The influence of iron concentration on the production of pyoverdine by *Pseudomonas aeruginosa* in mono and mixed biofilm cultures

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<tr>
<td>Extracelullar polymeric substances</td>
<td>EPS</td>
</tr>
<tr>
<td>American Type Culture Collections</td>
<td>ATCC</td>
</tr>
<tr>
<td>Tyrptone Soya broth</td>
<td>TSB</td>
</tr>
<tr>
<td>Tryptone Soya agar</td>
<td>TSA</td>
</tr>
<tr>
<td>Mannitol Salt Agar</td>
<td>MSA</td>
</tr>
<tr>
<td>Extracytoplasmic function</td>
<td>ECF</td>
</tr>
<tr>
<td>Ferric uptake regulator protein</td>
<td>Fur</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>CF</td>
</tr>
<tr>
<td>Phosphate buffered saline</td>
<td>PBS</td>
</tr>
<tr>
<td>Colony forming units</td>
<td>CFU</td>
</tr>
<tr>
<td>Relative fluorescent units</td>
<td>RFU</td>
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<tr>
<td>Deoxyribonucleic acid</td>
<td>DNA</td>
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III. Statement of authenticity

My work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at any other institution.

(Signature)
IV. Acknowledgements

First and foremost I would like to thank my supervisors, Dr. Michael Phillips and Dr. Mark Jones for their wisdom, patience, support and unwavering dedication to this project. I am truly grateful to them for allowing me this opportunity to learn and grow, knowing that I have their full support.

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Last but certainly not least, to my parents and sister. Thank-you Di, Chris & Claire for your support through this particularly stressful time. Thank-you for being my rock, my soundboard and an ear that’s always willing to listen, even if you don’t understand a word of what I am saying.

I could not have done it without you.
V. Abstract

Most species of bacteria have the ability to form biofilms, communities of bacterial cells that aggregate in a self-made matrix of extracellular polymeric substances (EPS), that allow them to adhere to inert and organic substances (Banin, Vasil and Greenberg, 2005; Lin et al., 2012). The biofilm is perceived to be the dominant form of bacterial life in the environment (Donlan and Costerton, 2002) and in comparison to planktonic or free-swimming cells, sessile or biofilm cells often have a higher tolerance to antibiotics and host defense mechanisms, alluding to their importance in human health and disease (Hentzer, Eberl and Givskov, 2005). One of the many factors regulating biofilm growth and formation is the presence or absence of iron (Lin et al., 2012). Iron is an essential nutrient for growth, in order to acquire iron many bacteria produce siderophores to sequester iron from host proteins and the environment (Rédly and Poole, 2003).

Pyoverdine (PVD) is one of two hydroxamate classed siderophores produced by P. aeruginosa (Meyer et al., 1997; Schalk et al., 2001) and regulated in part by Fur (Imperi, Banin). While iron concentration, pyoverdine production and biofilm formation have been studied in relation to P. aeruginosa, studies of this complex relationship has not yet been conducted in relation to mixed cultures. The aim of this research was to study the influence of iron concentration on the production of pyoverdine by Psuedomonas aeruginosa in mono and mixed biofilm cultures with Staphylococcus aureus Newman strain. An iron assay kit determined the concentration of iron in 1% TSB to be 1.62μM. Pyoverdine levels in P. aeruginosa biofilms were higher in 0.1% TSB biofilms than 1% TSB biofilms. In planktonic growth, pyoverdine concentration was higher in 1% TSB.
Crystal violet assays were used to determine the biofilm forming capabilities of *P. aeruginosa* and *S. aureus Newman* in differing iron conditions. *P. aeruginosa* formed more biofilm in 1% TSB, and formed more biofilm than *S. aureus Newman* in both media. *S. aureus*, surprisingly, formed more biofilm in 1% TSB as opposed to 0.1% TSB regardless of the fact that *S. aureus Newman* was been proven to promote biofilm formation only in low iron environments (Johnson, Cockayne and Morrissey, 2008; Lin et al., 2012). The amount of CFU of bacteria in mixed cultures was determined through sonicating biofilm cells into buffer and spread plating. *P. aeruginosa* dominated the biofilm growth in all 0.1% TSB mixed biofilms, however in 1% TSB mixed culture, *S. aureus Newman* produced more biofilm than *P. aeruginosa* when inoculated first. Due to interference within the co-cultures, the pyoverdine levels within the mixed culture biofilms were not established.

Further investigation of pyoverdine concentrations in mixed culture biofilms of different iron concentrations would allow for a better understanding of the complex interrelationship between pyoverdine production, biofilm formation and iron concentration. Three factors that contribute significantly to the bacteria’s ability to cause infection and mortality.
1. Introduction

1.1 Biofilms

Most species of bacteria have the ability to form biofilms, communities of bacterial cells that aggregate in a self-made matrix of extracellular polymeric substances (EPS), that allow them to adhere to inert and organic substances (Banin, Vasil and Greenberg, 2005; Lin et al., 2012). In comparison to planktonic, or free-swimming cells, sessile or biofilm cells often have a higher tolerance to antibiotics and host defence mechanisms, alluding to their importance in human health and disease (Hentzer, Eberl and Givskov, 2005). The biofilm is perceived to be the dominant form of bacterial life in the environment (Donlan and Costerton, 2002). In aquatic environments, microscopic observation and direct quantitative recovery techniques showed that more than 99.9% of bacteria form biofilms and establish on a wide variety of surfaces (Donlan and Costerton, 2002).

By forming biofilms, bacteria have a distinct advantage over their planktonic counterparts, as they are able to survive in nutrient deprived habitats, resist environmental stresses and flourish in an environment that facilitates genetic transfer (Kostakiotis, Hadjifrangiskou and Hultgren, no date; Donlan, 2002; Balcázar, Subirats and Borrego, 2015; Singh et al., 2017). In addition, the formation of a biofilm also provides the bacterium protection against the effects of many biocides and antibiotics (Davey and O’toole, 2000; Watnick and Kolter, 2000; Brown and Gilbert, 2018). In fact, Oliveira et al., (2015) states that for many bacterial species, gram-positive and negative alike, sub-lethal doses of antimicrobial agents induce the formation of a biofilm. According to Rabin et al., (2015) biofilms are more resistant to antibiotics by ‘several orders of magnitude’ in comparison to planktonic bacterium. This can be attributed to the altered bacterial physiology, gene transcription and metabolism observed in sessile cells (Lin et al., 2012).
Biofilms are an ‘ideal place’ for the exchange of genetic material, and also the maintenance of a ‘large and accessible gene pool’ (Flemming, 2008). As such, biofilms are a hot spot for genetic material exchange via horizontal gene transfer (HGT) (Stalder and Top, 2016) which allows for the population to maintain a 'large and accessible gene pool.' (Flemming, 2008). As the sessile cells are not completely immobilized and are near one another, conjugation, a mechanism which requires cell-to-cell contact can occur through HGT. In 1999, Hausner and Wuertz reported a significantly lower rate of conjugation in planktonic cultures as opposed to biofilms (Hausner and Wuertz, 1999; Flemming, Neu and Wozniak, 2007; Flemming, 2008). However, it has been shown that plasmid transfers in biofilms can also be problematic in mature biofilms due a decline in metabolic activity and division (Licht et al., 1999; Stalder and Top, 2016).

Additionally, biofilm-associated bacterium such as *P. aeruginosa* and *S. aureus* have been shown to have an increased rate of mutation compared to planktonic cells (Driffield et al., 2008; Ryder, Chopra and O’Neill, 2012), it is postulated that this is due to the higher levels of stress, particularly oxidative, in the biofilm environment (Cohen, Lobritz and Collins, 2013). Stress responses consistent with those involved in biofilm formation and maturation create a ‘suitable environment’ for transfer of genetic material and adaptive mutation to occur (Cohen, Lobritz and Collins, 2013). The formation of a biofilm is thus an effective survival strategy for bacteria and is influenced by a range of internal and external factors, such as changes in available nutrients, environmental conditions, host defenses and stress responses (Lin et al., 2012; Oliveira et al., 2015).
1.2 Biofilm formation and structure

The regulation of biofilm formation and structure vary greatly, depending on bacterial species and strains (D Monds and A O'Toole, 2009). One attribute common to all biofilms is the encapsulating extracellular or extracellular polysaccharide (EPS) matrix, a ‘highly sophisticated system’ which is as complex as it is successful (Branda et al., 2005; Flemming, Neu and Wozniak, 2007). The matrix consists of a variable combination of glycoproteins, proteins, glycolipids and under certain conditions or some bacterial strains, the presence of extracellular DNA (e-DNA) and polysaccharides in environmental biofilms (Donlan, 2002; Flemming, Neu and Wozniak, 2007). The exact nature of the matrix greatly depends on the growth conditions, medium, substrates and population of bacteria (Flemming, Neu and Wozniak, 2007).

The EPS is responsible for determining the attributes of the biofilms microenvironment, by affecting many abiotic characteristics including porosity, density, water availability and charge, mechanical stability, sorption and even content (Donlan, 2002; Flemming, 2008). The sorption properties of the EPS attach biofilms to surfaces and allows for the ‘sequestering of both dissolved and particulate substances from the environment’ (Flemming, Neu and Wozniak, 2007) while ‘hydrophobic interactions, cross-linking and entanglements of the matrix biopolymers’ provides sufficient mechanical stability for the biofilm over prolonged periods (Flemming, 2008).

Biofilm formation occurs in what is generally considered four distinct stages: (1) attachment of bacteria to the surface, (2) the formation of microcolonies, (3) biofilm maturation and (4) dispersal (also termed detachment) of bacteria for colonization of new areas (Crouzet et al., 2014; Rabin et al., 2015). Biofilm formation begins with the attachment of the bacteria to a
surface, where van der Waals forces between the bacteria and the surface assist the bacterial flagella and fimbriae in mechanically tethering to the surface (Rabin et al., 2015). This tethering in the first stage via the flagella is reversible and involves type IV pili-mediated motilities which enable ‘attached cells to aggregate and form microcolonies’ (Rabin et al., 2015) as demonstrated in the work by O’Toole and Kolter (2002) The bacteria orient themselves perpendicular to the substrate with their long axes and as the bacteria commit to semi-permanent surface attachment, they orientation because horizontal (Miller et al., 2012).

