefore poses a significant threat to public health. This is largely due to its ability to form biofilms which reduces its susceptibility to antimicrobials and host immune defences. Whilst antibiotic resistance (ABR) in *P. aeruginosa* biofilm has been studied extensively, less attention has been paid to the relationship between biofilm and alcohol-based disinfectants. This project aims to explore the extent to which alcohols contribute to biofilm formation in hospital settings by investigating the effect of sub-inhibitory concentrations of ethanol (EtOH) and isopropyl alcohol (propan-2-ol), both common active ingredients in hand sanitisers, on *P. aeruginosa* biofilm growth. This was achieved by establishing the tolerance of 58 clinical *P. aeruginosa* isolates to low concentrations of EtOH and propan-2-ol, quantifying biofilm formation in sub-inhibitory levels of EtOH and determining the effect of sub-inhibitory concentrations of EtOH on established biofilm. Results demonstrate that bacteria were more susceptible to propan-2-ol than EtOH (mean MBC=8.17% and 11.02% respectively), and statistically significant ($P< 0.05$) variation in response to low levels of alcohol exists between *P. aeruginosa* isolates belonging to ST111 and ST235. Most significantly, 19 isolates showed significant ($P< 0.05$) increases in biofilm formation in the presence of 1-3% EtOH compared to 0% EtOH. Finally, *P. aeruginosa* in established biofilms (post 24-hour incubation at 37°C) were able to withstand greater concentrations of EtOH than in the initial stages of biofilm formation. This suggests diluted alcohol-based disinfectants are capable of stimulating biofilm formation in hospital water systems.

1 Introduction

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is ubiquitous in nature and can be found in bodies of water and soils in addition to being a leading cause of human and animal infection and soft rot infection in plants. This is enabled by its inherent and acquired virulence and resistance to antimicrobials, including antibiotics and disinfectants, and an ability to thrive on different nutrient sources in a wide range of temperatures (Silby et al., 2011). This ability to exploit multiple different environmental niches and its relatively large genome enables *P. aeruginosa* to cause a wide range of infections, especially in immunocompromised individuals (Mulcahy et al., 2013). *P. aeruginosa* is a gram-negative, human opportunistic pathogen for high-risk patients including individuals with cystic fibrosis (CF), immunosuppression and chronic cases of obstructive lung disease, wounds, ventilator-associated pneumonia in intubated patients and urinary tract infections in patients with permanent bladder catheters. It has been classified by the World Health Organisation (WHO), Infectious Diseases Society of America and Centers for Disease Control as a major species associated with multiple antimicrobial resistance of urgent public health concern (Boucher et al., 2009), as it is a major cause of lethal nosocomial human infections and is the most common pathogen to cause lung infections in CF patients (Davies, 2002; Sousa and Pereira, 2014).

P. aeruginosa's ability to have multiple inherent and acquired mechanisms of resistance has led to the emergence and worldwide spread of high-risk, multidrug resistant (MDR) clones, posing a major threat to public health (Spagnolo et al., 2021). The past decade has seen the emergence of dominant MDR *P. aeruginosa* strains. A study by Guzvinec et al. (2014) described the clonal structure of 103 MDR *P. aeruginosa* strains found in Croatian health care settings and identified 12 sequence types (STs). ST235 was found to be the most frequent followed by ST111 and ST132. Subsequently, Treepong et al., (2018) reported that ST235 is the most prevalent high-risk clone associated with poor prognosis for patients and analysed the genomes of 79 ST235 isolates of *P. aeruginosa* with a wide range of global origins over a 27-year

period to further understand the epidemiology of this high-risk clone. They used clustering analysis to discover that ST235 *P. aeruginosa* could spread between continents as well as within and between countries. Interestingly, they found the most recent common ancestor of ST235 isolates to have arisen in 1984 which coincides with the beginning of the use of antipseudomonal fluoroquinolones as antibiotics (1984-1987). Furthermore, 22 highly conserved genes were identified that were specific to the ST235 *P. aeruginosa* lineage, associated with bacterial transformation, DNA processing and transmembrane efflux- a unique combination of genes thought to contribute to poor outcome associated with ST235 *P. aeruginosa* infections and enable the acquisition of mobile resistance elements (Table 1).

Table 1. Description of the 22 genes highly conserved in and specific to *Pseudomonas aeruginosa* ST235 lineage.

Table 2
Description of the 22 genes highly conserved in and specific to *Pseudomonas aeruginosa* ST235 lineage

Block number	Gene symbol in strain NCGM2.S1	Domain	Additional description	Accession no. of the closest homologue (name, bacterial species, % identity)
1	NCGM2_1826	Transposase	—	—
	NCGM2_1828	α/β hydrolase family protein	—	—
	NCGM2_1829	Pirin-related protein	Putative transcriptional regulation	—
	NCGM2_1830	Putative RND outer membrane protein (TolC family)	—	NP_417507 (TolC, *Escherichia coli*, 22%)
	NCGM2_1831	Putative RND membrane fusion protein (ErmA family)	—	NP_417170.1 (EmrA, *E. coli*, 50%)
	NCGM2_1832	Putative MFS multidrug efflux transporter (EmrB family)	—	NP_418166.1 (EmrB, *E. coli*, 24%)
	NCGM2_1836	Putative transporter membrane protein	—	WP_058142560.1 (*P. aeruginosa*, 99%)
	NCGM2_1837	Putative RND membrane fusion protein (HlyD/EmrA family)	—	—
	NCGM2_1838	PucR C-terminal helix-turn-helix	Probable transcriptional regulator	NP_391122.1 (*Bacillus subtilis*, 45%)
2	NCGM2_3761	Hypothetical protein	—	—
	NCGM2_3762	P-loop NTPase	Involved in replication	WP_025991883.1 (*P. aeruginosa*, 99%)
	NCGM2_3765	Type-I restriction endonuclease HsdR	Restriction-modification system	—
	NCGM2_3766	Type-I restriction endonuclease HsdS	Restriction-modification system	—
	NCGM2_3767	UvrD/REP helicase	—	—
	NCGM2_3768	SMC domain-containing protein	Replication, recombination and DNA repair	—
	NCGM2_3769	N-6 DNA methylase	—	—
	NCGM2_3770	N-7 DNA methylase	—	—
	dprA	DNA protection protein	Dedicated to natural bacterial transformation	—
	recQ	ATP-dependent DNA helicase	Involved in genome maintenance	—
	leuS	Leucyl-tRNA synthetase	—	—
3	NCGM2_6332	Hypothetical protein	—	—
	NCGM2_6333	DEAD/DEAH box helicase	—	—

Note. From "Global emergence of the widespread *Pseudomonas aeruginosa* ST235 clone", by Treepong et al., (2018), *Clinical Microbiology and Infection*, 24(3) pp. 265.

1.2 Biofilms

Like many bacterial pathogens, *P. aeruginosa* cells exist within complex microbial communities known as biofilms, which are found at surface-liquid, surface-air, and liquid–air interfaces (Redfern and Enright, 2020). Biofilm formation reduces the susceptibility of bacteria to both antibiotics and host immune defences by establishing a community of surface-associated bacterial cells enclosed in an extracellular polymeric substance matrix (EPS) (Donlan, 2002; Schroll et al., 2010). The EPS produced by *P. aeruginosa* consists of exopolysaccharides, matrix proteins, lipids and extracellular DNA which surround and adhere to bacterial cells to form a biofilm. Both the genetic makeup of *P. aeruginosa* isolates and their environment can affect biofilm development, as well as interaction between the two. The composition of a biofilm is also dependent on strain, growth conditions and age. It enables the bacteria to resist desiccation, mechanical removal, and the actions of antimicrobial agents (Rasamiravaka et al., 2015). Biofilms also promote persistence of MDR *P. aeruginosa* in the environment by acting as a site for transfer of virulence factors and ABR between cells.

Subsequently, this enables the colonisation of lungs in CF patients, indwelling medical devices and surgical wound infections leading to chronic infection and research has shown that 65-80% of pathogenic infections in hospitals are related to biofilms (Kumar et al., 2017; Shrestha et al., 2022). Furthermore, increased ABR as a result of biofilm formation has made *P. aeruginosa* infections notoriously difficult to treat with conventional single antibiotic therapy no longer being effective in most cases. Although there are numerous antibiotics available with differing modes of action, they are only able to treat the symptoms of *P. aeruginosa* infection rather than being able to eradicate the biofilm source (Li et al., 2020).

WHO guidelines (WHO, 2017) state that, to prevent the transmission of MDR *P. aeruginosa*, healthcare facilities should adhere to hand hygiene (including use of alcohol-based hand gels), contact precautions, patient isolation, environmental cleanliness and surveillance. Whilst ABR in *P. aeruginosa* biofilm has been widely

studied, less attention has been paid to the relationship between alcohols and biofilm. This is perhaps due to the fact alcohols cannot be used to treat human infections; however, they should not be ignored due to their extensive use as disinfectants in hospital settings. A study by Spagnolo et al. (2021) discovered biocide concentration gradients caused by inappropriate use of disinfectants and dilution in the environment after disposal. As a result, microorganisms, such as *P. aeruginosa*, are exposed to non-lethal concentrations of disinfectants which facilitates the development of ABR and resistance to disinfectants (Molina-González et al., 2014). There is also widespread concern that the overuse of disinfectants has attributed to ABR (Gerba, 2015).

In addition to disinfecting medical equipment and environmental surfaces, EtOH is one of the most common active ingredients in alcohol-based hand gels. Hand gels are essential for limiting human-to-human transmission of infections and are effective against bacteria, fungi and viruses by denaturing and coagulating proteins in their protective coatings (Gold et al., 2022). During the COVID-19 pandemic, scientists and governments urged the public to practice good hand hygiene, including the use of alcohol-based hand gels as a quick and effective alternative to hand washing, which caused a surge in demand for hand gels and cleaning supplies. Statistics Canada conducted a series of surveys on personal care item sales during the first few months of the pandemic and found that compared to sales in 2020, there was a dramatic increase in hand sanitizer sales, by March of 2020, sales of these products were up 800% from the previous year (Nicol, 2021).

Whilst such products were recommended with the best intentions, the public were not advised on responsible use of antimicrobials or made aware of the effects of overuse. As a result, resistance associated with hand sanitisers is now an area of concern; a study by Pidot et al. (2018) found that *Enterococcus faecium*, another leading cause of hospital-acquired infections, is showing increasing resistance to alcohol-based hand sanitisers. Furthermore, constant washing of traces of alcohol-based hand gels down hospital sinks results in environmental stress for *P. aeruginosa*, which has been found to trigger biofilm formation (Tashiro et al., 2014). Tashiro et

al. (2014) found that low concentrations of EtOH as residuals of disinfection (1% and 2%) increased biofilm and pellicle formation in *P. aeruginosa* via increased transcription of two extracellular polysaccharides, Pel and Psl, which each act as a structural scaffold in biofilm (Colvin et al., 2012).

In addition to Pel and Psl, alginate secreted by *P. aeruginosa* in biofilms plays a significant role in chronic infection (Mann and Wozniak, 2012). Increased production of alginate accounts for the slimy nature of mucoid biofilms which are more resistant to antibiotics and host immune defences than non-mucoid biofilms (Moradali and Rehm, 2019). Psl is the most commonly found polysaccharide in biofilm infections and is made up of a pentasaccharide-repeating unit of D-mannose, D-glucose and L-rhamnose which provides protection against immune defences and acts as a first line of defence during initial development of biofilms (Yin et al., 2022). Pel is involved in biofilm formation at air-liquid interfaces (Byrd et al., 2009) and is a positively charged exopolysaccharide made up of partially acetylated 1 → 4 glycosidic linkages of N-acetylglucosamine and N-acetylgalactosamine (Jennings et al., 2015)

1.3 Hospital water as infection reservoirs

Increased biofilm formation resulting from low concentrations of disinfectant is problematic due to the large number of *P. aeruginosa* outbreaks in hospitals, especially those linked to the water system; sinks and their associated components, drains and pipes (Lalancette et al., 2017). Such environments act as a refuge for *P. aeruginosa* by providing favourable conditions for biofilm growth, which subsequently enables them to survive levels of disinfectant up to 1000 times greater than their planktonic form (Araújo et al., 2011). Over time, corrosion of water pipes and subsequent crevices and fissures can exacerbate existing biofilms, causing *P. aeruginosa* to spread and further colonise the water system. A study by Venier et al. (2014) investigated *P. aeruginosa* infection in 10 intensive care units (ICUs) and found 32% of ICU patients colonised with the bacteria had been exposed to contaminated water in their room. Such cases are of great concern due to the increase in resistant

strains of *P. aeruginosa* that are associated with high mortality, morbidity and costs to healthcare services (Nathwani et al., 2014).

Whilst several reservoirs of *P. aeruginosa* have been identified in healthcare settings, water in hospitals is the most common cause of nosocomial infection (Spagnolo et al., 2021). Transmission can occur from direct contact with contaminated water during washing and splash back, or indirect contact with staff who have washed their hands in contaminated water, medical equipment that has been rinsed in contaminated water or contaminated surfaces (Spagnolo et al., 2016). A study by Hota et al. (2009) found that an ICU sink contaminated with MDR *P. aeruginosa* could create up to 1m of splash back whilst the tap was running. Furthermore, Lewenza et al. (2018) found *P. aeruginosa* was able to survive in water for over 145 days.

The people most likely to encounter the water system are those who may already have health issues which puts them at greater risk of infection. A study by Jensen et al. (1997) investigated *P. aeruginosa* infection in CF patients caused by contaminated dental equipment. Water samples were taken from syringes, turbines, handpieces and ultrasonic scalers and sputum samples were taken from CF patients before and after their treatment. This identified at least one case where genetically identical *P. aeruginosa* was found both in water from the dental equipment and the patient's sputum. Contaminated water systems have been attributed to numerous other outbreaks, including the widely reported incidents involving babies in Northern Ireland in 2011/12 (Walker et al., 2014). Following four neonatal deaths, guidance documents were produced by the Department of Health England to advise the NHS on how to manage *P. aeruginosa* outbreaks.

1.4 Genomic analysis of *P. aeruginosa*

The *P. aeruginosa* genome is relatively large comprising 6-7Mb of DNA. With the introduction of low-cost, high-throughput next generation sequencing, it is now possible to generate whole genome DNA sequence data from multiple different

bacterial isolates and decipher the *P. aeruginosa* pan genome - the full set of genes of a particular species or clade of bacteria (Freschi et al., 2019). Resources such as the International Pseudomonas Consortium Database (IPCD) have made it possible to generate draft genomes from >1100 *P. aeruginosa* isolates. IPCD is a source of thousands of environmental (water and soil), plant, animal, and human *P. aeruginosa* isolates with a particular focus on CF. It aims to aid the development of novel treatments for CF using metadata analysis, which enables the linking of bacterial phenotype, genotype and clinical data.

A recent study by Freschi et al. (2019) sequenced 619 *P. aeruginosa* isolates from 19 clinical (including patients with CF, wounds, burns, pneumonia, COPD and immunosuppression) and 7 environmental sources (including plants, soil, sand, rivers and animals) in order to improve and diversify genetic testing of *P. aeruginosa*. This enabled definition of the pan-genome size of *P. aeruginosa* as 54,272 genes, of which 665 were core genes. Core genes were shown to enhance cell division, translation, transcription, chromatin structure, RNA processing and metabolism and transport of amino acids, coenzymes, lipids and nucleotides. The accessory genome includes genes involved in virulence and ABR, so it is likely that biocide resistance varies greatly between strains. This highlights the importance of considering a range of isolates obtained from different clinical sources when investigating such traits.

2 Aims and Objectives

2.1 Aims

The aim of this research was to identify intraspecies phenotypic variation between clinical *P. aeruginosa* isolates, specifically their tolerance to EtOH and propan-2-ol and explore the extent to which sub-inhibitory concentrations of these alcohols contribute to biofilm formation.

2.2 Objectives

To achieve this aim, the following objectives were set:

- **Establish tolerance of 58 ST111 and ST235 *P. aeruginosa* isolates to EtOH and propan-2-ol**. Studies have shown ST235 to be the most common sequence type associated with MDR *P. aeruginosa* in healthcare settings, followed by ST111. EtOH and propan-2-ol are two active ingredients commonly used in hand sanitisers which are used extensively in healthcare settings.
- **Quantify *P. aeruginosa* biofilm formation in sub-inhibitory concentrations of EtOH using different biofilm growth models**. Due to disruption caused by COVID-19, there was only time to investigate biofilm formation in the presence of EtOH or isopropanol. Initial experiments showed that *P. aeruginosa* showed more resistance to EtOH than propan-2-ol, hence why it became the focus of the study.
- **Establish the effect of sub-inhibitory concentrations of EtOH on established biofilm**. To compare the effect of EtOH on *P. aeruginosa* at different stages of biofilm formation.