After the cells are loosely attached, exopolysaccharides are overproduced to help immobilize cells and hold the biofilm structure together (Davey and O’toole, 2000; Watnick and Kolter, 2000; Ahmad, Bari and Mohiuddin, 2012). Once attached, the cells produce 'mounds' referred to as microcolonies which are encased by the EPS matrix and separated from other microcolonies by interstitial water channels (Donlan, 2002). Two distinct sub-popolulations constitute the microcolonies; the cap-forming, motile population migrates to the top of the microcolonies which are formed by the non-motile, stalk-forming population which generates a mushroom-like structure in P. aeruginosa (Banin, Vasil and Greenberg, 2005; Miller et al., 2012).

These surface-attached microcolonies form the basic structural units of biofilms, and the intervening channels where water liquid flows, allowing for the diffusion and dispersal of nutrients, oxygen and even antimicrobial agents within the biofilm (Donlan, 2002; Ahmad, Bari and Mohiuddin, 2012). The proximity of cells within and between microcolonies also provides the ideal environment for genetic exchange, quorum sensing and cell-to-cell communication (Donlan, 2002).
After the biofilm has matured, the cells undergo a process of detachment and dispersal (Harmsen et al., 2010; Miller et al., 2012). In the center of the micro colony caps, a fluid-filled cavity is formed and populated with planktonic cells (Sauer et al., 2002; Boles, Thoendel and Singh, 2005; Miller et al., 2012). The cavity increases in size until the cap breaks open, allowing the planktonic cells to disperse and colonize new biofilms elsewhere (Sauer et al., 2002; Boles, Thoendel and Singh, 2005; Miller et al., 2012).

1.3 Clinical significance of biofilms

Biofilms are often associated with human health and disease. The rise of antibiotic resistance has meant that infections by bacteria are becoming harder to treat, especially when the infection involves biofilm formation. Already established is the advantages of biofilm formation by bacteria, against environmental stressors including antibiotics where structure and altered metabolism often renders antibiotics ineffective (Banin, Vasil and Greenberg, 2005; Flemming, 2008; Balcázar, Subirats and Borrego, 2015; Rabin et al., 2015).

For immunocompromised persons, infection by multi-drug resistant bacteria and biofilm forming bacteria can be detrimental to their health. For patients living with cystic fibrosis, S. aureus and P. aeruginosa are the primary pathogens involved in persistent infections, sometimes in a synergistic, Polymicrobial environment and others in competition (Haas et al., 1991; Fugère et al., 2014; Nguyen et al., 2014a; Ahlgren et al., 2015; Filkins et al., 2015).

1.4 Pseudomonas aeruginosa
Pseudomonas aeruginosa is a gram negative, opportunistic pathogen, particularly for immunocompromised persons. P. aeruginosa is a leading infectious agent for burn victims, chemotherapy recipients and most notably, CF patients (Imperi, Tiburzi and Visca, 2009; Oglesby-Sherrouse and Vasil, 2010; Oglesby-Sherrouse and Murphy, 2013; Ahlgren et al., 2015; Reinhart and Oglesby-Sherrouse, 2016). P. aeruginosa is also a major cause of chronic airway infections, Ahlgren et al., (2015) reports that an estimated 60-75% of adult CF patients are chronically infected by P. aeruginosa and is the predominant organism in sputum samples for at least half of patients.

Infections from P. aeruginosa, regardless of infection site, are associated with a decline in lung function, increased inflammation in children and significantly increased rates of morbidity and death of patients (Klare et al., 2016) complicating the treatment of P. aeruginosa infections are 'many innate and acquired resistance mechanisms' (Oglesby-Sherrouse et al., 2014) including biofilm formation. One of the many factors regulating biofilm growth and formation is the presence or absence of iron (Lin et al., 2012).

1.5 Iron

During infection, P. aeruginosa requires an abundance of iron (Oglesby-Sherrouse and Vasil, 2010). Iron is not only an essential nutrient for growth, as it is required for many respiratory enzymes for aerobic metabolism, but it is also required for bacterial energy production, oxygen transport and nucleotide synthesis (Lin et al., 2012, 2016; Pasqua et al., 2017). Iron is also a significant factor in gene expression and regulation (Lin et al., 2012) and as a stress signal that can alter biofilm morphology and regulate antibiotic sensitivity (Lin et al., 2016).
Lin et. al, (2016) demonstrates that iron can be used to regulate the formation of biofilms in numerous bacterial species by controlling the expression of adhesion factors crucial to the biofilms ability to attach to a surface. In addition to this, iron is also essential for the growth of bacterial cells, imperative in energy production, oxygen transport, nucleotide synthesis and the regulation of gene expression (Skaar, 2010; Lin et al., 2012).

According to Miller et al, 2012 low iron levels have been demonstrated to induce cell motility and inhibit biofilm formation, whilst sufficient intracellular iron concentrations (1uM) result in the characteristic, ‘mushroom like’ structures, indicative of *P. aeruginosa* biofilms (Banin, Vasil and Greenberg, 2005). Conversely, an overabundance of iron, at approximately 100uM, results in a ‘nebulous biofilm morphology and distinct lack of eDNA’ (Banin, Vasil and Greenberg, 2005; Miller et al., 2012). Biofilms that generate in the sputum of CF patients are 'unique in their structure' as they exist as intercellular aggregates near the air-surface interface of sputum, as opposed to the typical 'mushroom' shaped morphology (Klare et al., 2016).

Iron also serves as a stress signal that can alter biofilm morphology and regulate antibiotic susceptibility (Oglesby-Sherrouse et al., 2014; Lin et al., 2016). Whilst iron is therefore essential for the development of a biofilm where iron starvation can prevent bacterial growth, Lin et al., 2016 demonstrated that in high concentrations, iron can also be toxic (Banin, Vasil and Greenberg, 2005; Lin et al., 2016). Whilst essential for the development of a biofilm, and bacterial processes as aforementioned, where iron starvation can prevent bacterial growth, iron in high concentrations can be toxic to the bacteria (Banin, Vasil and Greenberg, 2005; Kim et al., 2009; Lin et al., 2012; Oglesby-Sherrouse and Murphy, 2013; Pasqua et al., 2017).
Iron metabolism must be regulated closely in order to prevent excess of free intracellular iron that can lead to the generation of toxic oxygen radicals (Ponraj et al., 2012; Oglesby-Sherrouse and Murphy, 2013). Iron is highly toxic for biologic substrates due to its high oxidative potential and its ability to generate Reactive Oxygen Species (ROS) according to the Haber-Weiss reaction (Ponraj et al., 2012; Symeonidis, 2012). High levels of free-iron catalyzes the formation of highly reactive compounds that can damage or destroy cells, like hydroxyl radicals. High levels of free iron catalyzes the formation of highly reactive compounds, such as hydroxyl radicals, that can damage or destroy the cell (Symeonidis, 2012).

Due to its importance to bacteria, one of the key defenses eukaryotic hosts employ to prevent the colonisation of bacterial pathogens is through limiting the availability of free iron (Cornelis and Dingemans, 2013). In the human body, free iron (Fe³⁺) tends to be sequestered into complexes with iron binding proteins such as haemoglobin, ferritin, transferrin and lactoferrin (Lin et al., 2012, 2016; Cornelis and Dingemans, 2013; Parrow, Fleming and Minnick, 2013; Reinhart and Oglesby-Sherrouse, 2016). Free iron concentration in the host environment is about, or lower than 10⁻¹⁵M and in some instances, as low as 10⁻²⁴ (Symeonidis, 2012)

Certain bacterial species have developed many iron acquisition mechanisms to be able to obtain iron from host cells and the iron-binding proteins, such as through the production of extracellular Fe³⁺ iron-chelating molecules termed siderophores (Iain L. Lamont et al., 2002; Miethke and Marahiel, 2007; Hannauer et al., 2012a; Lin et al., 2012; Cornelis and Dingemans, 2013; Parrow, Fleming and Minnick, 2013). Under iron deplete conditions, P.
*P. aeruginosa* employs a variety of mechanisms, including the synthesis of two siderophores; pyoverdine and pyochelin, to sequester this iron from host proteins (Voulhoux, Filloux and Schalk, 2006; Matilla *et al.*, 2007; Imperi, Tiburzi and Visca, 2009; Hannauer *et al.*, 2012a; Nguyen *et al.*, 2014a)

### 1.6 Pyoverdine

Siderophores, named after the Greek word for ‘iron carriers’, are low-molecular-weight excreted molecules that specifically chelate Fe$^{3+}$ from iron-binding proteins with a high affinity (Tsuda, Miyazaki and Nakazawa, 1995; I. L. Lamont *et al.*, 2002; Moon *et al.*, 2008; Cornelis and Dingemans, 2013). The siderophores compete with the host for available iron, bind to it creating a ferrisiderophore complex and are then taking up into the bacterial cell by specific membrane transporters (Schalk *et al.*, 1999; Iain L. Lamont *et al.*, 2002; Hannauer, Barda, *et al.*, 2010; Hannauer *et al.*, 2012b). Siderophores solubilize ferric iron of insoluble complexes for under aerobic conditions in order to make iron available for use by the bacteria (Clément *et al.*, 2004; Nguyen *et al.*, 2014a)

There are three major classes of microbial siderophores, the catecholate, the hydroxycarboxylate and the b-hydroxamate class. These substances exhibit extremely high affinity for iron, and hold it with three bidentate bonds. This affinity is specific for iron, and does not extend to other bivalent cations (Cox and Graham, 1979; Hantke *et al.*, 2003; Symeonidis, 2012). Siderophores have higher binding constants for iron, than Lactoferrin and transferrin, and thus are capable of detaching iron from these proteins (Nguyen *et al.*, 2014a; Reinhart and Oglesby-Sherrouse, 2016).
Pyoverdine (PVD) is one of two hydroxamate classed siderophores produced by *P. aeruginosa* from a total of three different classes (I-III), each distinguished by their peptides (Meyer *et al.*, 1997). PVD is a yellow-brown coloured, naturally fluorescent molecule that consists of a partly cyclic octapeptide linked to a quinoline-type chromophore that is derived from 2,3-diamino-6,7-dihydroxyquinoline and a short, 6-12 residue strain-specific peptide (McMorran *et al.*, 2001; Imperi, Tiburzi and Visca, 2009; Hannauer *et al.*, 2012a; Nguyen and Oglesby-Sherrouse, 2016). In PA01, this strain-specific peptide contains two, D-serine, two L-threonine, one L-arginine, one L-lysine and two N⁵-formyl- N⁵-hydroxyornithine (McMorran *et al.*, 2001). Pyochelin is a derivative of salicylic acid (Cox and Graham, 1979; Voulhoux, Filloux and Schalk, 2006; Minandri *et al.*, 2016).
Recent studies (Dumas, et al., 2013, others) have shown that \textit{P. aeruginosa} favours the production of pyochelin over pyoverdine in high-iron conditions, and switches to the energy-dependent production of pyoverdine when availability of iron declines. Ratledge and Dover, (2000) support this theory, they discovered that pyoverdine deficient mutants of \textit{P. aeruginosa} exhibit significantly restricted growth in human serum whereas pyochelin deficient \textit{P. aeruginosa} did not, suggesting that pyoverdine may be the key siderophore. Pyoverdine exhibits extremely high affinity for ferric iron at $10^{32}$ M$^{-1}$ giving it the ability to sequester iron from mammalian iron-sequestering proteins such as lactoferrin and ferritin (Ratledge and Dover, 2000; Kang, Turner and Kirienko, 2017).

In addition to its role in iron uptake and regulation, pyoverdine is also involved in cell-to-cell communication and the regulation of virulence factors such as Exotoxin A (Imperi, Tiburzi and Visca, 2009). A disruption in iron availability by using mutant strains of \textit{P. aeruginosa} preventing the use of pyoverdine, prevents cap formation in \textit{P. aeruginosa} biofilms (Yang et al., 2009). Pyoverdine has been shown to be produced solely by the stalk-forming subpopulation (Miller et al., 2012). Yang et al., (2009) hypothesize that the ferric-pyoverdine supplied by the stalk-forming subpopulation is necessary for the aggregation and development of the cap-forming subpopulation. Addition of ferric-citrate restored cap-forming ability in previously low-iron available conditions (Yang et al., 2009; Miller et al., 2012).

1.7 Pvd Locus

Almost all of the genes that are involved in the synthesis of pyoverdine are found in a single locus, the \textit{pvd} locus (Imperi, Tiburzi and Visca, 2009) however the \textit{pvdABCD} operon occurs elsewhere (Poole, 2003). In Pa01, these genes (See table 1) are located at about 47 min on the

Table 1: Genes involved in the synthesis and regulation of pyoverdine in Pa01 (Lamont and Martin, 2003)

<table>
<thead>
<tr>
<th>Gene</th>
<th>ORF*</th>
<th>Function</th>
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<tbody>
<tr>
<td>pvdA</td>
<td>PA2386</td>
<td>Ornithine hydroxylase (Visca et al., 1994)</td>
</tr>
<tr>
<td>fpvA</td>
<td>PA2387</td>
<td>ECF sigma factor required for expression of fpvA (Beare et al., 2003)</td>
</tr>
<tr>
<td>fpvR</td>
<td>PA2388</td>
<td>Anti-sigma factor for PvdS and FpvL (Lamont et al., 2002; Beare et al., 2003)</td>
</tr>
<tr>
<td>pvdF</td>
<td>PA2396</td>
<td>N5-Hydroxyornithine transformylase (McMorran et al., 2001)</td>
</tr>
<tr>
<td>pvdE</td>
<td>PA2397</td>
<td>ABC transporter (secretion) (McMorran et al., 1996)</td>
</tr>
<tr>
<td>fpvC</td>
<td>PA2398</td>
<td>Ferripyoverdine receptor protein (Poole et al., 1993)</td>
</tr>
<tr>
<td>pvdD</td>
<td>PA2399</td>
<td>Pyoverdine peptide synthetase (Merriman et al., 1995)</td>
</tr>
<tr>
<td>pvdI</td>
<td>PA2000/1</td>
<td>Pyoverdine peptide synthetase (Lehoux et al., 2000)</td>
</tr>
<tr>
<td>pvdJ</td>
<td>PA2002</td>
<td>Pyoverdine peptide synthetase (Lehoux et al., 2000)</td>
</tr>
<tr>
<td>pvdK</td>
<td>PA2426</td>
<td>ECF iron sigma factor (Canflifte et al., 1995; Miyazaki et al., 1995)</td>
</tr>
<tr>
<td>pvcABCD</td>
<td>PA2254-PA2257</td>
<td>Synthesis of the pyoverdine chromophore (Stintzi et al., 1996, 1999)</td>
</tr>
</tbody>
</table>


Both pvdA and pvdF encode for enzymes involved in the production of N5-formyl-N5-hydroxyornithine, a compound. PvdA encodes for ornithine hydroxylase which catalyses the synthesis of N5-hydroxyornithine (Meneely et al., 2009) whilst the pvdF gene product N5-hydroxyornithine tranformylase catalyses the reaction of N5-hydroxyornithine to give N5-formyl-N5-hydroxyornithine (Lamont and Martin, 2003). The pvdD gene encodes a peptide synthetase involved directly in the inclusion of two L-threonine residues into the PVD peptide, while pvdJ and pvdK, discovered to be part of a single gene (Lehoux, Sanschagrin and Levesque, 2000; Lamont and Martin, 2003) are also involved in the production of peptide synthesis. An ABC Transporter essential for pyoverdine production is the product of the pvdE gene (McMorran et al., 2001; Lamont and Martin, 2003; Smith et al., 2005).

The pvc gene cluster (pvcABCD) are reported to be involved in the synthesis of pyoverdine however these genes are not believed to be essential for synthesis to occur. This is demonstrated by Cornelis and Oschner (Lamont and Martin, 2003) in their study of pvc
mutants as the pvc knockout P. aeruginosa was able to synthesise pyoverdine. The alternative sigma factor protein, pvdS, is a transcriptional activator required for the expression of all pyoverdine-synthesis genes (Lamont and Martin, 2003) as well as the regAB and ptxR genes involved in the positive regulation of the toxA gene encoding exotoxin A (Leoni et al., 1996; I. L. Lamont et al., 2002; Lamont and Martin, 2003). PvdS incorporates structural, functional and biochemical properties of ECF alternative sigma factors and is believed to positively regulate the pvdA gene in conjunction with RNA polymerase (Leoni et al., 1996).

1.8 Synthesis and Mechanism of Pyoverdine

Synthesis of pyoverdine begins with the synthesis of the non-fluorescent precursor in the bacterial cytoplasm, through non-ribosomal peptide synthesis (Hannauer et al., 2012a). This non-fluorescent precursor is then transported across the inner membrane into the periplasm where ‘cyclisation of the chromophore moiety’ occurs, creating the fluorescent pyoverdine (Hannauer, Barda, et al., 2010; Hannauer, Yeterian, et al., 2010). After synthesis, pyoverdine is then transported into the extracellular medium through the outer membrane by the ATP-dependent PvdRR-OmpQ efflux system (Hannauer, Yeterian, et al., 2010; Hannauer et al., 2012a).

The siderophores compete with the host for available iron in the extracellular medium, and having bound to it they create a ferrisiderophore complex (FerriPVD) believed to occur through oxygen atoms that are present on the di-hydroxyquinoline and two hydroxamate units of pyoverdine (Merriman, Merriman and Lamont, 1995). FerriPVD are then taken up into the bacterial cell by specific membrane transporters (Hannauer, Yeterian, et al., 2010; Hannauer et al., 2012b). The iron loaded, ferrisiderophore complexes, bind to cognate receptors expressed at the bacterial surface. In gram-negative bacteria these receptors are present on the
outer membrane, external to the thin peptidolycan layer. For ferric-pyoverdine produced by *Psuedomonas aeruginosa*, the specific outer membrane receptor is FpvA (Clément *et al.*, 2004; Adams *et al.*, 2006).

FpvA is a transport protein belonging to a subfamily of siderophore outer membrane receptors. It is composed of two domains; a C-terminal B-barrel made of 22 b-strands and an N-terminal plug domain residing inside the barrel (Schalk *et al.*, 1999; Shen *et al.*, 2005; Adams *et al.*, 2006). FpvA and subfamily are differentiated from other families by an ‘additional 70-residue extension preceding the N-terminal plug domain’ of their structure (Clément *et al.*, 2004) involved in the regulation of the transcription of the fpvA operon (Adams *et al.*, 2006). Under iron limited conditions, the natural state of FpvA is an FpvA-Pvd complex (Schalk *et al.*, 1999, 2001; Shen *et al.*, 2005; Adams *et al.*, 2006). During the iron collection and uptake process, FpvA-Pvd disassociates to allow for the ferrisiderophore, FerriPVD complex to bind, the kinetics of which reaction are dependently controlled by TonB an inner membrane protein (Clément *et al.*, 2004; Adams *et al.*, 2006; Parrow, Fleming and Minnick, 2013). The ferrisiderophore complexes are large enough to pass through porins using these membrane transporters (Adams *et al.*, 2006; Symeonidis, 2012). The energy required for the transport of the ligands across the outer membrane is provided to the site through the inner membrane by a complex of cytoplasmic membrane proteins; TonB, ExbB and ExbD and the proton motive force (Hannauer, Barda, *et al.*, 2010; Symeonidis, 2012). Under iron-limited conditions the FpvA receptor is activated by TonB which results in a fast release of unbound pyoverdine, and the generation of an unloaded FpvA receptor in order to bind to the FerriPVD complex (Clément *et al.*, 2004).
Each class of siderophore has its own reciprocate periplasmic binding protein which shuttles the complex to the inner membrane (Clément et al., 2004; Hannauer et al., 2012a). When the ferrisiderophore complex arrives at the cytoplasmic membrane, periplasmic binding protein-dependent ABC transporters take the complexes across the membrane in an ATP-dependent process (Hannauer et al., 2012a; Ganne et al., 2017). The ABC transporters consists of two nucleotide binding domains that hydrolyse ATP and two transmembrane domains that form a channel that allows the siderophore complex to pass through. Once inside the bacterium, iron is released by proteolysis or through iron reducing enzymes. The ferrous iron is incorporated into metalloenzymes or it is stored through Dps proteins or bacterioferritin (Hannauer et al., 2012a).
1.9 Regulation of Pyoverdine

The production of pyoverdine is regulated by both fur-dependent mechanisms, through the ferric repressor protein, Fur (Banin, Vasil and Greenberg, 2005; Imperi, Tiburzi and Visca, 2009; Oglesby-Sherrouse and Vasil, 2010). Fur either directly or indirectly, the expression of all iron uptake genes, as well as the biosynthesis of siderophores, metabolism and virulence factors in response to intracellular iron concentrations (Hantke et al., 2003; Nguyen and Oglesby-Sherrouse, 2015, 2016; Lin et al., 2016; Pasqua et al., 2017) by regulating the extracytoplasmic function (ECF) sigma factor, which controls pyoverdine synthesis and secretion (Poole, 2003; Moon et al., 2008; Imperi, Tiburzi and Visca, 2009).