3 Methodology

3.1 Storage of *P. aeruginosa* Isolates

Fifty-eight *P. aeruginosa* isolates from two multi-locus sequence types (MLST) (ST111 and ST235) were obtained from clinical samples as provided by BioMérieux (Marcy-l'Étoile, France). Isolates were collected between 1985 and 2005 from a range of sources; burns, urine, blood, sputum, wounds and hospitals from the USA, France, Belgium, Colombia, Turkey, Greece & Bulgaria (Appendix 1). Isolates were stored in a freezer at -80°C until required. They were then streaked onto tryptone soya agar (TSA, Fisher Scientific) plates, incubated for 24 hours at 37°C and stored in a fridge at ~4°C for a maximum of 21 days.

3.2 Determination of Minimum Bactericidal Concentration (MBC)

Cultures of *P. aeruginosa* isolates were be prepared by inoculating 10 mL of sterile tryptone soya broth (TSB) with 3-5 *P. aeruginosa* colonies and incubating overnight at 37°C on an orbital shaker (Infors TR-225 at 200 rpm). Using a spectrophotometer, inocula were diluted with sterile saline (Fisher Scientific) to a standard absorbance of 0.6 at an optical density (OD) of 600nm. Two-fold serial dilutions of EtOH and propan-2-ol (Fisher Scientific) were prepared (100%, 50%, 25%, 12.5%, 6.25% and 3%) using pure alcohol and sterile water. 200 µL of either EtOH or propan-2-ol dilution was pipetted into wells 1-12 of rows A-F of a 96-well microtiter plate, then 200 µL of standardised inoculum was added to each well with one *P. aeruginosa* isolate per column (Fig. 1). Upon addition of an equal volume of inoculum, the dilution of alcohol in each well was halved (50%, 25%, 12.5%, 6.25%, 3% and 1.5%). Microtiter plates were sealed with Parafilm, placed in a plastic container with moistened tissue paper (to maintain humidity) and incubated overnight (~18 hours) at 37°C. After incubation, wells were mixed by pipette and 20 µL from each well was pipetted onto TSA plates following the Miles and Misra method (Miles et al., 1938) in triplicate to identify a range in which the MBC was located (e.g. between 6.25% and 12.5%). Once a more specific MBC range was established, the experiment was repeated using dilutions of

EtOH/propan-2-ol that increase in 1% increments to identify a specific MBC and variation between isolates; for EtOH this was 8-15% and 3-10% for propan-2-ol.

	1	2	3	4	5	6	7	8	9	10	11	12
A	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%
B	25%	25%	25%	25%	25%	25%	25%	25%	25%	25%	25%	25%
C	12.50%	12.50%	12.50%	12.50%	12.50%	12.50%	12.50%	12.50%	12.50%	12.50%	12.50%	12.50%
D	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%
E	3%	3%	3%	3%	3%	3%	3%	3%	3%	3%	3%	3%
F	1.50%	1.50%	1.50%	1.50%	1.50%	1.50%	1.50%	1.50%	1.50%	1.50%	1.50%	1.50%
G												
H												

Figure 1. Configuration of 96-well microtiter plates used to establish MBC of EtOH and propan-2-ol for *P. aeruginosa*. Column numbers 1-12 represent 12 different *P. aeruginosa* isolates.

3.3 Biofilm Assays

3.3.1 96-Well Plate Biofilm Assay

This method was adapted from Coffey and Anderson (2014). Cultures of *P. aeruginosa* isolates were prepared by inoculating 10 mL of sterile TSB with 3-5 *P. aeruginosa* colonies and incubating overnight at 37°C on an orbital shaker (Infors TR-225 at 200 rpm). Using a spectrophotometer, inocula were diluted with sterile TSB to a standard absorbance of 0.6 at an optical density (OD) of 600nm. 1-15% dilutions of EtOH were prepared using >99% EtOH and sterile TSB. In a 96-well U-bottom microtiter plate (Corning) (Figure 2); 200 µL of sterile TSB was placed in control 1 wells (negative control), 200 µL of sterile TSB inoculated with 1 µL of *P. aeruginosa* inoculum in control 2 wells (positive control), 200 µL of 1-15% EtOH inoculated with 1 µL of *P. aeruginosa* inoculum in test wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Control 1			Control 2			1%	1%	1%		
C		2%	2%	2%	3%	3%	3%	4%	4%	4%		
D		5%	5%	5%	6%	6%	6%	7%	7%	7%		
E		8%	8%	8%	9%	9%	9%	10%	10%	10%		
F		11%	11%	11%	12%	12%	12%	13%	13%	13%		
G		14%	14%	14%	15%	15%	15%					
H												

Figure 2. Configuration of 96-well microtiter plates used in biofilm assays

Microtiter plates were sealed with Parafilm, placed in a plastic container with moistened tissue paper to maintain humidity and incubated for 48 hours at 37°C. After the incubation period, test and control wells were rinsed twice with 250 µL of distilled water and remaining biofilm stained with 260 µL of 0.1% crystal violet (Fisher Scientific). Once non-adhered crystal violet was removed and residual stain rinsed away, plates were left to dry in a class II biosafety cabinet for ~20 minutes. Once dry, 270 µL of 30% acetic acid (Fisher Scientific) was added to test and control wells to solubilise the crystal violet, allowed to stand for 10 minutes and homogenised. Absorbance of the solubilised crystal violet in test and control wells was then measured at 550nm using the FLUOstar Omega Microplate Reader (BMG LABTECH, Ortenberg, Germany).

3.3.2 Minimum Biofilm Eradication Concentration Assay

The Minimum Biofilm Eradication Concentration (MBEC) Assay® was used to determine the efficacy of EtOH against established *P. aeruginosa* biofilms. *P. aeruginosa* cultures were prepared and standardised as described in 3.3.1. MBEC plates (Innovotech, Canada) were filled according to figure 3 where control 1 wells (negative control) contained 150 µL of sterile TSB and treatment, control 2 and control 3 wells contained 150 µL of bacterial inoculum

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		TSB			B			B				
C		B	B	B	B	B	B	B	B	B		
D		B	B	B	B	B	B	B	B	B		
E		B	B	B	B	B	B	B	B	B		
F		B	B	B	B	B	B	B	B	B		
G		B	B	B	B	B	B	B	B	B		
H												

Control 1
Control 2
Control 3

Figure 3. Configuration of MBEC **bacterial** plates (B= bacterial inoculum, grey wells= treatment wells)

Peg lids were attached and secured with Parafilm, then plates were placed in a plastic container with moistened tissue paper and incubated for 24 hours at 37°C on an orbital shaker (110 rpm). After incubation, pegs were rinsed in 200 µL of sterile saline for 10 seconds and transferred to a regular 96 well U-bottom plate (Corning) base containing EtOH dilutions according to figure 4; 200 µL of 1-15% EtOH in treatment wells, 200 µL of sterile TSB in control 1 and control 3 wells and 200 µL of sterile saline in control 2 wells. EtOH plates were the inverted orientation of MBEC bacterial plates due to the cut corner and subsequent fit of MBEC peg lids. Control 1 (negative control) was used to monitor contamination, control 2 (bacterial pegs placed in saline) was used to measure biofilm growth stunted at 24 hours incubation and control 3 (bacterial pegs placed in TSB) was used to measure biofilm growth in absence of EtOH.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			15%	15%	15%	14%	14%	14%	13%	13%	13%	
C			12%	12%	12%	11%	11%	11%	10%	10%	10%	
D			9%	9%	9%	8%	8%	8%	7%	7%	7%	
E			6%	6%	6%	5%	5%	5%	4%	4%	4%	
F			3%	3%	3%	2%	2%	2%	1%	1%	1%	
G			TSB			SALINE			TSB			
H												

Control 1
Control 2
Control 3

Figure 4. Configuration of **EtOH** plates containing 1-15% EtOH dilutions

Plates were returned to the incubator for a further 24 hours. After incubation, pegs were gently immersed in water to remove non-adhered cells and allowed to dry. Peg

lids were placed in 96 well plates containing 200 µL of 0.1% crystal violet for 10 minutes on an orbital shaker to stain the biofilm. Pegs were gently rinsed twice in water to remove excess stain and allowed to dry. Finally, the crystal violet was solubilised by placing pegs in 200 µL of 30% acetic acid for 10 minutes on an orbital shaker. Wells were mixed to homogenise the crystal violet, then absorbance was measured at 550nm using the FLUOstar Omega Microplate Reader (BMG LABTECH, Ortenberg, Germany).

3.3.3 CDC Biofilm Reactor

The CDC Biofilm Reactor (CBR; BioSurface Technologies Corp., Bozeman, MT, USA) was prepared and set up according to the standard test for quantification of *P. aeruginosa* (ASTM E2562, 2017). Each of the eight rods held three coupons and two rods were used for one of four materials: borosilicate glass, polycarbonate, 304 stainless steel and 316 stainless steel (Biosurfaces Technologies, USA). *P. aeruginosa* inoculum with a cell density of ~10^{-8} CFU/ mL was prepared by carrying out serial dilutions and plating. 1 mL of *P. aeruginosa* inoculum was injected into 500 mL of batch culture media (TSB) that was autoclaved within the reactor system. The baffle was set to rotate at 125 RPM for 24 hours, then a continuous flow of sterile TSB was pumped into the reactor at a flow rate of 11.67 (+/- 0.2) mL/ min for a further 24 hours at room temperature. At the end of the continuous flow phase, coupons were sonicated for three minutes to disrupt the biofilm and serial dilutions and spread plates were prepared on TSA to determine CFU/ mL.

3.5 Statistical Analysis

All statistical analysis was carried out using Prism 8 (GraphPad San Diego, USA); ANOVA followed by Tukey's multiple comparison tests were used to test for statistical significance between datasets. Statistical significance was assumed when P-values were less than 0.05.

4 Results

4.1 Minimum Bactericidal Concentration

4.1.1 Effect of EtOH on *Pseudomonas aeruginosa* in planktonic form

The most common MBC for *P. aeruginosa* isolates (n=58) in EtOH was 11% (n=23), followed by 10% (n=20) (figure 5). Data demonstrates no isolates had an MBC below 10% or above 13%, providing a 4% range in which all MBCs fell.

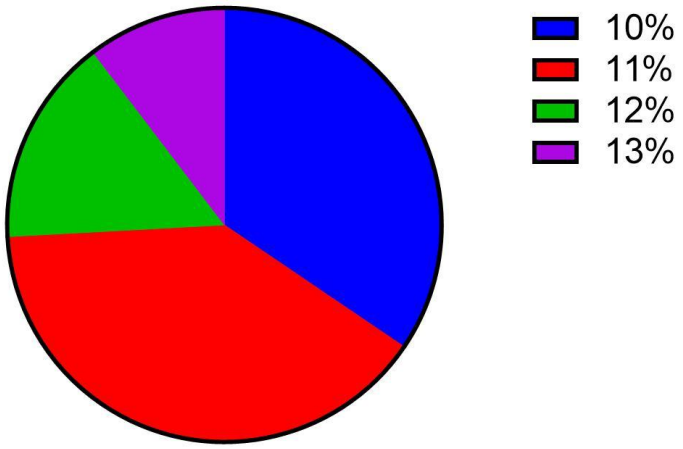

Figure 5. Overview of MBC for *P. aeruginosa* isolates (n=58) belonging to ST111 and ST235 in different concentrations of EtOH.

Variation in MBC between ST111 isolates of *P. aeruginosa* demonstrates intraspecies variation in response to EtOH (figure 6). Whilst MBC for ST111 isolates only ranged between 10% and 13%, this is a relative increase of 30% which is significant at such low concentrations of EtOH. Isolates 107751 and 115524 were the only isolates with an MBC of 13%, making them most resistant to EtOH. The majority of isolates (57%) had an MBC of 10% EtOH whilst just 32% had an MBC of 11%.

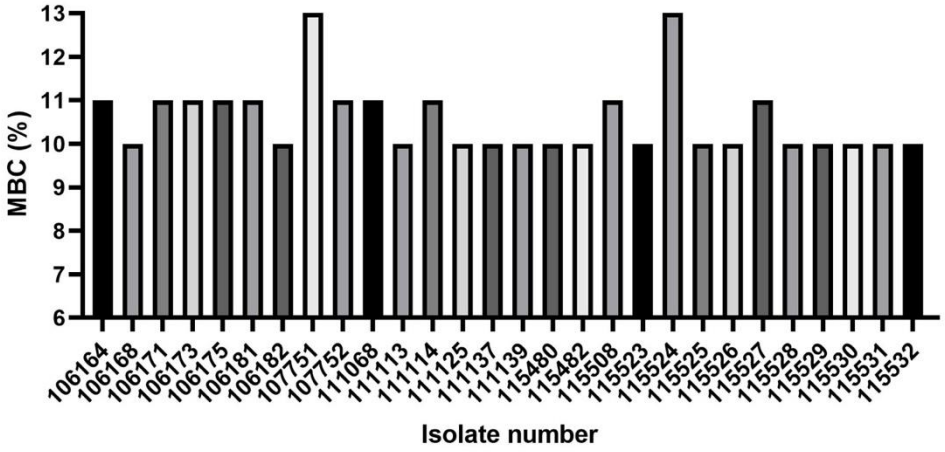

Figure 6. MBC of 28 *P. aeruginosa* isolates in EtOH belonging to ST111 (n=3)

In comparison to ST111 isolates, MBCs for ST235 isolates challenged with EtOH were higher overall, with four isolates at 13% compared to just two (figure 7). There are also 26 ST235 isolates that have an MBC of over 10% compared to just 12 ST111 isolates.

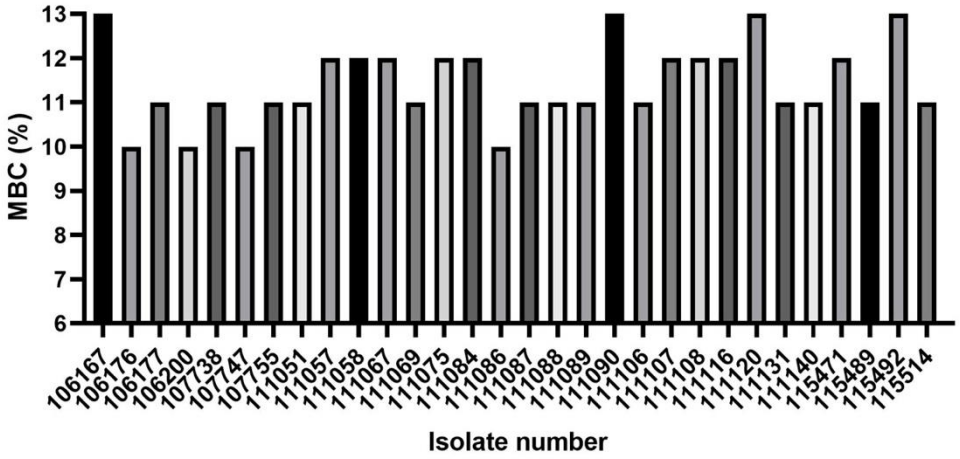

Figure 7. MBC of 30 *P. aeruginosa* isolates in EtOH belonging to ST235 (n=3)

4.1.2 Effect of propan-2-ol on *Pseudomonas aeruginosa* in planktonic form

The most common MBC for *P. aeruginosa* in propan-2-ol was 8% which constitutes 40% (n=23) of all isolates (figure 8). The number of isolates for which the MBC was 9% or 10% was equal, each accounting for 16% (n=9) of all isolates. Whilst all MBCs fell within a 4% range (7-10%), a similar pattern to figure 1, the range was 3% lower compared to EtOH.