Fur is a global regulator, having both a positive and negative regulatory effect on iron-responsive genes. Repression of the expression of these genes is achieved through the direct binding of the operators, while positive expression is controlled indirectly through two small regulatory RNA’s (rRNA’s) PrrF1 and PrrF2 (Banin, Vasil and Greenberg, 2005; Nguyen et al., 2014b). Under iron-rich conditions, Fur binds to its co-repressor, divalent iron, and changes configuration to form homodimers to bind to target DNA sequences known as Fur boxes in key promoters to inhibit the transcription of genes repressed by iron (Escolar, Pérez-Martín and de Lorenzo, 1999; Cornelis, Matthijs and Van Oeffelen, 2009; Pasqua et al., 2017).

1.10 Staphylococcus aureus

*Staphylococcus aureus* is a gram-positive, ubiquitous bacterial species. It is a cocci-shaped, facultative aerobe that is oven arranged in ‘grape like’ (Otto, 2008; Archer et al., 2011; Taylor and Unakal, 2018) clusters with typically yellow or golden colonies. *S. aureus* is an opportunistic human pathogen, as part of the general microbiota of the skin and mucous
membranes (Taylor and Unakal, 2018). It is estimated that 20-25% of the population also have *S. aureus* as normal flora in their nasal cavity (Kluytmans, van Belkum and Verbrugh, 1997). *S. aureus* is also a widely-known biofilm former, as frequent cases of nosocomial infection (Archer et al., 2011).

Like *P. aeruginosa*, *Staphylococcus aureus* is an opportunistic pathogen that can cause a range of infections including pneumonia. In addition, *S. aureus* is of great clinical significance in cystic fibrosis patients, and is recognised as the predominant cystic fibrosis pathogen in children. Polymicrobial lung infections of *S. aureus* and *P. aeruginosa* are common in CF patients. The ‘Newman’ strain of *S. aureus* was first isolated from a human infection in 1953, and has been used extensively in research since (Baba et al., 2008). The Newman strain has been studied in relation to biofilm formation and iron concentration due to the bacterium growing poor biofilms under high-iron concentration, which is not seen in other *Staphylococcus* spp. (Stintzi et al., 1999; Lin et al., 2012)

1.11 Iron metabolism in *S. aureus*

*S. aureus*, like *P. aeruginosa* also produces a siderophore, aureochelin, in order to gain iron for growth and development (Lin et al., 2012). In addition to aureochelin, *S. aureus* also has an iron acquisition system. The *Isd* contains 8 genes encoding for anchoring proteins, membrane transporters, a transpeptidase and cytoplasmic heme-degrading monooxygenases (*IsdG* and *IsdI*). This system is inhibited under iron-rich conditions through the use of a Fur box (Lin et al., 2012).

This system has not been adapted universally between *S. aureus* strains. In the case of *S. aureus Newman*, low iron concentrations promotes the growth of biofilms and high iron concentration represses biofilm formation. This is the opposite for most studied *S. aureus*
strains, such as SA113 used by Lin et al., 2012 where they demonstrated that low-iron concentrations repressed biofilm formation, and biofilm formation was only positively regulated under higher iron concentrations. Due to these results, it is postulated that S. aureus Newman is only positively regulated by Fur, not negatively (Lin et al., 2012).

1.12 Co-culture biofilms

Already established is the importance of an abundance of available iron for the establishment of a P. aeruginosa biofilm (Oglesby-Sherrouse and Murphy, 2013). However, it has also been noted that the need for iron in biofilm development in S. aureus is strain dependent (Lin et al., 2012). It has been suggested that one of the reasons P. aeruginosa and some strains S. aureus can survive in a shared environment is the ability of P. aeruginosa to sequester all the available iron from S. aureus, promoting biofilm growth of the latter (Fugère et al., 2014; Filkins et al., 2015).

Research into the synergistic co-cultures of P. aeruginosa and S. aureus have explored the virulent tactics that each bacteria employ in order to enhance their colonization (DeLeon et al., 2014; Filkins et al., 2015). In a co-infection between the two bacterium, P. aeruginosa employs various virulence factors in order to limit the growth of S. aureus. These virulence factors include various antistaphylococcal products and proteases, such as LasA, that can cause biofilms of S. aureus to disperse, and for cells to lyse (Vasil and Ochsner, 1999; DeLeon et al., 2014; Fugère et al., 2014; Filkins et al., 2015; Nguyen and Oglesby-Sherrouse, 2016)

In addition to this, P. aeruginosa produces several exoproducts, such as hydrogen cyanide, quinoline N-ocides and phenazine pyocanin, which serve to reduce the oxygen available to S.
aureus for growth, forcing it to metabolize anaerobically (Fugère et al., 2014; Filkins et al., 2015). Not all impacts of a co-culture environment between the two bacterial species are negative, it has been postulated that P. aeruginosa can benefit from a biofilm co-culture with S. aureus and vise versa (DeLeon et al., 2014; Fugère et al., 2014; Filkins et al., 2015). In terms of iron acquisition, P. aeruginosa has been shown to use S. aureus as a source of iron while certain strains of S. aureus can also potentially benefit from P. aeruginosa sequestering available iron, as it has shown to promote biofilm growth (Fugère et al., 2014; Filkins et al., 2015). The genetic diversity and virulence of P. aeruginosa in conjunction with the differing susceptibility of S. aureus strains to P. aeruginosa exotoxins all contribute to the likelihood of many unique and specific species interactions (Fugère et al., 2014).

1.13 Experimental Aims and Objectives

- Determine the effect of mixed population biofilms on the production on pyoverdine production
- Study the relationship between iron concentration, pyoverdine production and biofilm formation of P. aeruginosa in mono and mixed cultures with S. aureus Newman
- Determine if there is a relationship between pyoverdine and viability of S. aureus in mixed population biofilms
2. Methodology

2.1 Bacterial strain and culture conditions

The bacterial strain used in this work is a wildtype culture of *P. aeruginosa* PAO1 (Western Sydney University culture 377). And *Staphylococcus aureus* ‘Newman’ strain NTC8178 obtained from Public Health England (Western Sydney University Culture 189). Cultures were maintained in nutrient broth or on tryptone soya agar slopes, subculturing as required.

2.2 Microbiological Media

The Tryptone Soy Broth (TSB) media used in the biofilm experiments was chosen for its wide use in the literature for iron focused experiments. The low iron content of this media at 0.1% concentration was employed to promote biofilm growth and pyoverdine production. The media was also used at 1% concentration. Also used is Mannitol Salt Agar (Oxoid CM0085) made to manufacturers recommendations, for the selection of *S. aureus* growth whilst MacConkey Agar No.3 (Oxoid CM0115) also made to manufacturers recommendations was employed for selection of *P. aeruginosa* colonies from a mixed culture. Both MSA and MacConkey type 3 were used for viability counts.

2.3 Incubation setup

All 96-well microtiter plates were incubated inside a plastic chamber, custom designed to allow for air-flow across the plates and decrease variance between results. Air is passed into the chamber through a 0.2μm air filter via an air pump, into two beakers filled with water and glass wool where the air flows through a pumice stone outlet. Air then travels across the plates and presumably out the exit holes at the back, that are plugged with glass wool.
Before each use the apparatus is sterilized using UV light for 20 minutes before rotating and sterilizing again. The insides are also wiped down with 70% ethanol. (For photograph of incubator setup, see Appendix B)

2.4 Pyoverdine Standard Curve

Purified pyoverdine (~90% purity) from *Pseudomonas fluorescens* (Sigma-Aldrich, Castle Hill, Australia) was used to create a standard curve of pyoverdine concentration measured against fluorescence intensity. A serial dilution of the purified pyoverdine and RO water at concentrations of 0.05, 0.5, 1, 1.5 and 2μm were used to make the standard curve (1, 10). Three replicates of 200μL of each dilution were plated in black 96-well microtiter plates (Greiner, Kremsmünster, Austria) and imaged using the CLARIOstar Spectrometer (BMG LABTECH, Mornington, Australia). Measurements of fluorescence intensity were taken using an excitation of 398nm and emissions at 455nm (1, 2, 14) with a gain of 500 and a focus point of 7.0. Results were analysed through MARS Analysis Software (BMG LABTECH) and graphed using Microsoft Excel.

2.5 Pyoverdine quantification

Protocol was adapted from O’Toole (2011) and Alves et al., (2018). A 1:20 dilution of overnight broth culture of *P. aeruginosa* was mixed with chosen media, either 0.1% TSB or 1% TSB. 200uL of this mix was added to the wells of columns 1-10 of black 96-well microtiter plates. Column 11 contained the negative control, 200ul per well of sterile media. Microtiter plates were then incubated at 37°C for approximately 24 hours (±5 minutes) within incubation chamber. After removal from the incubator, a positive control, 200μL of 20μM pyoverdine (Sigma-Aldrich) was added to wells 12A-12C. Planktonic pyoverdine was then measured in the CLARIOstar with excitation of 398nm and emissions at 455nm (Braud et al.,
2.6 Pyochelin quantification
A 1:20 dilution of overnight broth culture of *P. aeruginosa* was mixed with chosen media, either 0.1% TSB or 1% TSB. 200μL of this mix was added to the wells of columns 1-10 of black 96-well microtiter plates with column 12 containing the negative control of uninoculated media. Microtiter plates were then incubated at 37°C for approximately 24 hours (±5 minutes) within incubation chamber. Plates were then measured in the CLARIOstar with excitation of 347nm and emissions at 420nm (Braud et al., 2009) with a gain of 500 and a focus point of 7.0. All results were analysed in Microsoft Excel.

2.8 Enumeration of bacteria
Coplin jars containing two microscope slides were filled with 30mL of the desired media, either 1% or 0.1% TSB, and sterilised in the autoclave for 15 minutes at 121°C. 5 different counts were completed. Single culture biofilms of *P. aeruginosa* and *S. aureus* were formed by inoculating coplin jars with 30μL of overnight culture. 3 variants of co-culture biofilms were also tested, where one of the single cultures was inoculated first and 24-hours later the other culture was added, and both cultures added together at the same time. All biofilms were incubated for a total of 48 hours at 37°C.

After incubation, slides were removed from the coplin jars and washed with ice-cold 1X PBS buffer by gently submerging the slides in the buffer and gently pouring the buffer down the
slide. Once washed, each slide was placed upside-down in a sterile 50mL Falcon tube containing 25mL of ice-cold 1X PBS. The Falcon tubes were sonicated for 20mL in ice-cold water at 40Hz. Serial dilutions were completed after sonication, taking 1mL from the 25mL 1X PBS in the Falcon Tube into 9mL of sterile 0.85% saline, and a further 1mL of the previously inoculated saline into another 9mL of sterile 0.85% saline. 0.1mL of the 25mL 1X PBS and each dilution was plated in triplicate on both Mannitol Salt Agar plates and MacConkey’s type 3 Agar plates. Plates were incubated for 24 hours at 37˚C and read using the Interscience Scan® 1200 plate counter and associated software.

2.9 Iron Assay and Quantification

Concentration of iron in media was ascertained using an Iron Colorimetric Assay Kit from BioVision (Milpitas, CA, USA). Protocol was followed as per manufacturer’s recommendations with minor adjustments. Assay buffer was warmed to 25˚C before protocol was commenced. Colorimetric assay was undertaken on bench under low light-levels, plate was left to rest at room temperature for approximately 90 minutes after the addition of the iron probe as opposed to the recommended 60 minutes to allow for bubbles in the reaction to dissipate. A standard curve was completed with every assay.

For the standard curve, 10μL of the 100nM iron standard was diluted with 990μL of RO water to generate 1nM of standard iron. 0, 2, 4, 6, 8 and 10μL of the diluted iron standard was added to different wells of a clear 96-well microtiter plate. Volumes of each well was brought to 100μL with warmed Assay Buffer. 5μL of iron reducer was added to each standard well. 50μL, 20μL, 10μL and 5μL of 5% TSB sample was added to wells in triplicates. Volume of each well was then brought to 100μL total with Assay buffer. 5μL of iron reducer was then added to each well and plate was incubated for 30min at 25˚C. After incubation, 100μL of iron probe was added to each well and mixed well with pipette tip. Plate was then wrapped in
aluminium foil and incubated for 90min at 25°C. OD was measured at 593nm by CLARIOstar microplate reader. For the calculation of iron concentrations, sample was blanked using the results from the ‘0’ standard reading and the standard curve was plotted.
3. Results

3.1 The iron concentration of 1% Tryptone Soy Broth

![Iron Assay Standard Curve](image)

**Figure 2:** A standard curve for the concentration of iron by colorimetric assay, equation of the line is $y = 0.0664x + 0.017$. n=3

The concentration of iron found in 1% TSB using this standard curve was 1.62μM. The average absorbance of a triplicate result of 5% TSB was used for the calculations of iron content, calculations were completed using the line equation from the standard curve and instructions from manufacturer.

3.2 Intensity of fluorescence by pyoverdine increases with concentration

The nature of fluorescence proposes that its intensity will increase or decrease as a direct result of the amount of fluorescence emitted. Using this principle, it is understood that the fluorescence intensity, also known as relative fluorescent units, measured in RFU, of pyoverdine will increase with a larger number of fluorescent molecules (Haas *et al.*, 1991;
Imperi, Tiburzi and Visca, 2009; Hannauer et al., 2012a). This directly proportional relationship is illustrated in Figure 1, where a standard curve and trend-line demonstrate that as the concentration, or abundance, of pyoverdine increases within the sample, so does the intensity of the fluorescence emitted (Haas et al., 1991).
Figure 3: A standard curve for the concentration of pyoverdine by fluorescence intensity.

Five concentrations of purified pyoverdine from *P. fluorescens* was analysed by fluorescence spectrometry, excitation at 398nm and emission at 455nm, and graphed using Microsoft Excel. The results show a positive correlative relationship between pyoverdine concentration and fluorescence intensity. Data points are the average taken from three replicates. Regression analysis, $R^2$ value = 0.9999, the equation of the line is $y=113469x - 2718.2$

3.3 Pyochelin levels in both planktonic and biofilm cultures

![Figure 4: The amount of pyochelin by relative fluorescence at excitation of 347nm and emission at 420nm and the amount of pyoverdine, excitation 398nm and emission at 455nm in planktonic cultures of *Pseudomonas aeruginosa* after 24hrs growth in a microtiter plate. N=264](image_url)
Figure 4 demonstrates the amount of both pyoverdine and pyochelin in a planktonic 24-hour biofilm culture of *P. aeruginosa*. Overall, the amount of pyoverdine is higher than the amount of pyochelin in both concentrations of media. Pyoverdine amount in 1% TSB is one fifth of the amount in 0.1%. Pyochelin also had a higher amount in 0.1% TSB compared to 1% TSB. T-tests determined this result significant, with a two-tailed p value of <0.001.
3.4 Levels of Pyoverdine differs between Planktonic and Biofilm Growth

**Figure 5:** The amount of pyoverdine by relative fluorescence at 455nm in planktonic versus biofilm cultures of Pseudomonas aeruginosa after 24hrs growth in a microtiter plate. Bars show standard deviation, n= 456.

The average fluorescence intensity, measured in relative fluorescent units (RFU) of both planktonic and biofilm growth of *P. aeruginosa* for both media tested is shown in Figure 5. Fluorescence intensity of pyoverdine of 24-hour cultures of *P. aeruginosa* grown in 96-well microtiter plates within the planktonic phase and a biofilm phase by washing with saline. It was found that there was a higher amount of relative fluorescence by pyoverdine in planktonic cultures (Figure 5) in comparison to the fluorescence detected in biofilm cultures (Figure 6).

While the difference depicted between the averages of both cultures is approximately 14-fold, an unpaired t-test shows a two-tailed P value is less than 0.0001.
Figure 6: Average pyoverdine concentrations of biofilm cultures of *P. aeruginosa* suspended in 0.85% saline and planktonic cultures between the two different media concentrations as determined by relative fluorescent units at an excitation of 398nm and emission at 455nm. Bars show standard error, N=264

Collectively, the results for planktonic and biofilm measurements of pyoverdine show the greatest production in the 1% TSB media with more than double the amount of relative fluorescence, 2.4 times higher in 1% TSB than 0.1% TSB (Figure 6). This result is despite the fact that the lesser concentrated media, 0.1% TSB contains only a tenth of the nutrient levels of 1% TSB.
Figure 7: Concentration of pyoverdine in planktonic cultures of *P. aeruginosa* determined by relative fluorescent units at an excitation of 398nm and emission at 455nm. Bars show standard error, N=264.

*P. aeruginosa* biofilm cells showed the greatest amount of pyoverdine production in the 1% TSB media, in comparison to the 0.1% media (Figure 7). This result is reversed when biofilm cultures were analysed, pyoverdine production of biofilms cultured in 0.1% TSB were on average, slightly increased over production in the higher nutrient content 1% TSB (Figure 8). A two-tailed t-test P value equals 0.0428.
Figure 8: Concentration of pyoverdine in biofilm cultures of *P. aeruginosa* suspended in 0.85% saline determined by relative fluorescent units at an excitation of 398nm and emission at 455nm. Bars show standard error, N=264

3.5 The growth of *Pseudomonas aeruginosa* in different nutrient conditions

The growth of *Pseudomonas aeruginosa* after 24-hours in the microtiter plate shows greater planktonic growth in the 1% TSB compared to 0.1% TSB (Figure 9). Average planktonic growth for the bacterium in 1% TSB is only 1.7 (To 1 decimal place) times higher than growth in 0.1% TSB despite a 10-fold difference in nutrient concentration and iron content.
Figure 9: Average planktonic growth of *P. aeruginosa* as determined by absorbance at 595nm for 24-hour cultures in 96-well microtiter plates. N=264

Both Figure 9 and 10 depict higher growth by *P. aeruginosa* in both planktonic and biofilm culture respectively, in the 1% TSB medium compared to the 0.1% TSB. A crystal violet assay on biofilm cultures of *P. aeruginosa* allows for both visualisation of the biofilm and a semi-quantitative assessment of the extent of biofilm formation. The difference between 1% and 0.1% TSB biofilm growth is larger in Figure 10 than the planktonic growth in Figure 8, where there is a 2.4-fold difference.
Figure 10: Average biofilm growth of *P. aeruginosa* as determined by crystal violet assay and absorbance at 595nm for 24-hour cultures grown in 96-well microtiter plates. N=264
3.7 The growth of *Staphylococcus aureus* in different nutrient conditions

![Graph showing mean absorbance of planktonic S. aureus growth by media concentrations]

**Figure 11:** Average planktonic growth of *P. aeruginosa* as determined by absorbance at 595nm for 24-hour cultures in 96-well microtiter plates. N=264

Figure 11 depicts the results of planktonic growth by *S. aureus* by the different media conditions. In comparison to Figure 9, the planktonic growth by *P. aeruginosa* there is a larger difference in growth between the two media. Growth in 1% TSB is 3.4 times higher than growth in 0.1% TSB. Biofilm growth in 1% TSB is 3.1 times higher than 0.1% TSB Biofilm growth, as shown in Figure 12.
Figure 12: Average biofilm growth of *S. aureus* as determined by crystal violet assay, absorbance at 595nm for 24-hour cultures grown in 96-well microtiter plates. N=264

3.8 A comparison of the growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in different nutrient conditions

Figure 13 compares the biofilm growth of *S. aureus* and *P. aeruginosa* between both media concentrations. Overall, *S. aureus* grew less biofilm than *P. aeruginosa* in both media.