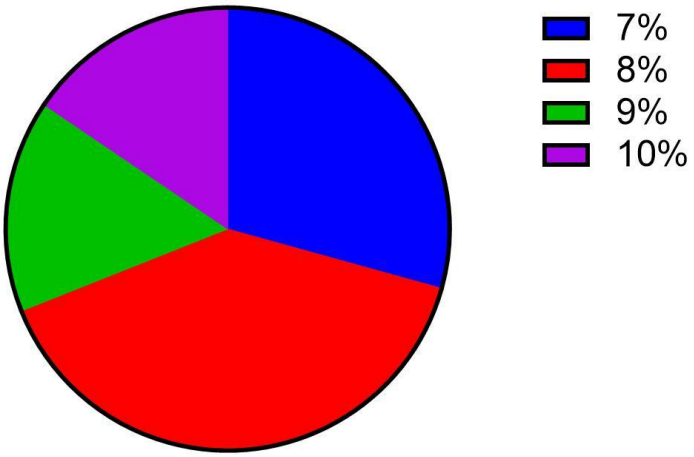

Figure 8. Overview of MBC for *P. aeruginosa* isolates (n=58) belonging to ST111 and ST235 in propan-2-ol

The majority (57%, n=16) of ST111 isolates had an MBC of 7%, however, isolates 106164, 107751 and 111137 (marked with yellow stars in figure 9) showed bacterial growth again in 10% propan-2-ol- bacterial growth was inhibited in 7-9% propan-2-ol. Isolate 111114 ceased to grow in the presence of 10% propan-2-ol (MBC=10%), whereas isolate 115524 (marked with a grey star in figure 9) showed consistent growth up to and including 10% propan-2-ol. MBC was not explored above 10% due to the 3-10% range being established in the initial doubling dilution experiments. 32% (n=9) of isolates had an MBC of 8% whilst 115482 was the only isolate with an MBC of 9%.

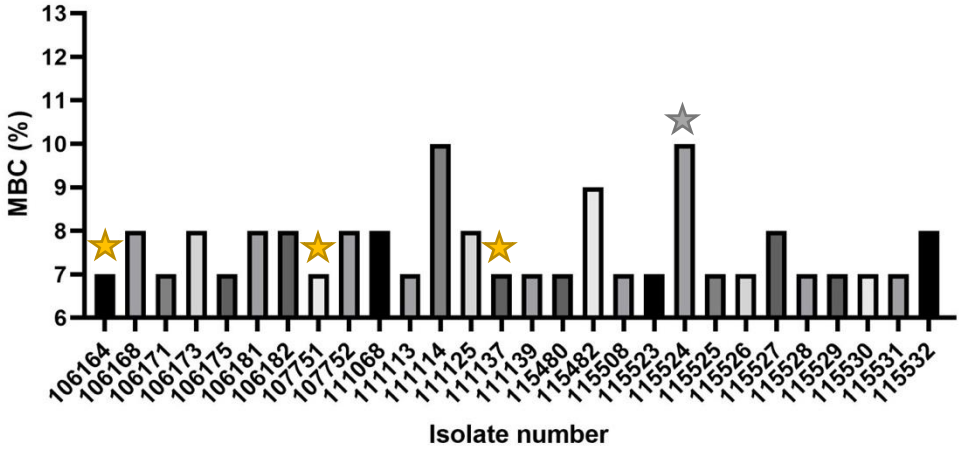

Figure 9. MBC of 28 *P. aeruginosa* isolates in propan-2-ol belonging to ST111 (n=3)

MBC for ST235 isolates was higher overall compared to ST111 isolates (figure 10). For example, 7 ST235 isolates (106167, 111051, 111058, 111067, 111075, 111120 and 115492) had the highest MBC of 10% compared to just two ST111 isolates (111114 and 115524) (figure 9). Isolates 106167, 111120 and 115492 also had the highest MBC for EtOH (figure 7). Similarly, 50% of ST235 isolates exceeded 8% propan-2-ol compared to just 10% of ST111 isolates. However, MBC did not drop below 7% propan-2-ol for ST111 or ST235 isolates. Isolates 111058 and 111067 (marked with a grey star in figure 10) indicate growth at 10% propan-2-ol that was not further investigated as explained above.

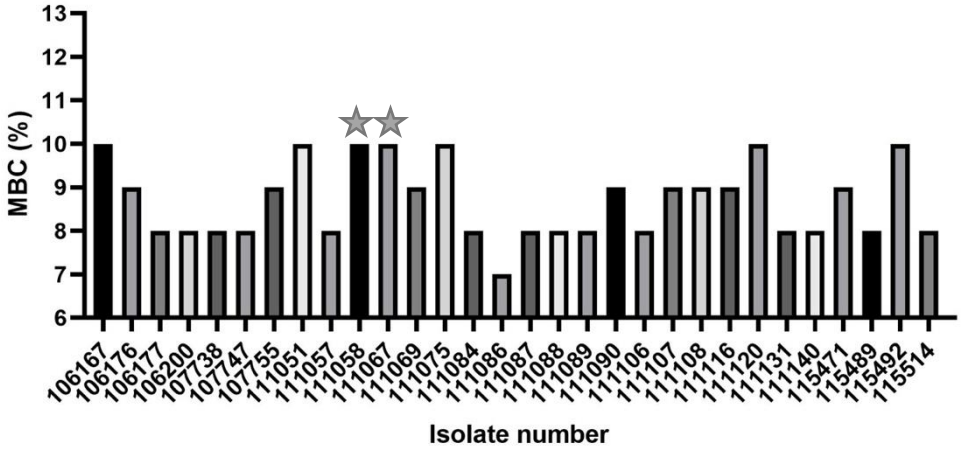

Figure 10. MBC of 30 *P. aeruginosa* isolates in propan-2-ol belonging to ST235 (n=3)

4.2 96-Well Plate Biofilm Results

4.2.1 Effect of EtOH on biofilm formation in ST111 Isolates

There was clear variation of optical density (OD), representing biofilm formation, between ST111 isolates when comparing OD at each percentage of EtOH (figure 11). Overall, the trend in biofilm formation decreased as concentration of EtOH increased (table 2), with the greatest biofilm growth occurring in 1% EtOH (OD=1.103 for 115525), and steadily decreased until 6% where it reached a plateau.

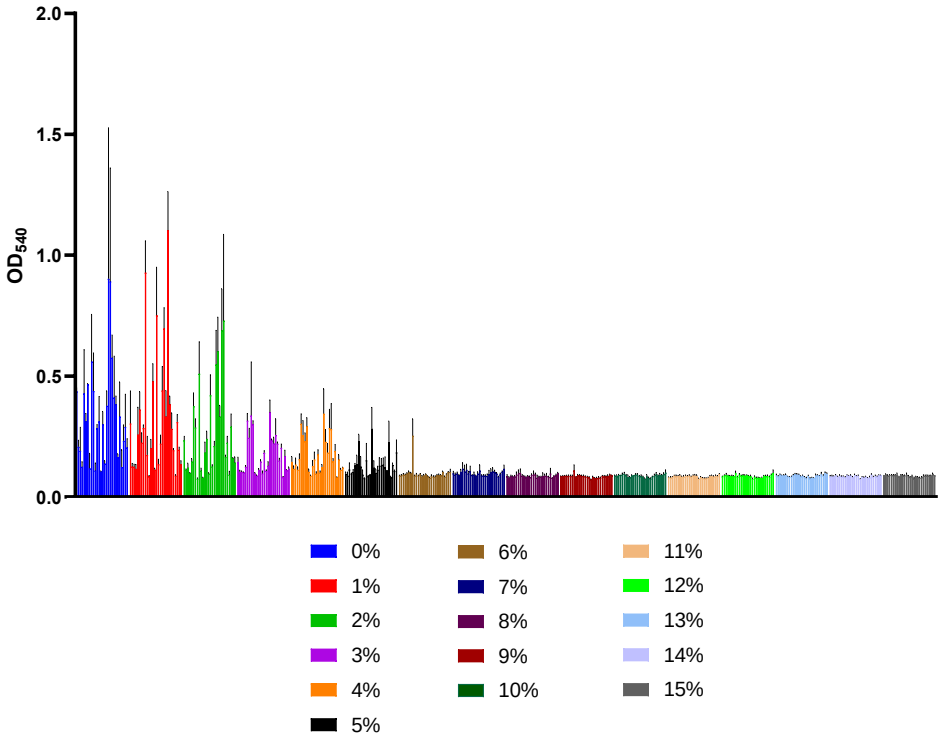

Figure 11. Optical density data demonstrating *P. aeruginosa* ST111 isolate 96-well plate biofilm after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours), grouped by concentration of EtOH. All data has been adjusted using average negative controls as the baseline. All mean and standard deviation data for each isolate and its treatment with different percentages of EtOH are described in appendix 2.

Table 2. Minimum, Maximum, Range, Mean and Standard Deviation of optical density values for combined ST111 isolate biofilm data grown in absence of, or in the presence of different concentrations of EtOH.

	Minimum	Maximum	Range	Mean	Std. Deviation
0%	0.1053	0.9027	0.7973	0.3371	0.2086
1%	0.08633	1.103	1.017	0.3237	0.2572
2%	0.07533	0.73	0.6547	0.2649	0.1923
3%	0.08367	0.3493	0.2657	0.1737	0.08145
4%	0.08233	0.3443	0.262	0.1737	0.07478
5%	0.078	0.282	0.204	0.1288	0.04828
6%	0.07967	0.2543	0.1747	0.09757	0.03132
7%	0.08467	0.1173	0.03267	0.0988	0.009062
8%	0.07967	0.1	0.02033	0.08833	0.005644
9%	0.073	0.1103	0.03733	0.08636	0.006255
10%	0.076	0.101	0.025	0.08873	0.005629
11%	0.07533	0.09533	0.02	0.08508	0.004473
12%	0.076	0.09933	0.02333	0.08737	0.005315
13%	0.07833	0.09933	0.021	0.08837	0.005305
14%	0.07567	0.09267	0.017	0.086	0.003972
15%	0.07733	0.09433	0.017	0.08817	0.003954

When comparing biofilm formation of each isolate at different concentrations of EtOH, there is clear variation within and between ST111 *P. aeruginosa* isolates (figure 12). The data also show variation within isolates such as the 91% OD increase from 1% to 2% in EtOH in 115526 (OD=0.383 to 0.730). In contrast, biofilm density was not affected by increasing EtOH concentration from 1-2% for a number of isolates (106173, 111113, 111137, 115480 and 115529), such as 106173 which displayed minimal variation from 0% to 15%. Whilst 6% EtOH appeared to inhibit biofilm formation by all isolates, there are instances where biofilm growth increased again at 5% before plateauing at 6% such as in 111139 and 115532.

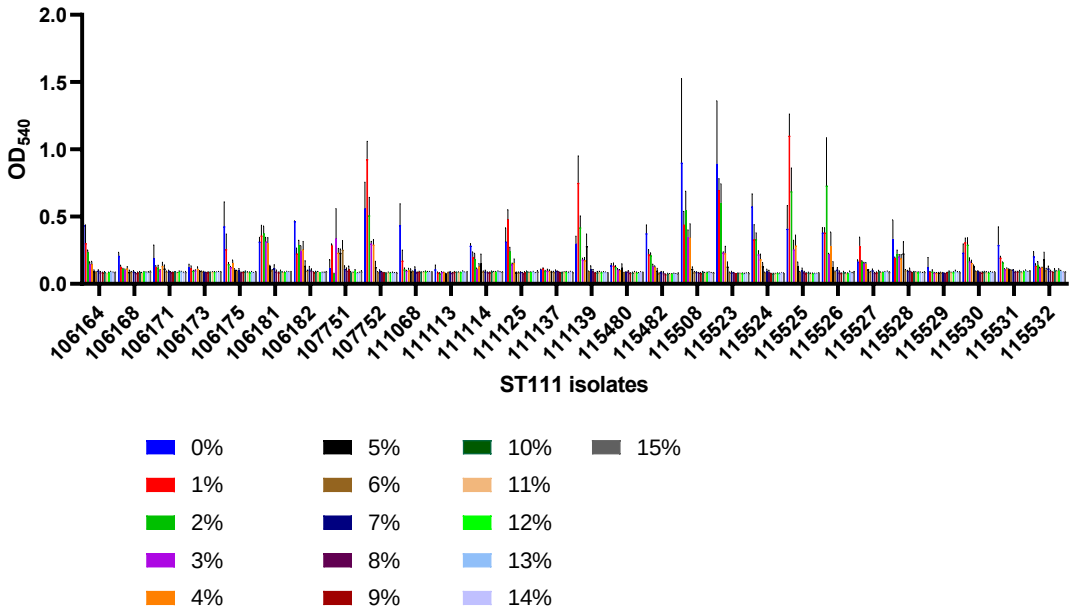

Figure 12. Optical density data demonstrating *P. aeruginosa* ST111 isolate 96-well plate biofilm formation after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours), grouped by isolate.

Whilst the majority of isolates produced most biofilm in 0% EtOH (n=18, figure 13), 35% of isolates grew more biofilm in the presence of EtOH (n=10). The highest OD value fell within the range of 0-3% EtOH, whilst the range for lowest OD reading was much wider (2-15%). However, the most common groups for lowest OD were 8%, and 11% which contained 6 and 7 isolates respectively.

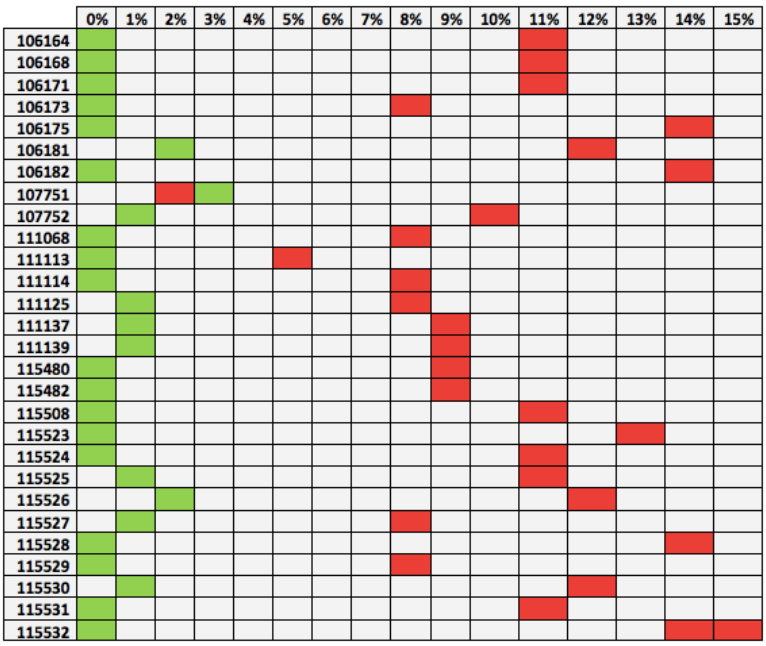

Figure 13. Minimum (red) and maximum (green) OD density readings for ST111 *P. aeruginosa* isolates representing 96-well plate biofilm production in the presence of 0-15% EtOH

Following statistical comparison using an ANOVA and Tukey post-hoc test, 20 isolates were identified to each have a statistically significant ($P<0.05$) variation of biofilm formation across the different EtOH concentrations (figure 14). Out of these isolates, 55% produced the most biofilm in the absence (0%) of EtOH (106164, 106175, 106182, 111068, 111114, 115482, 115508, 115523, 115524, 115528 and 115531) and showed a steady decline in biofilm formation upon addition of EtOH. Isolates marked with stars (n=9) indicate significant increases in biofilm formation in the presence of low concentrations (1-3%) of EtOH and can be viewed more clearly in figure 15.

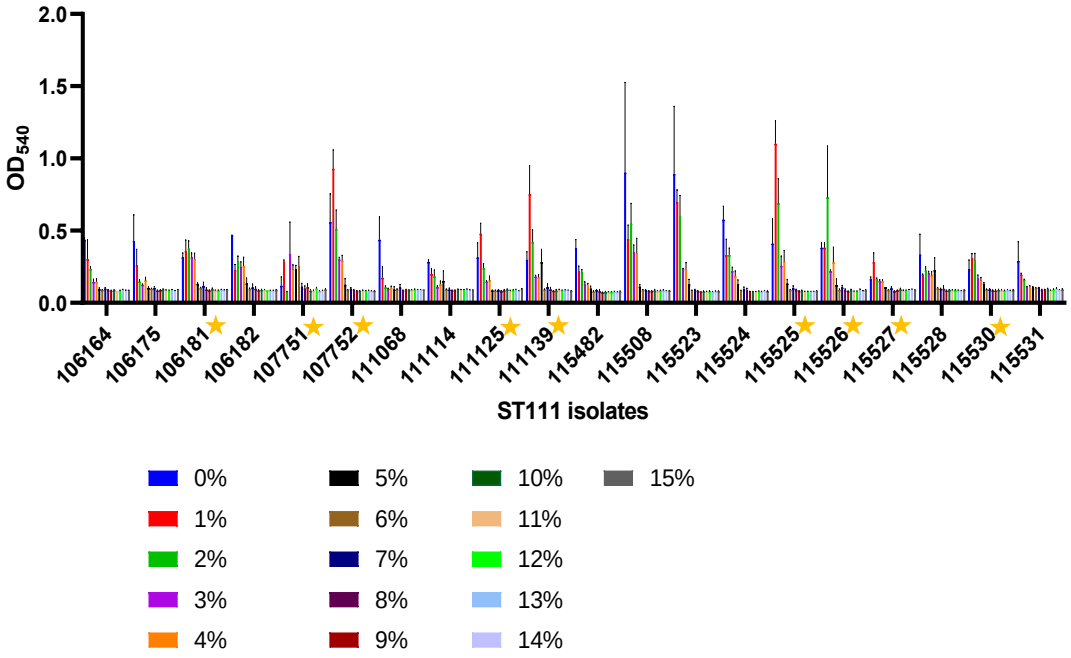

Figure 14. Optical density data demonstrating *P. aeruginosa* ST111 isolate 96-well plate biofilm formation after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours) that had statistically significant (P<0.05) variation in biofilm formation at different concentrations of EtOH.