In 0.1% TSB, *S. aureus* grew on average, half the amount of biofilm compared to *P. aeruginosa* whilst in 1% TSB, the difference between the two cultures is smaller. *S. aureus* grew, on average, approximately 62% of the amount of biofilm in comparison to *P. aeruginosa*. 
Figure 13: Average biofilm growth of *P. aeruginosa* and *S. aureus* as determined by crystal violet assay, absorbance at 595nm for 24-hour cultures grown in 96-well microtiter plates. N= 264

In planktonic growth, the difference between *S. aureus* and *P. aeruginosa* in 1% TSB is smaller than the difference in planktonic growth in 0.1% media, as presented in Figure 14. At a 1% concentration, *S. aureus* produces more planktonic growth than *P. aeruginosa* whereas at 0.1% concentration the opposite is depicted. Average recorded *S. aureus* planktonic growth is half that of *P. aeruginosa* growth.
Figure 14: Average planktonic growth of *P. aeruginosa* and *S. aureus* as determined by crystal violet assay, absorbance at 595nm for 24-hour cultures grown in 96-well microtiter plates. N=264

The ratio of growth between *P. aeruginosa* and *S. aureus* is the same in 0.1% TSB regardless of whether biofilm culture or planktonic culture is measured (Figure 13 and 14 respectively), both data sets indicate approximately double the amount of average growth of *P. aeruginosa* to *S. aureus*. In 1% TSB media however, the difference between the two bacteria is much smaller in planktonic growth compared to biofilm growth.

3.9 Enumeration and Viability of bacteria in mono and mixed cultures using coplin jars

Before performing viability counts, *P. aeruginosa* and *S. aureus* were screened for selective growth on agar. *S. aureus* grew on Mannitol Salt agar, whilst *P. aeruginosa* did not. *P. aeruginosa* grew on both MacConkey’s Type 3 Agar and Pseudomonas Cetrimide Agar with Glycerol and *S. aureus* did not. Mannitol Salt Agar and MacConkey’s Type 3 Agar were chosen for these experiments.
Figure 15: Estimated colony forming units (CFU) in 48-hour biofilms of mono-culture biofilms in 0.1% TSB media. Spread plates completed in triplicates of biofilms sonicated into 1X PBS from microscope slides within coplin jars. N=3

In mono culture, 48-hour biofilms of 0.1% TSB (Figure 15), the estimated CFU of *P. aeruginosa* is 2.3 times the estimated CFU of *S. aureus* Newman. This result is the opposite of what was recorded in the 24-hour biofilms cultivated in 96-well plates (Figure 11), where *P. aeruginosa* grew double the amount of biofilm of *S. aureus* Newman.
Figure 16: Estimated colony forming units (CFU) in 48-hour biofilms of mixed-culture biofilms in 0.1% TSB media. Three combinations of mixed cultures were tested, *P. aeruginosa* then *S. aureus* added 24-hours later, the reverse and both bacteria inoculated together. Spread plates on selective media for single cultures, completed in triplicates from biofilms sonicated into 1X PBS from microscope slides within coplin jars. N=3

In the mixed biofilms of 0.1% TSB (Figure 16), the calculated CFU of *P. aeruginosa* is higher in all combinations in respect to *S. aureus* Newman. CFU for *S. aureus* Newman in the *P. aeruginosa* + *S. aureus* biofilm was <250. *S. aureus* Newman had a higher CFU in the biofilm where it was added first in comparison to the CFU for *S. aureus* Newman when inoculated with *P. aeruginosa*. There is at least a logarithmic difference in the growth of *P. aeruginosa* to *S. aureus* Newman for all 0.1% TSB Biofilms.
Figure 17: Estimated colony forming units (CFU) in 48-hour biofilms of mono-culture biofilms in 1% TSB media. Spread plates completed in triplicates of biofilms sonicated into 1X PBS from microscope slides within coplin jars. N=3

Mono culture CFU’s of both *S. aureus* Newman and *P. aeruginosa* for 1% TSB is higher than the CFU’s for 0.1% biofilms. There is a decrease in the difference between the two cultures’ performance, with *S. aureus* Newman having a higher CFU.
Figure 18: Estimated colony forming units (CFU) in 48-hour biofilms of mixed-culture biofilms in 1% TSB media. Three combinations of mixed cultures were tested, *P. aeruginosa* then *S. aureus* added 24-hours later, the reverse and both bacteria inoculated together. Spread plates on selective media for single cultures, completed in triplicates from biofilms sonicated into 1X PBS from microscope slides within coplin jars. N=3

*S. aureus* performed better overall in 1% TSB (Figure 18) than in 0.1% (Figure 16) mixed cultures. When *P. aeruginosa* is added first, the viability of *P. aeruginosa* cells is higher than *S. aureus* cells, with *P. aeruginosa* CFU four times that of *S. aureus*. However, when *S. aureus* is added first, there is a log difference in the CFU and *S. aureus* produced more biofilm cells. In the mixed-culture biofilm where both cultures were inoculated simultaneously, *P. aeruginosa* dominated. There is a 2-log difference between the amount of *P. aeruginosa* CFU and the *S. aureus* CFU.
4. Discussion

Most species of bacteria have the ability to form biofilms (Banin, Vasil & Greenberg), in fact it is believed that the biofilm is the dominant form of bacterial life (Donlan & Costerton). Biofilms provide bacteria protection from environmental stresses, nutrient depravation and competition through the production and maintenance of the EPS matrix that encases the cells (Kostakioti, Hadjifrangiskou and Hultgren, no date; Donlan, 2002; Lin et al., 2012; Balcázar, Subirats and Borrego, 2015; Rabin et al., 2015; Singh et al., 2017; Brown and Gilbert, 2018). Already established is the importance of iron on the maintenance of bacterial growth and development, including the regulation of biofilm formation (Banin, Vasil and Greenberg, 2005; Imperi, Tiburzi and Visca, 2009; Miller et al., 2012; Lin et al., 2016).

Pyoverdine is the main siderophore produced by *P. aeruginosa* in order to acquire iron from the environment, and other bacterial species in a mixed culture (Hannauer et al., 2012a; Peek et al., 2012; Dumas, Ross-Gillespie and Kümmerli, 2013). It has been revealed that the production and mechanism of pyoverdine is regulated by genes located in a *pvd* locus and also a *pvdABCD* operon that also has a regulatory effect on the production of virulence factors such as Exotoxin A (Tsuda, Miyazaki and Nakazawa, 1995; Vasil and Ochsner, 1999; Lehoux, Sanschagrin and Levesque, 2000; Lamont and Martin, 2003). Furthermore, differences in iron concentration has significant influence over biofilm production, siderophore production and virulence. This investigation focused on the complex relationship between iron concentration, biofilm formation and the production of pyoverdine, in both mono-cultures of *P. aeruginosa* and mixed cultures with *S. aureus* ‘Newman.’
4.1 Iron concentration of TSB

An important part of this experimental work was to utilise a media of low iron content. Kim & Park defined the iron concentrations required for optimal bacterial growth to be between 0.3 and 1.8μM. The 1% TSB media used in this study is at a concentration within this range, at 1.62μM total iron content. Due to the sensitivity limit of the assay used, the concentration of 0.1% TSB could not be determined by analysing a sample of the media. Therefore, the assumed concentration of 0.1% TSB is 0.162μM due to the 1:10 ratio of nutrients. Wang (2011) performed a phenanthroline iron assay according to (Komadel and Stucki, 1988) and determined the total iron concentration in 1% TSB (Bacto, 3g/L) to be 0.2μM. Both measurements place 0.1% TSB to be below the required iron content for bacterial growth, defining 0.1% TSB as a low-iron content media.

An iron chelating molecule, such as 1,2,3,4,6-Penta-O-galloyl-b-D-glucopyranose (PGG) or 2,2-dipyridyl chelates iron from the environment in a similar fashion to siderophores, reducing the available free iron for the bacteria to utilise (Imperi, Tiburzi and Visca, 2009; Lin et al., 2012). There were plans to incorporate iron chelating molecules into the experiments, in order to remove all free iron from the media and study the effects on pyoverdine production and biofilm production. However, due to the high affinity of pyoverdine for both free iron and iron bound within complexes, it was believed that these molecules would not be able to compete, thus making the use of the iron chelator ineffective.

4.2 Development of an incubation chamber for 96-well microtiter plates

*P. aeruginosa* is a strictly anaerobic organism, requiring oxygen for all metabolic processes to survive (Fugère et al., 2014). An issue that was encountered in using the 96-well microtiter
plates initially, was the high level of variance in the results for both pyoverdine concentration and both biofilm and planktonic growth. It is believed that by incubating with the lid-on, airflow to the bacteria is restricted, causing patterns of growth across the plate. Distinct regions of increased bacterial growth was found in the wells closest to the perimeter of the plate, as opposed to the cells in the center of the plate which had significantly lower growth (Appendix A). This observation led to the creation of a custom incubation chamber which dramatically improved consistency in results and lowered standard deviation. Initially, plates were grown within a UV-sterilised steel tray wrapped in aluminium foil without the lids, however maintaining the sterility of the system proved difficult, as it was suspected that certain areas of the apparatus were not being touched by the UV-light in the sterilisation process.