Of these 9 isolates, 106181 is the only instance where biofilm production was comparable at 4% (OD=0.304) to 0-3% EtOH, before there was a 57% decline at 5% (OD=0.131) (figure 15). Isolate 107751 is another example of an irregular growth pattern with a sharp decline in biofilm production at 2% EtOH (OD=0.075) followed by peak production at 3% (OD=0.338). Biofilm production remained high until reaching 7% EtOH where OD dropped by 56% (OD=0.113).

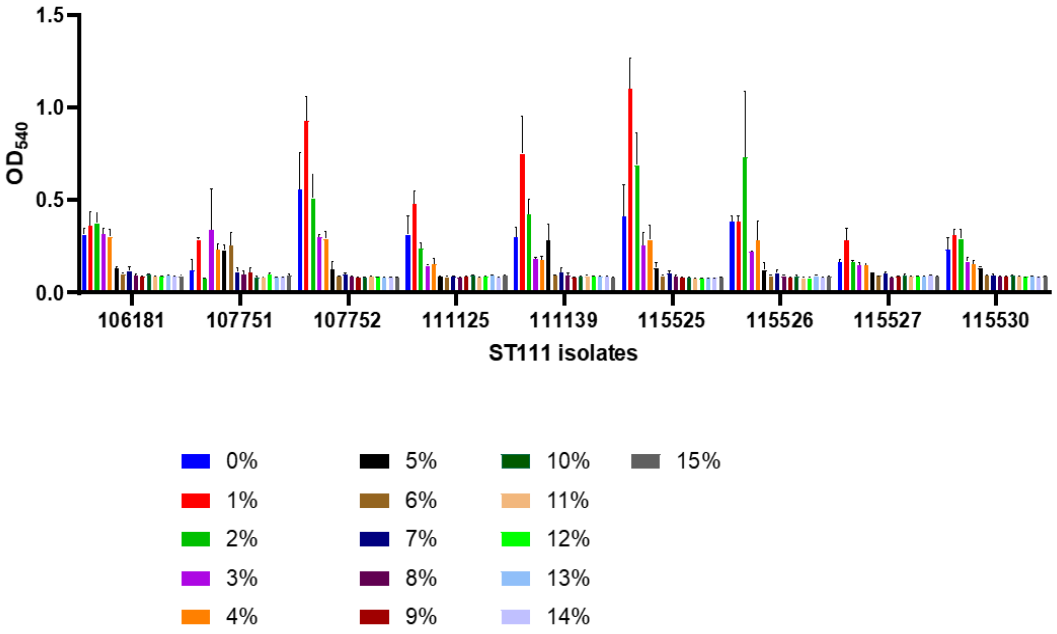

Figure 15. Optical density data demonstrating *P. aeruginosa* ST111 isolate 96-well plate biofilm formation after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours) that had statistically significant (P<0.05) variation in biofilm formation at different concentrations of EtOH, where an increase in biofilm was demonstrated in sub-inhibitory concentrations of EtOH compared to 0% control.

4.2.2. Effect of EtOH on biofilm formation in ST235 Isolates

Whilst the general pattern of biofilm production was similar to that of ST111 isolates (described above), there is noticeably greater variation of optical density between ST235 isolates when comparing OD at each percentage of EtOH each percentile (figure 16). For example, OD data at 0% EtOH ranged from 0.090 to 1.376, indicating a 1429% difference in biofilm production between isolates. Variation within percentiles was observed in higher concentrations of EtOH (6-15%), whereas OD was consistently low in ST111 isolates. Furthermore, the ST235 isolates producing greater biofilm in 6-15% EtOH were largely the same and have been indicated with gold arrows. Whilst the greatest biofilm production of all ST235 isolates also occurred in

1% EtOH (OD=1.718), such as for ST111 isolates, it is 56% greater than the maximum value for ST111 isolates (OD=1.103).

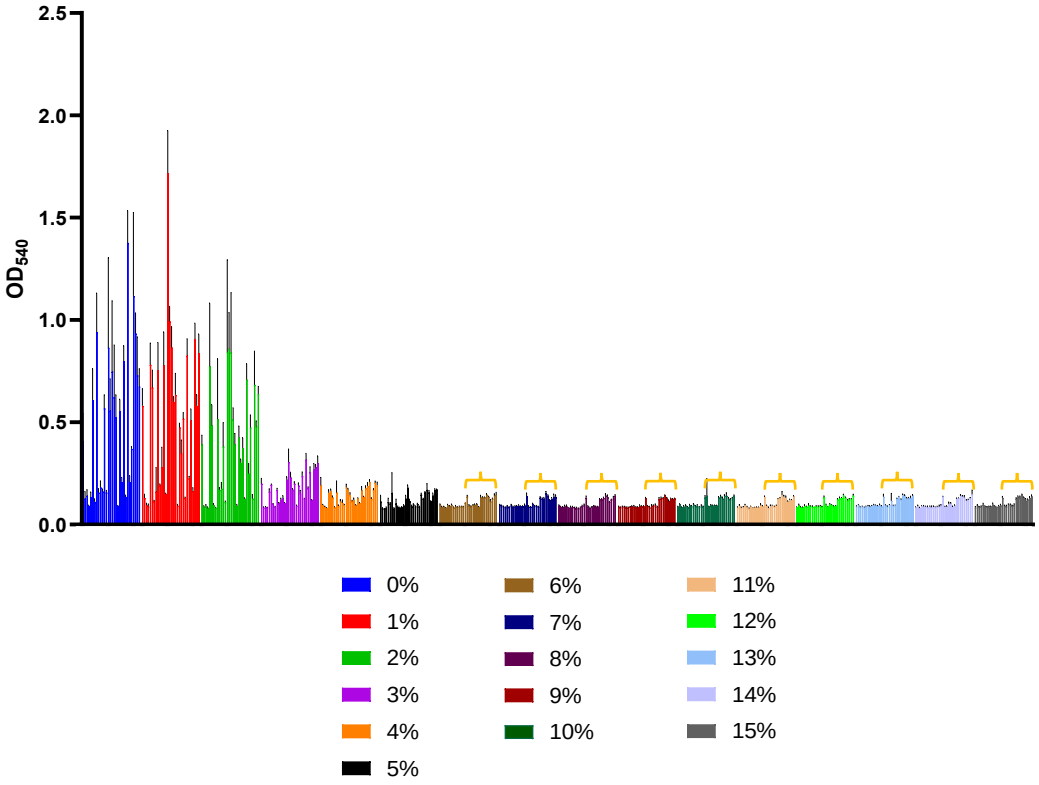

Figure 16. Optical density data demonstrating *P aeruginosa* ST235 isolate 96-well plate biofilm after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours), grouped by concentration of EtOH. All data has been adjusted using average negative controls as the baseline. All mean and standard deviation data for each isolate and its treatment with different percentages of ethanol are described in appendix 3.

Table 3. Minimum, Maximum, Range, Mean and Standard Deviation of optical density values for combined ST235 isolate biofilm data grown in absence of, or in the presence of different concentrations of EtOH.

	Minimum	Maximum	Range	Mean	Std. Deviation
0%	0.08967	1.376	1.286	0.4766	0.3517
1%	0.08933	1.718	1.629	0.5065	0.3738
2%	0.07933	0.8583	0.779	0.3854	0.2606
3%	0.08267	0.3177	0.235	0.1757	0.07334
4%	0.08133	0.2083	0.127	0.1392	0.03975
5%	0.07667	0.1797	0.103	0.1162	0.03291
6%	0.082	0.1463	0.06433	0.1056	0.02217
7%	0.085	0.153	0.068	0.1047	0.02139
8%	0.08	0.142	0.062	0.1004	0.02116
9%	0.08367	0.1427	0.059	0.1012	0.01965
10%	0.085	0.1407	0.05567	0.1066	0.02161
11%	0.07967	0.145	0.06533	0.1013	0.02113
12%	0.08333	0.1433	0.06	0.1034	0.02001
13%	0.08333	0.145	0.06167	0.106	0.02048
14%	0.081	0.1477	0.06667	0.1031	0.02173
15%	0.08533	0.1457	0.06033	0.1044	0.0198

Increased biofilm production within isolates marked by gold arrows in figure 16 can also be observed in figure 17, which groups biofilm production by isolate rather than concentration of EtOH. Here, the gold dotted line acts as a threshold and demonstrates increased biofilm formation in isolates 111108-115514 in 6-15% EtOH, compared to isolates 106167-111107. Further variation between ST235 isolates was observed by comparing isolate 106200, which displayed minimal variation in biofilm production in 0-15% EtOH, to 111084 which saw a 1875% difference between its minimum and maximum OD values (OD=1.718 and 0.087 at 1% and 12% EtOH respectively). There were two instances where biofilm growth dramatically dropped between 0% and 1% EtOH (111051 and 115471).

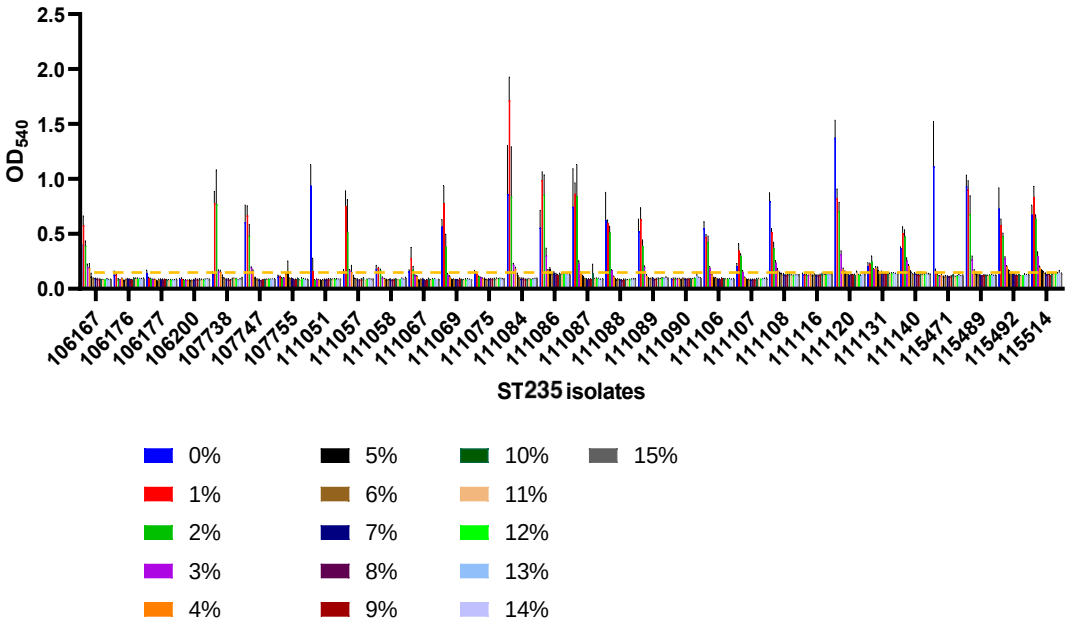

Figure 17. Optical density data demonstrating *P. aeruginosa* ST235 isolate 96-well plate biofilm formation after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours), grouped by isolate.

Whilst 37% of ST235 isolates produced the most biofilm in the absence (0%) of EtOH (n=11; 106177, 106200, 111051, 111075, 111088, 111106, 111108, 111120, 115472, 115489 and 115492), 63% of isolates grew more biofilm in the presence of EtOH (n=19; 106167, 106176, 107738, 107747, 107755, 111057, 111058, 111067, 111069, 111084, 111086, 111087, 111089, 111090, 111107, 111116, 111131, 111140 and 115514) (Figure 18). The highest OD value fell within a wide range 0-13% EtOH, however, 50% of isolates produced the most biofilm in 1% EtOH which is double that of ST111 isolates (figure 13). Furthermore, 107755 was the only isolate to produce the most biofilm in 5% EtOH of all ST235 and ST111 isolates (OD=0.155). There were two instances where maximum OD was much higher at 10% and 13% EtOH (111116 and 111090 respectively), however, statistical comparison using an ANOVA and Tukey post-hoc test showed these isolates had no significant variation in biofilm growth within their datasets. Finally, similarly to ST111 isolates, 8% was the most common group for minimum OD across ST235 isolates (n=11; 107747, 111057, 111058, 111067, 111069, 111088, 111089, 111106, 111107, 111108 and 111116).

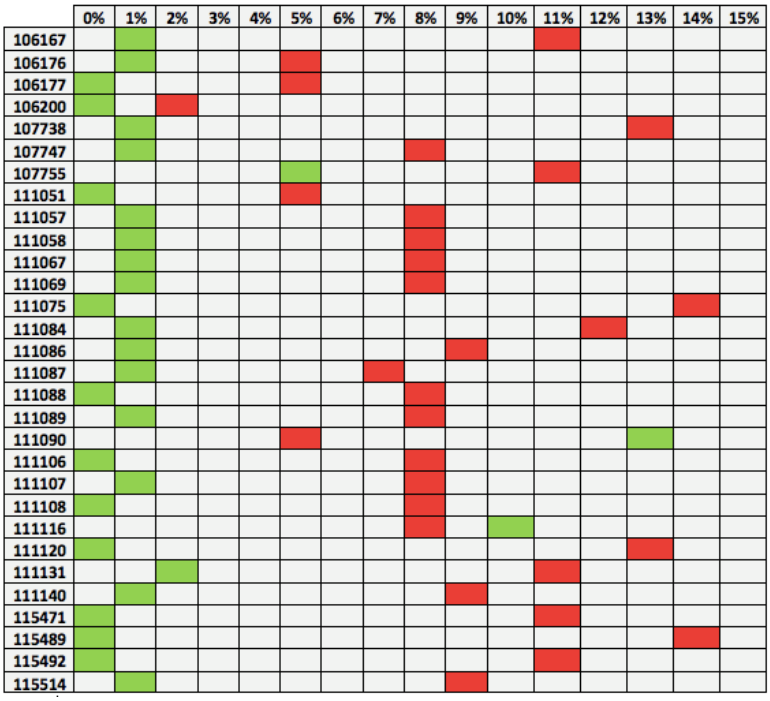

Figure 18. Minimum (red) and maximum (green) OD density readings for ST235 *P. aeruginosa* isolates representing 96-well plate biofilm production in the presence of 0-15% EtOH

Following statistical comparison using an ANOVA and Tukey post-hoc test, 20 isolates were identified to each have a statistically significant ($P<0.05$) variation of biofilm formation across the different EtOH concentrations (figure 19). Of these isolates, 40% produced the most biofilm in the absence EtOH (n=8; 111051, 111088, 111106, 111108, 111120, 115471, 115489 and 115492), whilst all remaining isolates (n=12) produced more biofilm in the presence of EtOH (1%). Isolates marked with stars in figure 19 (106167, 107738, 111057, 111067, 111069, 111084, 111086, 111089, 111140 and 115514) indicate statistically significant increases in biofilm production in the presence of EtOH and can be seen more clearly in figure 20. There was also high biofilm growth at 2% EtOH, with 6 isolates (107738, 111057, 111067, 111086, 111087 and 111140) producing more biofilm at 2% EtOH than 0%.

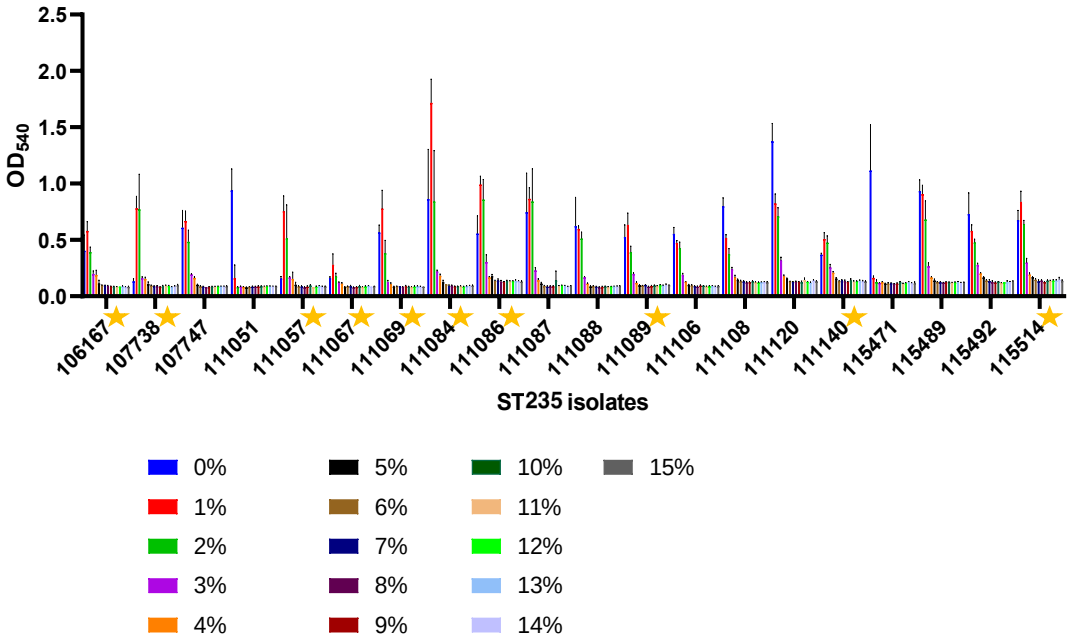

Figure 19. Optical density data demonstrating *P. aeruginosa* ST235 isolate 96-well plate biofilm formation after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours) that had statistically significant ($P<0.05$) variation in biofilm formation at different concentrations of EtOH.

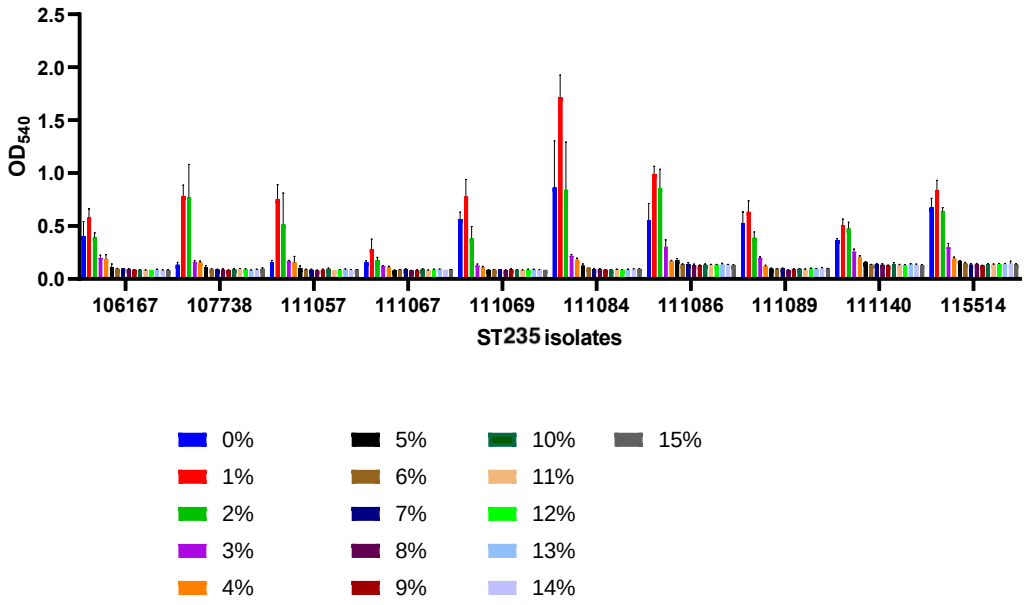

Figure 20. Optical density data demonstrating *P. aeruginosa* ST235 isolate 96-well plate biofilm formation after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours) that had statistically significant (P<0.05) variation in biofilm formation at different concentrations of EtOH, where an increase in biofilm was demonstrated in sub-inhibitory concentrations of EtOH compared to 0% control.

4.3 MBEC Plate Biofilm Results

4.3.1 Effect of EtOH on established biofilm- ST111 Isolates

Established biofilm (post 24-hour incubation on MBEC plates) was exposed to 0-15% EtOH to determine how the addition of EtOH affects biofilm growth mid-incubation. There was clear variation between isolates when comparing optical density at each percentage, as well as comparing each individual isolates growth at different concentrations of EtOH (figure 21). For example, the OD reading at 0% EtOH was 1650% higher in 107752 compared to 115526 (OD=3.5 and 0.2 respectively) and therefore 1650% more biofilm was present. However, the data also shows that biofilm formation was not prohibited in the presence of EtOH. For example, there are multiple instances where biofilm growth increased in the presence of EtOH

compared to the 0% EtOH control – namely isolates 106164, 106181, 107751, 111068, 111114, 111125, 111137, 111139, 115480, 115482, 115508, 115524, 115525, 115526, 115527, 115528, 115529 and 115531. Isolate 115508 produced the highest amount of biofilm in 1% EtOH, which mirrors a similar pattern to the 96-well plate data (see section 4.2), where biofilm growth gradually decreases until a plateau is reached at 7% EtOH. Whilst the amount of biofilm formation differs between isolates, this pattern is generally consistent throughout the data.

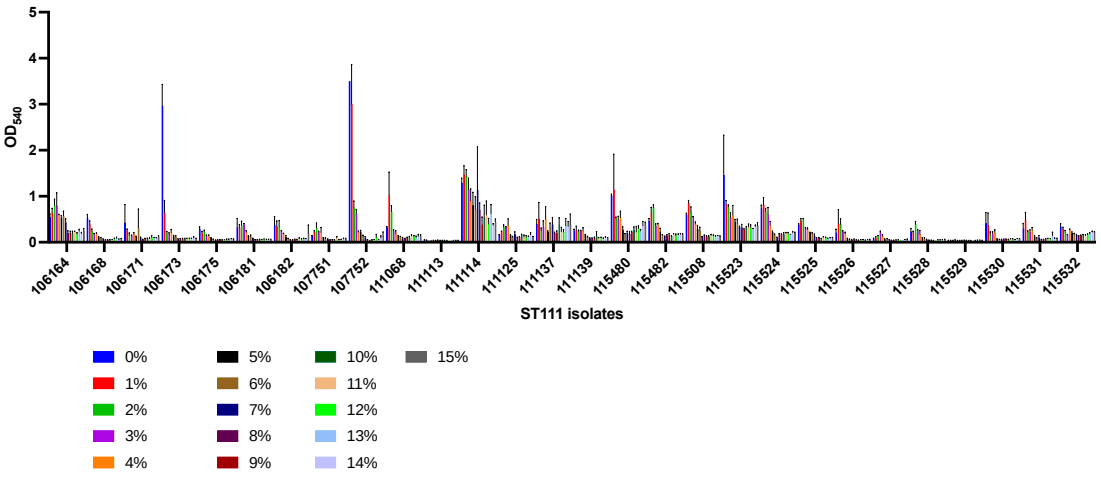

Figure 21. Optical density data demonstrating *P. aeruginosa* ST111 isolate MBEC biofilm formation after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours).

Following statistical comparison using an ANOVA and Tukey post-hoc test, 18 isolates were identified to each have a statistically significant ($P<0.05$) variation of biofilm formation across the different EtOH concentrations (figure 22). Just one-third of these isolates (n=6; 106168, 106171, 106173, 107752, 115523 and 115530) produced the most biofilm in the absence (0%) of EtOH, whilst the remaining 12 isolates (106164, 106181, 111068, 111114, 115480, 115482, 115508, 115524, 115525, 115526, 115528 and 115531) produced more biofilm upon addition of low-concentrations of EtOH. Whilst OD reduced after 1% EtOH in the majority of cases (106168, 106171, 106173, 107752, 111068, 111114, 115480, 115508, 115523, 115524, 115526, 115530 and 115531), there are also isolates (106164, 106181, 115482, 115525 and 115528) which continued to produce notable amounts of

biofilm in higher concentrations of EtOH. For example, a steady decrease in OD after 2% EtOH can be observed in isolate 106164 before a 54% decline between 7% and 8% EtOH, demonstrating the variation of biofilm phenotype in the presence of sub-inhibitory concentrations of EtOH.

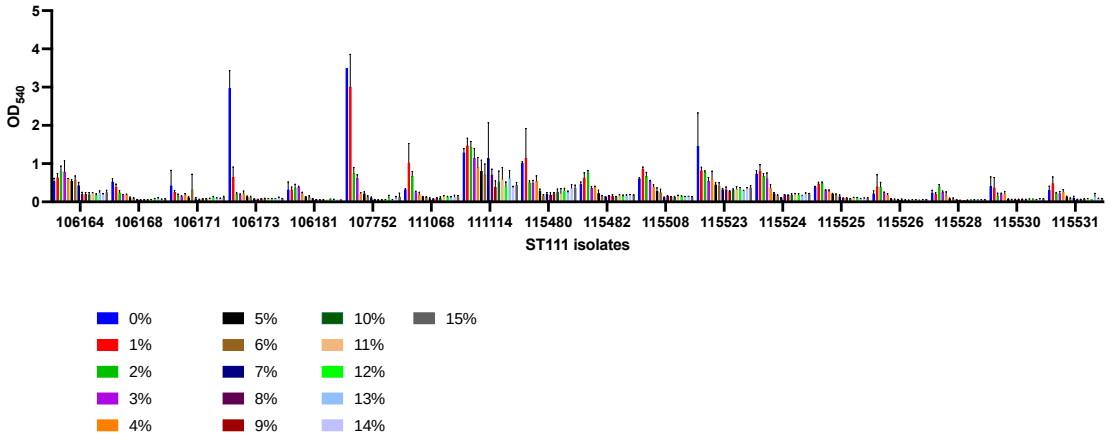

Figure 22. Optical density data demonstrating *P. aeruginosa* ST111 isolate MBEC biofilm formation after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours) that had statistically significant ($P<0.05$) variation in biofilm formation at different concentrations of EtOH.

Of the isolates that demonstrated a statistically significant difference in biofilm production (figure 22), 11 isolates demonstrated increases in biofilm production upon exposure to low concentrations of EtOH, producing the highest amount of biofilm in 1-2% EtOH (Figure 23). However, these isolates still showed variation between isolates, as maximum OD values ranged from 1.47 for isolate 111114 to just 0.39 for 115528. There were also instances where biofilm production increased again in EtOH concentrations that were higher than that of the minimum OD value within an isolate. For example, OD started to increase after reaching its minimum value at 6% EtOH for isolate 115480 (OD=0.166).

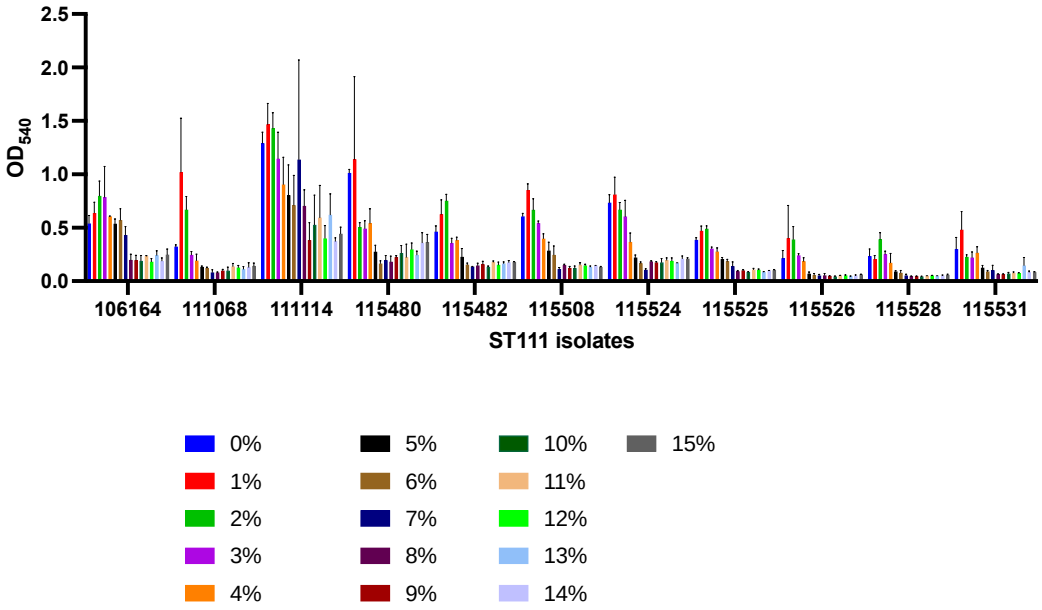

Figure 23. Optical density data demonstrating *P. aeruginosa* ST111 isolate MBEC biofilm formation after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours) that had statistically significant (P<0.05) variation in biofilm formation at different concentrations of EtOH, where an increase in biofilm was demonstrated in sub-inhibitory concentrations of EtOH compared to 0% control.

4.3.2 Effect of EtOH on established biofilm- ST235 Isolates

Patterns of biofilm growth where ST235 isolates were exposed to 0-15% EtOH mid-incubation were more variable (figure 24) than ST111 isolates described above. For example, OD readings for isolate 107747 fluctuate across the differing concentrations of EtOH and it was the only isolate to produce the most biofilm in 6% EtOH. A similar pattern was seen for isolate 115471 which produced the most biofilm at 11% EtOH. However, the majority of isolates still produced the most biofilm in low concentrations of EtOH (0-2%) followed by a steady decrease before plateauing at ~5-6%, (106167, 106176, 106177, 107738, 107755, 111051, 111058, 111075, 111084, 111086, 111087, 111088, 111089, 111106, 111107, 111108, 111120, 111131, 111140, 115489, 115492 and 115514). Data also shows variation in

capability to produce biofilm between ST235 isolates, with maximum OD values ranging from 3.02 in 111067 to 0.047 in 111116.

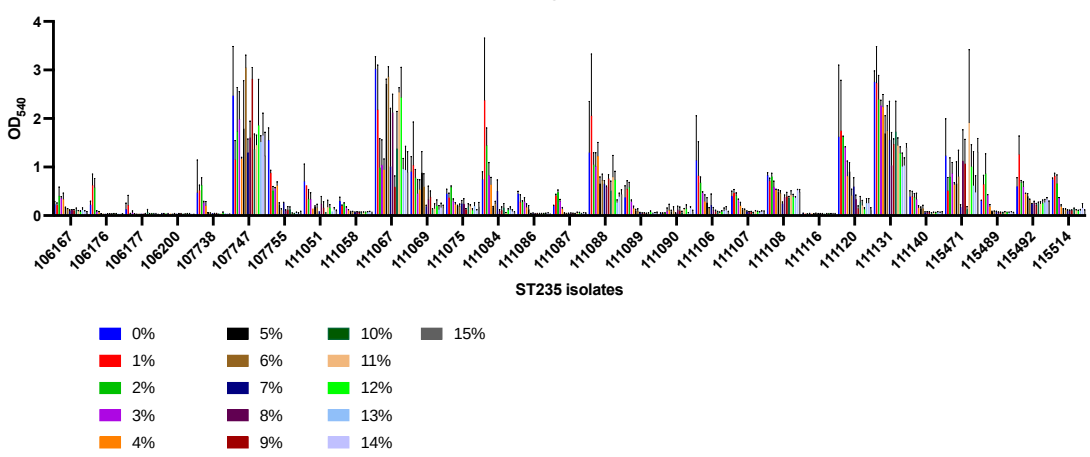

Figure 24. Optical density data demonstrating *P. aeruginosa* ST235 isolate MBEC biofilm formation after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours).

Following statistical comparison using an ANOVA and Tukey post-hoc test, just 12 isolates were identified to each have a statistically significant ($P<0.05$) variation of biofilm formation across the different EtOH concentrations (figure 25). These include those with OD readings that fluctuated across all concentrations of EtOH such as 111131, and those with a more standard biofilm pattern, decreasing in OD as the EtOH concentration increased - as described above. Biofilm production across ST235 isolates was at its highest amongst these fluctuating isolates with multiple OD readings >2.0, such as 0%, 6% in and 9% 107747 (OD= 2.48, 3.05 and 2.82 respectively).