4.3 The production of siderophores in *P. aeruginosa* planktonic and biofilm cultures

Along with pyoverdine, *P. aeruginosa* spp. produce another siderophore, pyochelin, for iron chelation (Poole, 2003; Dumas, Ross-Gillespie and Kümmerli, 2013; Hoegy, Mislin and Schalk, 2014). Although pyochelin has a lower affinity to iron than pyoverdine, pyochelin production is not an energy-dependent process (Dumas, Ross-Gillespie and Kümmerli, 2013) and as such, is often produced before pyoverdine. To determine the levels of production of pyochelin in cultures between 1% TSB and 0.1% TSB, 96-well plates were scanned and scanned twice, for pyoverdine fluorescence and pyochelin fluorescence. The average result over three plates is shown in Figure 5. As expected, whilst there is pyochelin production within the cultures, the levels in comparison to pyoverdine production were significantly less, P=0.0016 in 0.1% TSB and P=0.0024 in 1% TSB.
The production of pyoverdine is tightly regulated by Fur, and as such, directly controlled by intracellular iron content (Leoni et al., 1996; Pasqua et al., 2017). This relationship would suggest that the concentration or amount of pyoverdine would be higher in *P. aeruginosa* cultures in lower-iron environments. Additionally, the production of pyoverdine also has a positive regulatory effect on the formation of a biofilm. Kang, Turner and Kirienko (2017) describes this relationship as a ‘complex, bidirectional regulatory relationship.’ Pyoverdine is tasked with scavenging iron which is essential for biofilm production across many bacterial species, *S. aureus* and *P. aeruginosa* included (Lin et al., 2012; Kang, Turner and Kirienko, 2017). The presence of pyoverdine is required for ‘full biofilm formation’ under iron-deplete conditions (Banin, Vasil and Greenberg, 2005; Kang, Turner and Kirienko, 2017) and the inverse of this relationship also stands, where pyoverdine is down-regulated by biofilm formation under high-iron concentrations (Kang, Turner and Kirienko, 2017). Kang, Turner and Kirienko (2017) also demonstrated that compromising biofilm formation severely decreases pyoverdine production, and lowers the pathogenicity of *P. aeruginosa* against the fungus *Caenorhabditis elegans* (Kang, Turner and Kirienko, 2017).

The concentration of pyoverdine in planktonic cultures was overall, higher in comparison to biofilm cultures (Figure 6, 7 and 8). Across both media, a 14-fold difference in fluorescence was observed, and between the differing media concentrations, the average pyoverdine determined by fluorescence was 2.5 times higher in 1% TSB than in 0.1% TSB (*P*<0.0001). Considering the relationship between biofilm growth, pyoverdine production and iron concentration, this result is not unexpected. Whilst biofilms have a higher cell density, planktonic cultures can have significantly more cells (Spoering and Lewis, 2001). This higher number of cells requires increased nutrients, including iron, and other essential growth factors in order to sustain planktonic growth.
A high level of pyoverdine production in these planktonic cultures after 24-hours of growth, could suggest that the bacteria have utilized a significant amount of the iron present in the growth medium and thus require the production of high-affinity siderophores in order to acquire the residual iron from the environment. To a certain extent, the slower growth of sessile cells within the biofilm (Spoering and Lewis, 2001; Donlan, 2002; Branda et al., 2005) may be advantageous in that resources deplete slower, suggesting that biofilms may not require the production of siderophores as quickly as planktonic cells. On the other hand, as a consequence of the greater cell density of biofilms, cells are likely to encounter limitations to oxygen and nutrients and experience higher level of waste, secreted factors and secondary metabolites (Mikkelsen, Sivaneson and Filloux, 2011).

The production of pyoverdine is an energy-dependent process (Dumas, Ross-Gillespie and Kümmelerli, 2013), as such it is under tight regulation and only synthesizes pyoverdine under iron-deplete conditions (Vasil and Ochsner, 1999; Banin, Vasil and Greenberg, 2005; Johnson, Cockayne and Morrissey, 2008; Ochsner et al., 2018). The results identified in this study (Figure 9) demonstrate a significantly higher level of pyoverdine in 0.1% TSB Biofilms over 1% TSB biofilms ($P = 0.0428$), where the iron content is also a tenth the concentration in 0.1% TSB at 0.162μM. This finding is also consistent with what is currently understood as the mechanisms for pyoverdine regulation. Fur is an ‘iron-responsive, DNA—binding repressor protein’ (Vasil and Ochsner, 1999) which employs ferrous iron as a cofactor. In order to bind to the DNA sequences and repress gene expression, Fur binds to Fe$^{2+}$ and undergoes a conformational change (Oglesby-Sherrouse and Murphy, 2013). When Fur is unbound, transcription takes place. Under low intracellular iron concentrations, Fur remains unbound to the DNA due to, in part, decreased concentration of Fe$^{2+}$ (Leoni et al., 1996;
Stintzi et al., 1999; Ganne et al., 2017; Ochsner et al., 2018). This strongly suggests that the production of pyoverdine is in response to the low iron concentration of 0.1% TSB.

4.4 Biofilm formation by *P. aeruginosa* and *S. aureus* mono-cultures

As aforementioned, biofilm formation in *P. aeruginosa* is heavily influenced by iron concentration and siderophore production (Banin, Vasil and Greenberg, 2005; Lin et al., 2012, 2016). The characteristic ‘mushroom’ like structures seen in 3D representation of *P. aeruginosa* biofilms are formed under sufficient iron concentration. An overabundance of iron results in a ‘nebulous’ structure (Miller et al., 2012). Biofilm formation is also heavily reliant on the synthesis of pyoverdine, as without the ability to sequester iron *P. aeruginosa* forms thin, flat biofilms (Banin, Vasil and Greenberg, 2005). Banin et al., (2005) demonstrates this through confocal microscopy of the biofilms of pyoverdine and fur knockout mutants of *P. aeruginosa*. Using green fluorescent proteins and Z-stacks, 3D representations of the grown biofilms were created, demonstrating the characteristics of *P. aeruginosa* biofilms under differing iron conditions and siderophore production. In these experiments, the biofilm forming capacity of the bacteria is analysed through a crystal violet assay.

Figure 10 depicts the average biofilm mass by absorbance for *P. aeruginosa* in 0.1% TSB and 1% TSB, where biofilm formation in 1% TSB is, on average, 2.4 times higher than in 0.1% TSB. When considering the importance of iron concentration in the positive regulation of biofilm formation, and the effect of pyoverdine production on biofilm formation. Whilst low-iron concentrations and the production of siderophores are believed to promote biofilm formation, several studies have demonstrated that biofilm formation favours high iron concentrations (Banin, Vasil and Greenberg, 2005; Oglesby-Sherrouse and Vasil, 2010;
Reinhart and Oglesby-Sherrouse, 2016; Singh et al., 2017) and during infection, *P. aeruginosa* requires an ‘abundance of iron’ (Cox and Graham, 1979; Meyer et al., 1997; Oglesby-Sherrouse and Vasil, 2010). Ponraj et al., (2012) demonstrates the importance of iron uptake through siderophores on the fitness of the bacterial biofilm, as it ‘significantly influences biofilm attachment and formation.’ The planktonic growth of *P. aeruginosa* in 0.1% TSB is also lower than the growth in 1% TSB, with a 1.7-fold difference between the two results.

A factor that could be affecting biofilm growth is the limited nutrients available to the bacteria, in addition to low iron availability. The recommended preparation of TSB media is a ratio of 30 grams of media to 1L of water, however 0.1% TSB only has 0.3g/L which is 1% of the recommended nutrient concentration. The exact quantities of nutrients is unknown, therefore determining whether this will have a positive or negative impact on biofilm formation is also unknown.

Many studies have demonstrated that *S. aureus* has poor-biofilm forming capabilities under low iron concentrations (Baba et al., 2008; Johnson, Cockayne and Morrissey, 2008; Lin et al., 2012; DeLeon et al., 2014). However, *S. aureus* Newman has been shown to produce high levels of biofilm under low iron concentrations and low levels under high iron concentration. Johnson compared *S. aureus* Newman’s biofilm formation requirements against another strain of *S. aureus* (SA113), which requires iron for biofilm formation. Johnson et al., (2008) found that biofilms by *S. aureus* Newman biofilms were significantly repressed by iron. It was postulated that this is due to Fur not demonstrating any negative regulatory activity despite Fur demonstrating a positive regulatory effect on biofilm formation in low iron conditions (Johnson, Cockayne and Morrissey, 2008). These results
were confirmed by Lin et al., (2012) whom repeated the experiments by culturing S. aureus Newman biofilms in CRPMI (Chelex 100 resin-treated RPMI) media, which is iron-restricted, and getting the same results. When iron was added to the media, biofilm formation was inhibited (Johnson, Cockayne and Morrissey, 2008; Lin et al., 2012).

However, this result was not demonstrated in this study. Figure 11 presents the results for S. aureus Newman biofilm formation in 0.1% TSB and 1% TSB, where approximately three times more biofilm was produced in 1% TSB over 0.1% TSB. Planktonic growth in Figure 10 displays a similar result. The average biofilm growth of P. aeruginosa is twice that of S. aureus Newman for biofilms in both 1% and 0.1% TSB (Figure 13) however in comparing planktonic growth, S. aureus Newman had more growth than P. aeruginosa in 1% TSB and half the amount of P. aeruginosa in 0.1% TSB (Figure 14).

4.5 Biofilm formation in mixed cultures of P. aeruginosa and S. aureus

The co-cultures of P. aeruginosa and S. aureus are a complex dynamic of competition and synergism where each bacteria employ their own virulence tactics to ensure the survival of their culture and to limit the growth of the other. This study looks at the viability of bacterial cells in mixed culture biofilms through enumeration of cells via spread plate techniques.

The mono culture results in Figures 15 and 17 demonstrate that S. aureus is able to produce large numbers of viable cells for both 0.1% TSB and 1% TSB, in comparison to P. aeruginosa, which in both experiments produces fewer CFU. In 0.1% TSB there is a logarithmic difference between the CFU of P. aeruginosa and S. aureus. T-tests indicate that there is a significant difference between the CFU of S. aureus in 0.1% TSB and 1% TSB (P
and also a significant difference between the CFU of \textit{P. aeruginosa} in both media (P=0.0437).