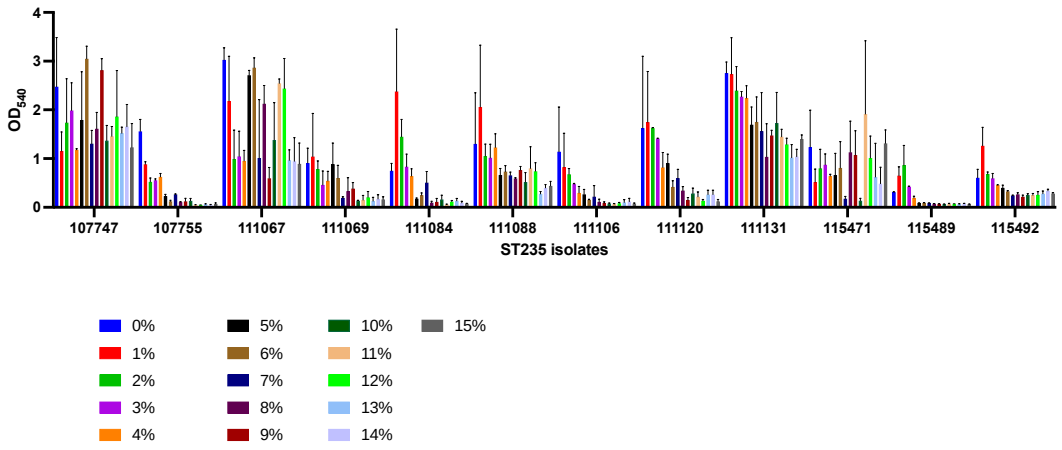

Figure 25. Optical density data demonstrating *P. aeruginosa* ST235 isolate MBEC biofilm formation after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours) that had statistically significant ($P<0.05$) variation in biofilm formation at different concentrations of EtOH.

Of the isolates that demonstrated a statistically significant difference in biofilm production (figure 25), 8 isolates demonstrated increases in biofilm production upon exposure to low concentrations of EtOH (figure 26). There are multiple instances where biofilm production continued in high concentrations of EtOH, for example, isolates 107747 and 115471 continued to produce biofilm in the presence of 15% EtOH (OD= 1.23 and 1.31 respectively) without reaching a plateau.

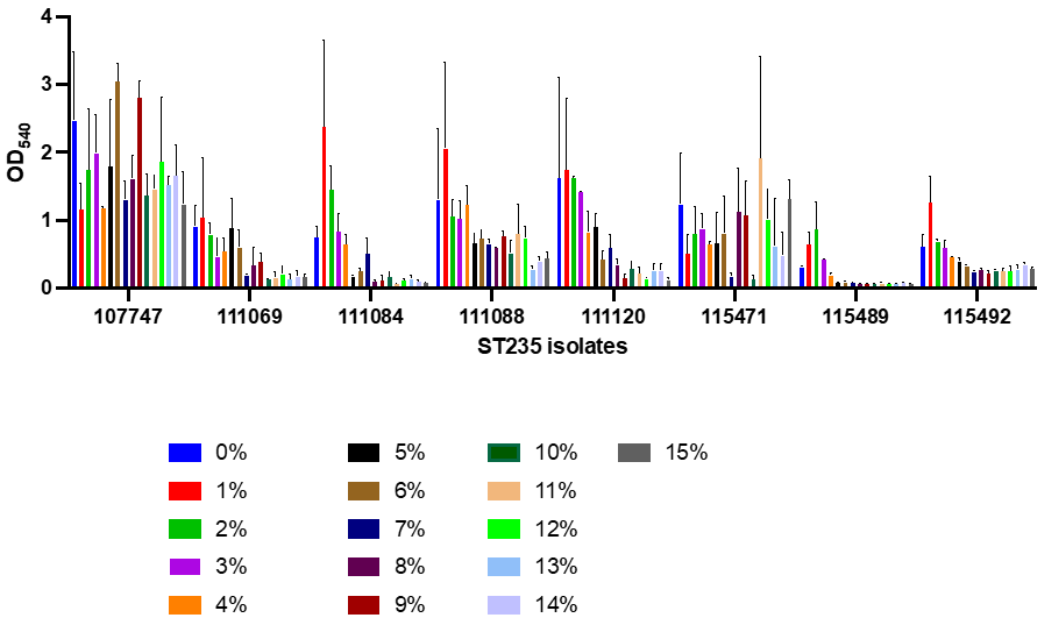

Figure 26. Optical density data demonstrating *P. aeruginosa* ST235 isolate MBEC biofilm formation after 48 hours with addition of 0-15% EtOH mid-incubation (24 hours) that had statistically significant ($P<0.05$) variation in biofilm formation at different concentrations of EtOH, where an increase in biofilm was demonstrated in sub-inhibitory concentrations of EtOH compared to 0% control.

4.4 Effect of material on biofilm formation

In addition to the 96-well plate and MBEC biofilm methods, there was intention to use the CDC biofilm reactor as an example of a more realistic biofilm model to determine differences in *P. aeruginosa* biofilm formation on 4 materials found in hospital settings: borosilicate glass, polycarbonate, 304 stainless steel and 316 stainless steel. However, due to fungal contamination of the system, no reproducible data was produced. Other limitations to the method included a reliance on technical staff to autoclave the entire system on a weekly basis which was not possible due to COVID restrictions at the time. Furthermore, the method was extremely time consuming so only a few select isolates would have been investigated, however, this would be an interesting study for a PhD student.

5 Discussion

5.1 Minimum Bactericidal Concentration

Differences in susceptibility to alcohols were observed both within and between ST111 and ST235 isolates by comparing MBC in EtOH and propan-2-ol. Results demonstrated that, overall, *P. aeruginosa* isolates were more susceptible to propan-2-ol than EtOH. This suggests that EtOH and propan-2-ol have differing modes of action against *P. aeruginosa,* and that propan-2-ol is more effective. The most common mode of action of alcohols against bacteria is denaturation of proteins (Boyce, 2018); however, a study by Haft et al. (2014) suggests that EtOH causes inhibition of messenger ribonucleic acid (mRNA) and protein synthesis via direct action on ribosomes and RNA polymerase. This provides a mechanism for the differing effectiveness of alcohols as a disinfectant even at equal concentrations. Bakht et al (2022) also assessed the susceptibility of biofilm producing and non-biofilm producing *P. aeruginosa* isolates to five common hospital disinfectants (70% EtOH, 5% sodium hypochlorite, 4.8% Dettol, 2% Saya Sept-HP and 2% chlorhexidine) and found 70% EtOH to be the least effective.

Variation in MBC within ST111 and ST235 groups was evident and suggests there were significant differences in the ability of isolates to resist the antimicrobial action of EtOH and propan-2-ol. Whilst differences in MBC only ranged by 1-4%, it is interesting to consider whether an association between increased tolerance to disinfectants and antibiotic resistance exists. In theory, this could help inform clinicians on how best to treat *P. aeruginosa* infections. However, for this to be a viable option, extensive further classification of *P. aeruginosa* strains is required.

Whilst differences were observed between MBC results for EtOH and propan-2-ol, of the 16 ST111 isolates that had the lowest MBC for propan-2-ol (7%), 11 also had the lowest MBC for EtOH (10%). This suggests they were the most susceptible to the antimicrobial effects of alcohol. Furthermore, isolate 111086 was the only ST235 isolate with the lowest MBC for propan-2-ol (7%) and was also amongst the most susceptible isolates in EtOH with an MBC of 10%.

Another interesting observation was whilst there was not a direct correlation between alcohol concentration and MBC, there was a general trend towards the decrease of bacterial growth as concentration of EtOH/propan-2-ol increased. However, more isolates (ST111 and ST235 combined) had an MBC of 11% than 10% EtOH and more isolates had an MBC of 8% propan-2-ol than 7%. This suggests that a 1% increase in alcohol at these specific concentrations does not make a significant difference to planktonic growth of *P. aeruginosa*. However, this is unlikely as fewer isolates survive 1% increases at higher concentrations of alcohol. It is more likely attributed to the subjective nature of the Miles and Misra method, where very minimal growth can be difficult to detect and is subject to human error. Alternatively, it is possible living cells existed in the well, but were not dropped onto the agar plate, due to only 20 µL of the solution being pipetted.

Another example of unusual results was the 3 instances (106164, 107751 and 111137) where *P. aeruginosa* growth was halted in 7-9% propan-2-ol but showed growth again at 10%. This suggests that 7-9% propan-2-ol inhibits a specific mechanism involved in cell growth that is not triggered at 10% propan-2-ol. Such results could be attributed to a stress response if the isolates had been grown in biofilm; however, their planktonic form makes this unlikely. Isolate 107751 also had the highest MBC in EtOH (13%) which supports its growth at 10% propan-2-ol and suggests it is one of the most resistant isolates. Isolate 115524 was the only other isolate with an MBC of 13% in EtOH and was the only isolate to grow consistently up to and including 10% propan-2-ol- it would be interesting to explore MBC above this range in future work. These isolates (107751 and 115524) would also be good examples of relatively more resistant *P. aeruginosa* for use in future studies.

In contrast, there were two isolates (111058 and 111067) that continued to grow in 10% propan-2-ol but did not have the highest MBC for EtOH. This suggests that isolates react differently to different alcohols and the most resistant isolates will not necessarily be consistent across studies. This is analogous to bacteria displaying variety in their resistance to different types of antibiotics. For example, *P. aeruginosa* is known to show resistance to nearly all antibiotics, including carbapenems, but can

be treated using a combination of two anti-pseudomonal antibiotics from different classes (Bassetti et al., 2018).

In both EtOH and propan-2-ol results, ST235 isolates generally survived higher concentrations of alcohol than ST111 isolates. This suggests that as a group they are more resistant to antimicrobial action and subsequently pose a higher risk to public health. This is significant as ST235 *P. aeruginosa* has been associated with MDR and more virulent infections in CF patients (Lee and Jones, 2018), and it is likely that ST235 isolates would also be able to tolerate higher concentrations of antibiotics compared to ST111 isolates. Increased tolerance to antibiotics in ST235 has also been suggested by Kocsis et al. (2021), who described colistin resistance in ST235 isolates despite *P. aeruginosa* remaining largely susceptible to colistin, including ST111 isolates. However, it is not clear whether the small increases in alcohol ST235 *P. aeruginosa* are able to tolerate would translate to more severe infection, or if they are too small to make a significant difference to the patient. Furthermore, despite ST235 isolates surviving better in EtOH and propan-2-ol, their MBCs were still in the same range as ST111 isolates. This suggests *P. aeruginosa* as a species is unlikely to be able to survive concentrations of EtOH above 12% and propan-2-ol above 10%.

5.2 96-Well Plate Biofilm

Due to time constraints as a result of the COVID-19 pandemic, biofilm experiments were carried out using EtOH only. EtOH was chosen over propan-2-ol due to more comparable studies existing in the literature. It would be interesting to repeat these experiments using propan-2-ol in future work.

Biofilm data generated using the 96-well plate method suggest that overall, *P. aeruginosa* isolates survive higher concentrations of EtOH (10-13%) in their planktonic form than in biofilm, where growth generally plateaus from 6% onwards. However, there were major differences in these methods (see section 3) so patterns of growth should be valued over growth at specific concentrations. Furthermore, it

is not clear from the biofilm data if at 6-15% EtOH there is no growth at all, or just minimal growth, which was easier to distinguish in the MBC method. Whilst isolates appear to form small quantities of biofilm in 6-15% EtOH (figure 11), it is likely this is cell debris from the 24-hour incubation prior to addition of EtOH that was subsequently detected by the plate reader. On reflection, this method could be improved by plating a sample from each well onto agar to determine if there was any living *P. aeruginosa* present at these concentrations.

Whilst *P. aeruginosa* isolates generally followed a similar trend of growth in this biofilm model, there were significant differences in biofilm formation between isolates within each group. This is a reflection of the inherent heterogeneity of *P. aeruginosa* and demonstrates how they are able to successfully colonise such a variety of environmental niches. For example, biofilm growth in some ST111 isolates varied significantly depending on EtOH concentration, whereas others showed minimal variation from 0-15%. The isolates that did not appear to be affected by EtOH concentration did not form extensive biofilm in the absence of EtOH, which suggests they are poor biofilm-producers. It is interesting to consider the factors that influence *P. aeruginosa* isolates to be good or poor biofilm producers. This can be investigated by gene expression analysis, as it is likely that good biofilm producers express similar sets of genes. A study by Müsken et al. (2010) screened the Harvard *P. aeruginosa* PA14 mutant library for mutants exhibiting an altered biofilm phenotype and identified 394 and 285 genetic determinants of reduced and enhanced biofilm production which were mostly involved in survival under microaerophilic growth conditions, arginine metabolism, pH homeostasis, alkyl-quinolone signalling and the DNA repair system. This exemplifies the vast range of genes involved in biofilm formation and offers an explanation for the variation in ability of ST111 and ST235 isolates to produce biofilm.

Similarities exist between the 96 well plate biofilm data and the MBC data where *P. aeruginosa* were in their planktonic form. Interestingly, isolates 111139 and 115532 demonstrated increases in biofilm formation at 5% after an initial decline following their peak growth. Whilst they are different isolates, this is a similar pattern to MBC

results for 106164, 107751 and 111137, where *P. aeruginosa* growth was halted in 7-9% propan-2-ol but began again at 10% (discussed above). Alone, these results appear unusual and may be dismissed as an artefact, although each example of this growth pattern makes the other more credible. A study by Łapińska et al. (2022) identified a subset of fast-growing phenotypic variants of *P. aeruginosa* that were able to avoid macrolide accumulation and survive antibiotic treatment without genetic mutations. Such variants displayed significantly higher expression of ribosomal promotors compared to slow-growing variants. This suggests isolates that survived higher concentrations of EtOH may have a similar mechanism of AMR.

By mapping minimum and maximum OD readings for each isolate, it became clear that the majority of ST111 isolates produced more biofilm in the absence of EtOH (figure 13), whilst the majority of ST235 isolates produced more biofilm in the presence of EtOH (figure 18). This suggests ST235 isolates are more sensitive to sub-inhibitory concentrations of alcohol and increase biofilm production as a response. Tango et al. (2018) found *P. aeruginosa* PA01 biofilm formation to be induced by 1-2% EtOH and found 2.5-3.5% EtOH to stimulate biofilm formation *in Staphylococcus aureus* ATCC 13150. Sub-inhibitory concentrations of EtOH have subsequently been recognised as a crucial environmental factor influencing biofilm formation (He et al., 2022). Furthermore, a study by Lewis et al (2019) investigated microbes that produce low concentrations of EtOH proposed that EtOH acts as a signal pathway to rapidly repress *P. aeruginosa* swimming and swarming motility and induces matrix production to promote biofilm initiation.

There was greater variation in minimum OD readings which ranged from 2-15% EtOH and notably, 8% was a common group for both ST111 and ST235 isolates.
It is interesting to consider why biofilm formation wasn't lowest at the highest concentration of EtOH (15%); perhaps biofilm is eradicated by 8% EtOH and therefore subsequently higher concentrations of EtOH have no greater effect. However, this would mean it was a coincidence that minimum OD readings for 17 isolates fell at 8% which is unlikely. Therefore, it would be interesting to generate a genetic tree of isolates from these data to determine if there is any shared ancestry between these

17 isolates. Furthermore, it would also be interesting to investigate if there is shared genetic ancestry between isolates 111108-115514 (n=9) which produced relatively more biofilm in 6-15% EtOH compared to isolates 106176-111107 (figure 17).

Whilst it's interesting to compare biofilm growth of all isolates, it was important to consider the statistical significance of the results, especially when comparing very minimal differences in biofilm production. Therefore, using ANOVA and Tukey post-hoc tests were crucial to the integrity of the results as they allowed separation of isolates which showed statistically significant variation in biofilm production between EtOH concentrations from those that did not. Furthermore, they were used to determine which isolates showed a significant increase in biofilm production in the presence of EtOH; 32% of ST111 and 33% of ST235 isolates. It is remarkable these percentages are so similar, and it suggests one could expect 32-33% of other *P. aeruginosa* strains to increase biofilm production in the presence of 1-3% EtOH, particularly those known to show antimicrobial resistance. Of the isolates that produced significantly more biofilm in EtOH, one from each group (107751 in ST111 and 106167 in ST235) also had the highest MBC for EtOH (13%), which suggests they are the most resistant to the antimicrobial effects of EtOH of all 58 isolates.