Interestingly, the result for \textit{S. aureus} in 0.1\% TSB is contrasting to the result gathered from the Crystal Violet assays for biofilm growth (Figures 10 and 12) where \textit{P. aeruginosa} performed better than \textit{S. aureus}. As aforementioned, \textit{S. aureus} is known for it’s ability to produce biofilms in low-iron conditions, a trait that is contrasting to the general characteristics of \textit{Staphylococcus spp.} which tend to favour higher iron concentrations (Johnson, Cockayne and Morrissey, 2008; Lin \textit{et al.}, 2012). So, whilst this result is as expected when examined in relation to the literature, it is contrasting to previous biofilm experiments in this study.

The mixed culture results for 0.1\% TSB show overall, higher CFU and biofilm for \textit{P. aeruginosa} (Figure 16). In a mixed culture where \textit{P. aeruginosa} was inoculated 24-hours prior to the inoculation of \textit{S. aureus}, 250 CFU by \textit{S. aureus} was detected, suggesting the value is too low for the sensitivity of the plate count method. The significant difference in CFU by the bacteria in this experiment suggests that the 24-hour period \textit{P. aeruginosa} had to develop a biofilm and utilise nutrients was enough to establish dominance of \textit{S. aureus} after inoculation. In the promotion of pyoverdine synthesis in \textit{P. aeruginosa}, is the production of the virulence factor Exotoxin A through the \textit{toxA} gene (Ochsner \textit{et al.}, 2018). Due to the high concentration of pyoverdine that has been previously demonstrated in biofilm cultures within 0.1\% TSB (Figure 8), a plausible theory for the lack of biofilm growth by \textit{S. aureus} is a high concentration of Exotoxin A within the media led to a decrease in CFU by \textit{S. aureus}. Additionally, \textit{P. aeruginosa} also has the ability to produce several other exoproducts, including antistaphylococcal proteases such as LasA which can cause cell lysis for \textit{S. aureus}.
bacteria (Sauer et al., 2002; Fugère et al., 2014; Filkins et al., 2015). The pyoverdine concentration of both the planktonic culture within the coplin jar, and the biofilm cells was measured, however due to interference from something within the mixed cultures and *S. aureus* monoculture, the results were erratic, unreproducible and almost reached the sensitivity limit of the ClarioSTAR.

The CFU for *S. aureus* is higher in mixed cultures where the media is inoculated with *S. aureus* first (Figure 16). *S. aureus* has many biological advantages over *P. aeruginosa*, including the ability to grow aerobically and anaerobically, meaning that whilst *P. aeruginosa* biofilms are limited to the liquid-air interface of the slide, *S. aureus* can potentially grow a biofilm from the liquid-interface to the bottom of the slide submerged in media. A study by Alves et al., (2018) demonstrates that *S. aureus* predominates over *P. aeruginosa* in the early stages of biofilm formation in terms of aggregation, attachment and growth. Early biofilm development for both bacterial species begins with the formation of bacterial aggregates in suspension, which then attach to a surface (Alves et al., 2018). By comparing the autoaggregation of each bacterium in single cultures and in mixed cultures, it was found that *S. aureus* had the largest proportion of aggregates and even promoted aggregation by *P. aeruginosa* (Alves et al., 2018).

When both bacteria are inoculated together in 0.1% TSB, the resulting cell viability is predominantly *P. aeruginosa*, with a 2-log, 124-fold difference in CFU between *P. aeruginosa* and *S. aureus*. However, in comparison to the *P. aeruginosa* then *S. aureus* mixed culture, there is still CFU of *S. aureus* in the biofilm. The result of the mixed culture biofilm in 1% TSB where both cultures were inoculated at the same time is comparable to the results
of the same experiment for 0.1% TSB. There is 51 times the CFU of \( P. \text{aeruginosa} \) cells in comparison to \( S. \text{aureus} \).

When cultured in 1% TSB, and as previously mentioned, the CFU of both \( P. \text{aeruginosa} \) and \( S. \text{aureus} \) were significantly higher than the CFU of their respective cultures in 0.1% TSB (Figure 17). The rise in viability of these cells could be attributed to the higher level of nutrients in the media, promoting more growth by the bacteria, however without further experimentation these are only conjecture. In the 1% mixed biofilm where \( P. \text{aeruginosa} \) was inoculated first, the CFU of \( P. \text{aeruginosa} \) was higher than both \( S. \text{aureus} \) and the CFU of \( P. \text{aeruginosa} \) from the same mixed culture setup on 0.1% TSB. In this experiment, the CFU of \( S. \text{aureus} \) cells is approximately 4 times less than \( P. \text{aeruginosa} \), however there is still viability of \( S. \text{aureus} \) biofilm. This result could possibly be explained by a lower concentration of pyoverdine in 1% TSB, as demonstrated in Figure 8, increase in nutrients allowing for less competition between bacterial species.

In 1% TSB Mixed culture with the addition of \( S. \text{aureus} \) 24-hours before \( P. \text{aeruginosa} \), the CFU of \( S. \text{aureus} \) in comparison to \( P. \text{aeruginosa} \) is much higher with 3.7 times the amount of CFU (Figure 18). The 24-hour incubation of \( S. \text{aureus} \) as a monoculture appears to have been advantageous in competition with \( P. \text{aeruginosa} \). Similarly to \( P. \text{aeruginosa} \), \( Staphylococcal \text{spp.} \) also produce virulence factors against competing species, such as the production of nucleases that can prevent and also disrupt biofilm formation and growth of \( P. \text{aeruginosa} \) among other species as well as the Staphylococcal protein A which ‘impairs biofilm formation’ of \( P. \text{aeruginosa} \) (Yang et al., 2009; Tang et al., 2011; Alves et al., 2018). The production of any or all of these virulence factors could affect the viability of \( P. \text{aeruginosa} \) biofilm formation, resulting in a lower CFU.
5. Conclusions and Future Work

This research determined that both pyoverdine concentration and biofilm production respond to the iron concentration in the ambient environment within single culture bacterial species. There are multiple genes involved in the synthesis and regulation of pyoverdine. Studies show that iron concentration has a direct effect on the transcription of pyoverdine synthesis genes through the Ferric uptake regulator protein, Fur. In measuring the amount of pyoverdine in planktonic and biofilm cultures between two media concentrations, 1% TSB with an iron content of 1.62uM and 0.1% TSB with an iron content of 0.162uM determined in this study through an Iron Assay Kit (Figure 2), it was found that the amount of pyoverdine was higher in 0.1% TSB biofilm cultures of \textit{P. aeruginosa} than in 1% TSB (Figure 8).

It was also identified that biofilm formation in \textit{P. aeruginosa} was higher in 1% TSB (Figure 10), as was biofilm formation by \textit{S. aureus} (Figure 12). \textit{P. aeruginosa} also grew more biofilm in both media in comparison to \textit{S. aureus} (Figure 13), however in planktonic cultures, \textit{S. aureus} once again has the highest growth (Figure 14). The results of this research project indicate that low iron concentrations favours pyoverdine production in \textit{P. aeruginosa} and higher iron concentrations favours biofilm production for both \textit{S. aureus Newman} and \textit{P. aeruginosa}. Experiments by Johnson, Cockayne and Morrissey (2008 and Lin et al., 2012) however, demonstrated that under low-iron concentrations, biofilm growth by \textit{S. aureus Newman} was promoted, whereas the result collected in these experiments suggest the opposite. An investigation into the effect of iron concentration on viability of biofilm cells in mixed cultures further demonstrated \textit{S. aureus Newman} producing more biofilm in higher iron concentrations as opposed to low-iron concentration.
Biofilm production by *P. aeruginosa* was most effective in 0.1% TSB even in polymicrobial biofilms (Figure 16). *P. aeruginosa* dominated the 0.1%TSB co-cultures, evident in the CFU counts, regardless of whether *S. aureus* was inoculated first, *P. aeruginosa* was inoculated first or the species were inoculated together. No result was recorded for *S. aureus* cells in the *P. aeruginosa* inoculated first biofilm, as no growth occurred on the spread plates (Figure 16). In 1% TSB mixed biofilms, *P. aeruginosa* has the same effect on *S. aureus* cells when inoculated at the same time (Figure 18). However, 1%TSB mixed biofilms, the bacteria inoculated first for 24-hours has the highest CFU after 48 hours. Due to interference by something within the co-cultures, the pyoverdine levels within the mixed culture biofilms were not established.

Further work in this area would include establishing pyoverdine concentration in various mixed biofilms of *P. aeruginosa* and *S. aureus* in different iron concentrations and establishing the mechanism of *S. aureus* Newman biofilm regulation in different iron concentrations. Additionally, repeats of these experiments with more iron concentrations, including an iron-deplete media and addition of different iron concentrations to observe repression or promotion of both biofilm growth and pyoverdine production. Further investigation in this area would allow for a better understanding of the complex interrelationship between pyoverdine production, biofilm formation and iron concentration. Three factors that contribute significantly to the bacteria’s ability to cause infection and mortality.
6. Bibliography


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7. Appendicies

Appendix A: Major variance in results of biofilm formation assays.

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Figure A1: BitMap of a 24-hour, 96-well microtiter plate inoculated with *P. aeruginosa* imaged in the CLARIOstar for planktonic growth.
Figure 1B: Photograph of incubator setup described in section 2.3. Not pictured, air pump and tube running air into the chamber.
Figure C1: Photograph taken of coplin jars used to culture 1% TSB Mixed culture biofilms after 48 hours of growth, demonstrating differences in colour of media where uninoculated Is light brown, suggesting different concentration of pyoverdine production. On the left is *P. aeruginosa* with *S. aureus* added 24 hours later. On the right is *S. aureus* with *P. aeruginosa* added 24 hours later.
Figure C1: Photograph taken of coplin jars used to culture 1% TSB Mixed culture biofilms after 48-hours of growth, demonstrating differences in colour of media where uninoculated Is light brown, suggesting different concentration of pyoverdine production. Jar contains mixed culture inoculated together.