In contrast, it is also crucial to consider those isolates in which biofilm formation was not induced by sub-inhibitory concentrations of EtOH. For example, isolates 111051 and 115471 showed a dramatic drop in biofilm formation between 0% and 1% EtOH, which suggests they are not able to tolerate EtOH at all.

5.3 MBEC Plate Biofilm

Whilst the 96-well plate biofilm assay determined if *P. aeruginosa* isolates could produce biofilm in the presence on 0-15% EtOH, the MBEC assay investigated the effect of 0-15% EtOH on established biofilm. Like the 96-well plate data, there was variety both within and between ST111 and ST235 groups, however the extent of variation between isolates from the same group was much greater in the MBEC

results. This suggests presence of EtOH has a greater effect on established biofilm than in the development of biofilm. Furthermore, in the majority of isolates, the presence of EtOH did not inhibit biofilm production and actually stimulated growth in sub-inhibitory concentrations. Following statistical analysis, isolates that had significant variation within their dataset were identified and the majority of these isolates (63%) also produced the most biofilm in the presence of EtOH. This was not the case in the 96 well plate data, where 48% of isolates had a significant increase in biofilm production in the presence of EtOH.

Amongst the isolates that produced significantly more biofilm in the presence of EtOH, there were instances where biofilm continued to form at all concentrations including 15% EtOH. This suggests that these isolates would have been able to tolerate higher concentrations of EtOH in established biofilm which would be interesting to investigate in future work. This was not the case is the 96-well plate biofilm model which shows *P. aeruginosa* in established biofilm are able to withstand greater concentrations of EtOH than in the initial stages of biofilm formation.

Whilst 107751 (ST111) and 106167 (ST235) had the highest MBCs (figs. 6 & 7) and showed a statistically significant ($P<0.05$) increase in biofilm formation in the presence of EtOH in the 96-well plate method (figs. 15 & 20), neither of them showed significant increases in biofilm formation in the presence of EtOH in the MBEC method. However, isolate 115524 from ST111 and isolates 111120 and 115492 from ST235 did show significant increases using the MBEC method and also had the highest MBC for EtOH (13%). It is interesting that the same isolates are not consistently better biofilm producers throughout different methods. However, there were two instances within each group (115525 and 115526 from ST111 and 111069 and 111084 from ST235) where biofilm production significantly increased in the presence of EtOH in both the 96-well plate and MBEC biofilm methods. This perhaps makes them the most interesting yet dangerous isolates.

5.4 Future Work

In order to build upon the findings of this study, it would be interesting to repeat the biofilm experiments using sub-inhibitory concentrations of propan-2-ol to determine if it also promotes biofilm formation in *P. aeruginosa*. This could help to advise on the disinfectants used in hospital settings. It is important to replicate the environments in which biofilms are found in hospital settings, so methods such as the CDC reactor are key to generating accurate biofilm data in addition to the 96-well/MEBC plate methods. Future studies are likely to focus on mechanisms of biofilm formation in order to develop novel treatments that not only eradicate the infection but prevent disease progression by targeting said mechanisms in their tracks. This has the potential to limit the damage caused to patients.

6. Conclusion

This study has established that statistically significant variation in response to low levels of alcohol exists between *P. aeruginosa* isolates belonging to ST111 and ST235. MBC results showed that *P. aeruginosa* were more susceptible to propan-2-ol than EtOH and that there was variation in the ability of isolates to resist the antimicrobial action of alcohols, with ST235 isolates generally surviving better than ST111 isolates. The 96-well plate biofilm model demonstrated significant differences in the ability of isolates to form biofilm in the presence of EtOH. The majority of ST111 isolates produced more biofilm in the absence of EtOH whilst the majority of ST235 isolates produced more biofilm in low levels of EtOH. Furthermore, it showed 19 instances of biofilm formation significantly increasing in the presence of sub-inhibitory concentrations of EtOH. Similarly, sub-inhibitory concentrations of EtOH increased biofilm production of 19 isolates where biofilm had already been established in MBEC plates. Finally, *P. aeruginosa* in established biofilm were able to withstand greater concentrations of EtOH than in the initial stages of biofilm formation.

The variety exhibited by *P. aeruginosa* isolates in this study is testament to the need for a more targeted approach in the treatment of biofilm infections. Understanding the environmental and genetic prerequisites of biofilm formation is key to achieving this. MDR is the most pressing threat to public health associated with *P. aeruginosa* infection, therefore research and development of novel treatments is essential in limiting mortality associated with *P. aeruginosa* biofilm infections. Furthermore, it is essential to address the role of alcohol-based disinfectants in the stimulation of biofilm formation as a side effect of overuse and the role of the water system in contamination of hospital settings.

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8 Appendix

Appendix 1. Table of isolate origin information for 58 *P. aeruginosa* isolates

BM NAME	NAME	MLST	ANATOMICAL SITE	DATE OF ISOLATION	COUNTRY OF ORIGIN
VTK_106164	WH-SGI-V-07168	111	Hospital	1992	France
VTK_106168	WH-SGI-V-07171	111	Hospital	1992	France
VTK_106171	WH-SGI-V-07173	111	Hospital	1992	France
VTK_106173	WH-SGI-V-07174	111	Hospital	1992	France
VTK_106175	WH-SGI-V-07175	111	Hospital	1992	France
VTK_106181	WH-SGI-V-07180	111	Hospital	1991	France
VTK_106182	WH-SGI-V-07221	111	Hospital	1992	France
VTK_107751	WH-SGI-V-07391	111	Hospital	1992	France
VTK_107752	WH-SGI-V-07392	111	Hospital	1992	France
VTK_111068	WH-SGI-V-07497	111	Hospital	2005	USA
VTK_111113	WH-SGI-V-07651	111	Hospital	2005	USA
VTK_111114	WH-SGI-V-07652	111	Hospital	2005	USA
VTK_111125	WH-SGI-V-07688	111	Hospital	2005	USA
VTK_111137	WH-SGI-V-07700	111	Hospital	2005	USA
VTK_111139	WH-SGI-V-07702	111	Hospital	2005	USA
VTK_115480	WH-SGI-V-07256	111	Hospital	1997	Belgium
VTK_115482	WH-SGI-V-07259	111	Burn	1998	Belgium
VTK_115508	WH-SGI-V-07296	111	Urine	1985	Belgium
VTK_115523	WH-SGI-V-07320	111	Clinical non-CF	2004	Belgium
VTK_115524	WH-SGI-V-07322	111	Blood	2003	Colombia
VTK_115525	WH-SGI-V-07323	111	Burn	1997	Turkey
VTK_115526	WH-SGI-V-07324	111	Sputum	1994	Greece
VTK_115527	WH-SGI-V-07325	111	Urine	1999	Colombia
VTK_115528	WH-SGI-V-07326	111	Clinical non-CF	1993	Belgium
VTK_115529	WH-SGI-V-07327	111	Sputum	1999	Belgium
VTK_115530	WH-SGI-V-07328	111	Sputum	1999	Belgium
VTK_115531	WH-SGI-V-07329	111	Wound	1994	Greece
VTK_115532	WH-SGI-V-07330	111	Wound	1997	Bulgaria
VTK_106167	WH-SGI-V-07170	235	Hospital	1992	France
VTK_106176	WH-SGI-V-07176	235	Hospital	1992	France
VTK_106177	WH-SGI-V-07177	235	Hospital	1992	France
VTK_106200	WH-SGI-V-07186	235	Hospital	1992	France
VTK_107738	WH-SGI-V-07378	235	Hospital	1988	France
VTK_107747	WH-SGI-V-07387	235	Hospital	1991	France
VTK_107755	WH-SGI-V-07406	235	Hospital	1992	France
VTK_111051	WH-SGI-V-07425	235	Hospital	2005	USA
VTK_111057	WH-SGI-V-07486	235	Hospital	2005	USA
VTK_111058	WH-SGI-V-07487	235	Hospital	2005	USA

VTK_111067	WH-SGI-V-07496	235	Hospital	2005	USA
VTK_111069	WH-SGI-V-07498	235	Hospital	2005	USA
VTK_111075	WH-SGI-V-07504	235	Hospital	2005	USA
VTK_111084	WH-SGI-V-07622	235	Hospital	2005	USA
VTK_111086	WH-SGI-V-07624	235	Hospital	2005	USA
VTK_111087	WH-SGI-V-07625	235	Hospital	2005	USA
VTK_111088	WH-SGI-V-07626	235	Hospital	2005	USA
VTK_111089	WH-SGI-V-07627	235	Hospital	2005	USA
VTK_111090	WH-SGI-V-07628	235	Hospital	2005	USA
VTK_111106	WH-SGI-V-07644	235	Hospital	2005	USA
VTK_111107	WH-SGI-V-07645	235	Hospital	2005	USA
VTK_111108	WH-SGI-V-07646	235	Hospital	2005	USA
VTK_111116	WH-SGI-V-07679	235	Hospital	2005	USA
VTK_111120	WH-SGI-V-07683	235	Hospital	2005	USA
VTK_111131	WH-SGI-V-07694	235	Hospital	2005	USA
VTK_111140	WH-SGI-V-07703	235	Hospital	2005	USA
VTK_115471	WH-SGI-V-07247	235	Burn	1997	Germany
VTK_115489	WH-SGI-V-07268	235	Burn	1997	Hungary
VTK_115492	WH-SGI-V-07276	235	Burn	1997	Turkey
VTK_115514	WH-SGI-V-07309	235	Burn	1997	Bulgaria

Appendix 2. Mean and standard deviation of OD values for ST111 isolate biofilm data grown in absence of, or in the presence of different concentrations of EtOH.

		106164	106168	106171	106173	106175	106181	106182	107751	107752	111068	111113	111114	111125	111137	111139	115480	115482	115508	115523	115524	115525	115526	115527	115528	115529	115530	115531	115532
0	Mean	0.43733	0.20833	0.19167	0.12533	0.42933	0.31367	0.467	0.119	0.56067	0.43833	0.10967	0.285	0.314	0.10533	0.3	0.139	0.378	0.30267	0.89233	0.576	0.41167	0.383	0.166	0.33367	0.12433	0.23267	0.29033	0.20567
	St Dev	0.01595	0.0261	0.0968	0.02026	0.18007	0.03213	0.002	0.06149	0.1947	0.15758	0.03092	0.01473	0.10153	0.00306	0.05408	0.01179	0.06173	0.6239	0.4687	0.09358	0.17053	0.03387	0.01323	0.14199	0.07073	0.06453	0.13413	0.03553
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
1%	Mean	0.30467	0.127	0.125	0.11633	0.25867	0.36133	0.22533	0.28567	0.929	0.174	0.08633	0.20167	0.479	0.11333	0.75233	0.13633	0.221	0.441	0.698	0.333	1.10333	0.383	0.282	0.194	0.08933	0.31067	0.19567	0.13633
	St Dev	0.13364	0.01136	0.01058	0.0179	0.1125	0.07454	0.04067	0.01168	0.13066	0.07692	0.00231	0.03856	0.07219	0.00551	0.19798	0.01922	0.03503	0.0985	0.08461	0.10704	0.15972	0.03387	0.06564	0.00529	0.00252	0.03024	0.01097	0.01328
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
2%	Mean	0.23533	0.112	0.12133	0.098	0.147	0.37633	0.28833	0.07533	0.51033	0.10967	0.07967	0.18433	0.24033	0.09767	0.42133	0.12667	0.21167	0.54833	0.60233	0.33233	0.68967	0.73	0.166	0.22467	0.093	0.292	0.16033	0.142
	St Dev	0.0155	0.00346	0.01861	0.00361	0.01212	0.05348	0.0351	0.00351	0.13167	0.00907	0.00208	0.04244	0.03201	0.00252	0.0842	0.00702	0.01834	0.14034	0.1413	0.0468	0.17123	0.35608	0.00656	0.02603	0.01345	0.05069	0.00321	0.02427
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
3%	Mean	0.146	0.104	0.10367	0.10167	0.12533	0.316	0.24567	0.33767	0.30133	0.09733	0.089	0.11133	0.14733	0.09767	0.183	0.10767	0.129	0.34933	0.23467	0.2133	0.257	0.22067	0.152	0.20367	0.08367	0.169	0.11133	0.16133
	St Dev	0.01825	0.007	0.00462	0.00208	0.00493	0.02946	0.03859	0.22113	0.0138	0.00379	0.002	0.00569	0.00643	0.01079	0.00854	0.00493	0.01735	0.05164	0.00473	0.02579	0.06751	0.00681	0.008	0.01501	0.00153	0.02427	0.00153	0.00808
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
4%	Mean	0.14533	0.11667	0.13467	0.113	0.15633	0.30367	0.25633	0.23167	0.29233	0.10433	0.08833	0.12867	0.166	0.09733	0.177	0.10133	0.124	0.34433	0.22833	0.18367	0.28533	0.28167	0.14867	0.19067	0.08233	0.15533	0.11233	0.12267
	St Dev	0.02219	0.01168	0.02639	0.01253	0.02139	0.0398	0.06014	0.03317	0.03602	0.01115	0.00153	0.0223	0.03012	0.00709	0.01852	0.00666	0.01044	0.10333	0.05021	0.03787	0.07759	0.10454	0.01102	0.02658	0.00115	0.02021	0.00611	0.00115
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
5%	Mean	0.09533	0.08833	0.114	0.09767	0.10367	0.13133	0.13567	0.23067	0.126	0.09267	0.078	0.15233	0.087	0.09233	0.282	0.12167	0.1	0.113	0.12967	0.131	0.134	0.12333	0.10933	0.22733	0.08367	0.13267	0.10767	0.185
	St Dev	0.0085	0.01447	0.02707	0.00208	0.00907	0.00702	0.0371	0.02857	0.04158	0.01986	0.001	0.06921	0.00265	0.00153	0.08802	0.02829	0.01652	0.014	0.03317	0.02848	0.02821	0.0429	0.00058	0.08667	0.00252	0.00808	0.00404	0.05151
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
6%	Mean	0.089	0.089	0.093	0.09233	0.09667	0.102	0.098	0.25433	0.089	0.09367	0.08833	0.094	0.08467	0.08967	0.09367	0.08667	0.07967	0.087	0.08533	0.08533	0.09067	0.09	0.09133	0.10333	0.085	0.094	0.10267	0.10367
	St Dev	0.001	0.00346	0.00361	0.00306	0.00379	0.00624	0.004	0.0685	0.001	0.00416	0.00153	0.00265	0.00451	0.00416	0.00252	0.00058	0.00208	0.0052	0.00252	0.00252	0.00252	0.00529	0.0115	0.00404	0.00173	0.00173	0.00289	0.01159
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
7%	Mean	0.099	0.093	0.09733	0.09	0.10467	0.11733	0.10667	0.113	0.09767	0.107	0.09133	0.093	0.08867	0.097	0.10933	0.08933	0.08967	0.08933	0.08767	0.095	0.10267	0.10667	0.103	0.097	0.08467	0.094	0.10567	0.11667
	St Dev	0.00721	0.00608	0.00577	0.00361	0.00902	0.02483	0.02454	0.02234	0.00896	0.0203	0.00153	0.00964	0.00153	0.00721	0.02421	0.00231	0.00289	0.00208	0.00723	0.0148	0.01504	0.01498	0.00781	0.00458	0.00115	0.005	0.00252	0.01504
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
8%	Mean	0.087	0.081	0.09667	0.08533	0.08567	0.09267	0.09833	0.1	0.08867	0.087	0.08333	0.08733	0.08233	0.09167	0.09467	0.09233	0.08	0.08467	0.084	0.08667	0.08867	0.08767	0.08333	0.09767	0.07967	0.08767	0.091	0.09833
	St Dev	0.00608	0.003	0.00379	0.00208	0.00379	0.01079	0.01358	0.01682	0.00379	0.00265	0.00208	0.00115	0.00404	0.00451	0.01361	0.00651	0.01039	0.00153	0.00173	0.01361	0.00569	0.01026	0.00252	0.02043	0.00058	0.00115	0.00361	0.00404
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
9%	Mean	0.086	0.08633	0.088	0.08733	0.08867	0.08767	0.087	0.11033	0.08467	0.09	0.09033	0.08767	0.08767	0.086	0.084	0.082	0.073	0.08267	0.07933	0.07933	0.081	0.08033	0.08733	0.08567	0.08667	0.08633	0.09267	0.09
	St Dev	0.00173	0.00252	0.001	0.00289	0.00252	0.00231	0.00361	0.02223	0.00058	0.00346	0.00252	0.00231	0.00321	0.00173	0.00173	0.00173	0.0073	0.00321	0.00058	0.00231	0.00361	0.00231	0.00208	0.00351	0.00058	0.00513	0.00153	0.001
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
10%	Mean	0.08967	0.08767	0.09167	0.09067	0.093	0.09633	0.09067	0.08367	0.08333	0.089	0.091	0.09333	0.093	0.088	0.08633	0.08433	0.076	0.085	0.07933	0.07767	0.08467	0.08767	0.09367	0.08933	0.092	0.09133	0.095	0.101
	St Dev	0.00153	0.00404	0.00153	0.00058	0.00529	0.00586	0.00289	0.00808	0.00153	0.00265	0	0.00306	0.00361	0.001	0.00586	0.00252	0.003	0.00755	0.00306	0.00153	0.00231	0.00651	0.00723	0.00252	0.00608	0.00208	0.005	0.00127
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
11%	Mean	0.08233	0.08033	0.084	0.089	0.08633	0.08733	0.08833	0.08433	0.08533	0.08833	0.087	0.08933	0.08467	0.089	0.08833	0.08733	0.07533	0.082	0.079	0.077	0.078	0.07967	0.08633	0.08767	0.08567	0.088	0.087	0.09533
	St Dev	0.00058	0.00493	0.001	0.001	0.00493	0.00153	0.00473	0.00252	0.00351	0.00252	0.002	0.00252	0.00208	0.00265	0.00513	0.00451	0.00252	0.003	0.002	0.00265	0.002	0.00208	0.00321	0.00289	0.00208	0.003	0.00173	0.00153
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
12%	Mean	0.086	0.09033	0.091	0.08967	0.08767	0.087	0.08767	0.099	0.085	0.093	0.08833	0.09	0.088	0.091	0.08767	0.08567	0.076	0.084	0.08	0.08067	0.07967	0.07933	0.088	0.087	0.08767	0.08533	0.09233	0.09933
	St Dev	0.001	0.00153	0.007	0.00058	0.00153	0.003	0.00058	0.00781	0.001	0.00361	0.00153	0.00265	0.00361	0.001	0.00153	0.00404	0.00173	0.00361	0.002	0.00153	0.00115	0.00321	0.001	0.00346	0.00289	0.00153	0.00321	0.01286
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
13%	Mean	0.09033	0.08667	0.09233	0.09	0.08967	0.09167	0.09633	0.08333	0.08533	0.08467	0.09033	0.09467	0.09267	0.09267	0.09633	0.08867	0.08333	0.07833	0.08733	0.07833	0.07967	0.08	0.089	0.09067	0.088	0.09467	0.08967	0.09933	0.09567
	St Dev	0.00404	0.00208	0.00252	0.001	0.00306	0.00321	0.00058	0.00153	0.00208	0.00289	0.00351	0.00462	0.00208	0.00153	0.00208	0.00153	0.00289	0.00289	0.00289	0.00115	0.00153	0.001	0.00608	0.0115	0.001	0.00764	0.00208	0.00473	0.00503
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
14%	Mean	0.086	0.08467	0.08633	0.09	0.084	0.08867	0.085	0.08567	0.08267	0.09067	0.088	0.089	0.08333	0.092	0.08733	0.08867	0.07567	0.083	0.08033	0.085	0.08	0.08167	0.09267	0.08333	0.08733	0.086	0.091	0.09
	St Dev	0.003	0.00551	0.00153	0.00265	0.00173	0.00289	0.00265	0.00153	0.00208	0.00153	0.001	0.00265	0.00058	0.002	0.00115	0.00252	0.00115	0.002	0.00252	0.001	0.001	0.00379	0.00306	0.00289	0.00404	0.001	0.001	0.001
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
15%	Mean	0.08867	0.09233	0.08867	0.09167	0.09067	0.09033	0.09067	0.092	0.08433	0.09233	0.09033	0.09033	0.092	0.08667	0.08367	0.08767	0.07733	0.085	0.08333	0.081	0.08233	0.08733	0.08867	0.08933	0.08933	0.08833	0.09433	0.09
	St Dev	0.00058	0.00379	0.00321	0.00058	0.00208	0.00231	0.00208	0.00781	0.00153	0.00058	0.00252	0.00058	0.00656	0.00231	0.00115	0.00231	0.00493	0.001	0.00058	0.001	0.00153	0.00451	0.00252	0.00208	0.00115	0.00208	0.00404	0
	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3

Appendix 3. Mean and standard deviation of OD values for ST235 isolate biofilm data grown in absence of, or in the presence of different concentrations of EtOH.

		106167	106176	106177	106200	107738	107747	107755	111051	111057	111058	111067	111069	111075	111084	111086	111087	111088	111089	111090	111106	111107	111108	111116	111120	111131	111140	115471	115489	115492	115514
Mean		0.40567	0.12767	0.14167	0.091	0.134	0.60867	0.11167	0.94033	0.15667	0.18233	0.16133	0.568	0.15733	0.86433	0.55633	0.74733	0.62333	0.52533	0.08967	0.555	0.208	0.79867	0.135	1.376	0.20733	0.36967	1.11733	0.93367	0.72967	0.675
St Dev		0.13726	0.03406	0.02868	0.00346	0.02427	0.15274	0.01328	0.19029	0.01858	0.03101	0.01201	0.06514	0.00971	0.43916	0.15645	0.34604	0.25391	0.10825	0.00351	0.05709	0.02252	0.07481	0.00866	0.15839	0.03197	0.01172	0.40764	0.10043	0.18762	0.08648
N	0%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.58	0.13	0.09467	0.06933	0.78133	0.67	0.11367	0.16133	0.75533	0.192	0.28	0.77833	0.14933	1.78	0.993	0.86533	0.599	0.63333	0.092	0.47433	0.34867	0.517	0.13033	0.826	0.22667	0.509	0.163	0.90667	0.58033	0.83833
St Dev		0.08302	0.01735	0.00981	0.01115	0.1057	0.08466	0.00462	0.11752	0.13459	0.00557	0.03707	0.16256	0.00416	0.20762	0.07318	0.10132	0.026	0.10531	0.00624	0.02001	0.06353	0.02972	0.00416	0.08205	0.0095	0.05667	0.01652	0.07724	0.0546	0.09311
N	1%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.392	0.085	0.09	0.07933	0.77367	0.494	0.09533	0.083	0.51533	0.16967	0.17567	0.382	0.11067	0.843	0.85833	0.84167	0.51433	0.33133	0.09067	0.42533	0.30133	0.37433	0.12533	0.70867	0.24967	0.475	0.128	0.68233	0.47833	0.63833
St Dev		0.04453	0.007	0.00721	0.00681	0.30803	0.1025	0.00709	0.001	0.29536	0.01041	0.02991	0.17544	0.00404	0.45	0.17733	0.29115	0.0559	0.05335	0.0085	0.05564	0.01834	0.05029	0.00551	0.07714	0.04826	0.0608	0.01825	0.1652	0.02868	0.03664
N	2%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.19967	0.08367	0.08567	0.08267	0.161	0.19467	0.102	0.087	0.16867	0.10367	0.12133	0.128	0.10467	0.219	0.306	0.23033	0.17033	0.19967	0.09367	0.19067	0.153	0.238	0.12767	0.31767	0.17933	0.257	0.119	0.26833	0.27933	0.29967
St Dev		0.02589	0.00321	0.00252	0.00208	0.01311	0.00351	0.001	0.00265	0.00833	0.00473	0.00681	0.01311	0.00231	0.014	0.06317	0.02538	0.00513	0.01007	0.00208	0.01305	0.01386	0.01908	0.00115	0.02928	0.00493	0.02516	0.00346	0.03014	0.01332	0.03581
N	3%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.19133	0.096	0.086	0.08133	0.155	0.16367	0.13267	0.08433	0.156	0.09233	0.11233	0.10633	0.09533	0.18233	0.16967	0.145	0.11367	0.12033	0.093	0.12933	0.10433	0.16733	0.134	0.178	0.18867	0.20833	0.12533	0.168	0.204	0.193
St Dev		0.0388	0.00265	0.003	0.00115	0.0151	0.00945	0.00702	0.00306	0.0573	0.00306	0.00961	0.01332	0.00306	0.01419	0.00666	0.00115	0.00493	0.00896	0.00458	0.00153	0.00289	0.01909	0.00458	0.01136	0.01528	0.01106	0.00586	0.00755	0.00819	0.0151
N	4%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.11267	0.07933	0.07667	0.08233	0.11	0.10267	0.15533	0.07933	0.104	0.086	0.08233	0.086	0.09233	0.12667	0.17967	0.11367	0.089	0.09833	0.09	0.10067	0.08767	0.146	0.12633	0.15733	0.17067	0.157	0.11333	0.141	0.16767	0.176
St Dev		0.02977	0.00289	0.00115	0.00416	0.01852	0.00503	0.00844	0.00306	0.02088	0.00436	0.00252	0.00173	0.00569	0.01607	0.01447	0.01002	0.00529	0.00379	0.001	0.00379	0.00208	0.007	0.00208	0.00404	0.02926	0.00866	0.00252	0.01311	0.00635	0.00265
N	5%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.09733	0.085	0.087	0.082	0.09533	0.089	0.09633	0.08467	0.08767	0.08567	0.089	0.089	0.08767	0.107	0.13767	0.092	0.08967	0.09633	0.095	0.09767	0.09667	0.133	0.13267	0.13367	0.14533	0.136	0.11367	0.12733	0.141	0.146
St Dev		0.00351	0.00458	0.002	0.001	0.00351	0.00436	0.00306	0.00153	0.00551	0.00115	0.001	0.001	0.00153	0	0.00462	0.0052	0.00252	0.00115	0.00624	0.00451	0.00153	0.00964	0.00321	0.00153	0.00473	0.00173	0.0095	0.00231	0.00854	0.01126
N	6%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.098	0.093	0.08733	0.085	0.09033	0.086	0.094	0.08733	0.08767	0.08967	0.09133	0.09033	0.09	0.09367	0.13967	0.08733	0.087	0.10167	0.09267	0.091	0.08667	0.13167	0.12733	0.126	0.153	0.14	0.11767	0.12533	0.136	0.13367
St Dev		0.001	0.00265	0.00252	0.001	0.00321	0.002	0.0052	0.00252	0.00416	0.00351	0.00503	0.00058	0.002	0.00666	0.0145	0.00513	0.001	0.00208	0.00115	0.002	0.00208	0.00404	0.00306	0.00721	0.00917	0.00608	0.00306	0.00611	0.01249	0.01193
N	7%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.09367	0.096	0.08533	0.088	0.08767	0.08267	0.08367	0.09567	0.08067	0.08233	0.08	0.08433	0.09167	0.094	0.13033	0.08767	0.08033	0.087	0.092	0.08733	0.087	0.123	0.12367	0.12967	0.142	0.13433	0.11133	0.12333	0.12867	0.14
St Dev		0.00321	0.00361	0.00252	0.00624	0.00737	0.00058	0.00586	0.00351	0.00451	0.00306	0.002	0.00153	0.00252	0.00557	0.00929	0.00473	0.00416	0.001	0.001	0.00306	0.002	0.00458	0.00306	0.00473	0.00854	0.01021	0.00153	0.00058	0.00833	0.00624
N	8%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.08767	0.084	0.08833	0.08667	0.08533	0.08367	0.087	0.08867	0.08733	0.08633	0.08367	0.08967	0.08933	0.089	0.12867	0.091	0.08433	0.092	0.09333	0.09467	0.08733	0.12533	0.129	0.13	0.14267	0.129	0.114	0.126	0.12467	0.12867
St Dev		0.00208	0.00265	0.00153	0.00231	0.00153	0.00208	0.001	0.00351	0.00611	0.00153	0.00321	0.00666	0.00351	0.002	0.00379	0.00265	0.00462	0.00436	0.00306	0.00635	0.00153	0.00611	0.00608	0.002	0.00208	0.00361	0.006	0.004	0.00289	0.00058
N	9%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.087	0.09833	0.085	0.08667	0.09267	0.08633	0.09433	0.09	0.09733	0.09067	0.091	0.086	0.09033	0.088	0.13333	0.14067	0.088	0.096	0.09233	0.09433	0.092	0.13333	0.13667	0.126	0.14067	0.14033	0.125	0.12333	0.13167	0.14033
St Dev		0.002	0.00321	0.001	0.00666	0.00379	0.00208	0.00404	0.00346	0.00611	0.00153	0.00656	0.00173	0.00596	0.00265	0.00862	0.0843	0.00265	0.002	0.00321	0.00115	0.003	0.00289	0.00666	0.00755	0.00473	0.01537	0.007	0.00473	0.00153	0.00404
N	10%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.084	0.091	0.083	0.08533	0.09433	0.08467	0.07967	0.089	0.08233	0.08333	0.084	0.085	0.09333	0.09033	0.13533	0.092	0.082	0.09067	0.093	0.088	0.09233	0.126	0.12833	0.145	0.13633	0.13033	0.111	0.12167	0.12033	0.13767
St Dev		0.00265	0.007	0.002	0.00252	0.00473	0.00404	0.00231	0.00173	0.00058	0.00153	0.002	0.001	0.00833	0.00231	0.00416	0.00361	0.00361	0.00451	0.001	0.002	0.00351	0.002	0.00473	0.01646	0.00586	0.0085	0.005	0.00231	0.00153	0.00404
N	11%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.08633	0.093	0.09533	0.08333	0.09167	0.088	0.093	0.08967	0.08733	0.087	0.08867	0.087	0.09133	0.087	0.13433	0.09567	0.08767	0.09833	0.09667	0.09	0.09233	0.12367	0.12867	0.1067	0.14333	0.13233	0.11767	0.12333	0.12567	0.14133
St Dev		0.00058	0.00633	0.00153	0.00289	0.00513	0.00173	0.00954	0.00289	0.00404	0	0.00351	0.00529	0.00208	0.00173	0.00462	0.00404	0.00058	0.00306	0.00153	0.00173	0.00058	0.00231	0.00586	0.00115	0.00404	0.00321	0.00404	0.00451	0.00058	0.00611
N	12%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.089	0.09633	0.086	0.08933	0.08333	0.089	0.09133	0.087	0.09233	0.09433	0.09433	0.09	0.095	0.09033	0.13633	0.095	0.08833	0.09667	0.116	0.093	0.09467	0.126	0.136	0.122	0.145	0.13933	0.12633	0.12833	0.129	0.14033
St Dev		0.003	0.00153	0.001	0.00058	0.00231	0.00173	0.00252	0.005	0.00153	0.00416	0.00115	0.00361	0.00436	0.00208	0.01201	0.00173	0.00306	0.00153	0.03559	0.00173	0.00115	0.003	0.001	0.003	0.00346	0.00586	0.00681	0.00473	0.00872	0.00666
N	13%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.08533	0.09033	0.081	0.08833	0.088	0.08433	0.08867	0.08367	0.08633	0.08633	0.08733	0.08667	0.093	0.137	0.08667	0.08633	0.09967	0.10233	0.08733	0.09133	0.12767	0.12967	0.14067	0.13767	0.133	0.11733	0.11967	0.12833	0.14767
St Dev		0.00208	0.00416	0.003	0.00173	0.00493	0.00265	0.00596	0.00153	0.00723	0.00503	0.00058	0.00208	0.00737	0.00436	0.001	0.00252	0.00603	0.01007	0.00473	0.00321	0.00503	0.00231	0.00153	0.00643	0.00416	0.00854	0.00503	0.00416	0.00306	0.02023
N	14%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Mean		0.08533	0.09233	0.08633	0.089	0.097	0.08767	0.08733	0.08867	0.08767	0.09633	0.09	0.087	0.09133	0.094	0.13033	0.092	0.08867	0.09833	0.098	0.092	0.09533	0.12667	0.13333	0.13467	0.14567	0.132	0.115	0.12267	0.12967	0.13767
St Dev		0.00252	0.00586	0.00208	0.002	0.00755	0.00416	0.00115	0.00153	0.00231	0.00839	0.001	0	0.00208	0.005	0.00702	0.002	0.00586	0.00231	0.00265	0.00265	0.00643	0.00451	0.00666	0.00473	0.00321	0.00346	0.01212	0.00321	0.00651	0.00751
N	15%	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3